

Repeated Episodes of Hypoglycemia as a Potential Aggravating Factor for Preclinical Atherosclerosis in Subjects With Type 1 Diabetes

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OBJECTIVE — To evaluate through early preclinical atherosclerosis assessment whether repeated episodes of hypoglycemia represent an aggravating factor for macrovascular disease in type 1 diabetes.

RESEARCH DESIGN AND METHODS — After sample-size calculation, a case-control study of 25 patients with type 1 diabetes and repeated severe/nonsevere hypoglycemia (H-group) compared with 20 age- and sex-matched type 1 diabetes control subjects (C-group) was designed. Assessment of preclinical atherosclerosis consisted of flow-mediated brachial dilatation (FMD) and carotid and femoral intima-media thickness (IMT) studies. To consider hypoglycemia awareness, two different questionnaires and symptomatic response to an acute induction to hypoglycemia were used. Evaluation of the glycemic profile was obtained from continuous glucose monitoring. Endothelial function/inflammation markers were measured in euglycemia/hypoglycemia. A multivariate linear regression analysis was performed to test whether repeated hypoglycemia was independently associated with atherosclerosis.

RESULTS — H-group subjects displayed hypoglycemia unawareness and presented a higher percentage of continuous glucose values and area under the curve <70 mg/dl compared with the C-group (14.2 ± 8.9 vs. $6.3 \pm 7.1\%$, $P < 0.02$ and 2.4 ± 1.8 vs. 0.6 ± 1.0 mg/dl/day, $P < 0.01$). The percentage of maximal FMD was lower in the H-group than in the C-group (6.52 ± 2.92 vs. $8.62 \pm 3.13\%$, $P < 0.05$). A significantly higher IMT was observed at both carotid and femoral sites in the H-group (carotid 0.53 ± 0.09 vs. 0.47 ± 0.08 mm, $P < 0.05$ and femoral 0.51 ± 0.17 vs. 0.39 ± 0.09 mm, $P < 0.05$). Baseline inflammation and endothelial function markers were higher in the H-group (leukocytes 7.0 ± 1.8 vs. $5.6 \pm 1.4 \times 10^3$ /ml, von Willebrand factor 119 ± 29 vs. $93 \pm 26\%$, fibrinogen 2.82 ± 0.64 vs. 2.29 ± 0.44 g/l, and soluble intercellular adhesion molecule-1 408 ± 224 vs. 296 ± 95 ng/ml; $P < 0.05$ for all).

CONCLUSIONS — In addition to the induction of hypoglycemia unawareness and an increased risk for severe hypoglycemia, repeated hypoglycemia could be related to and considered an aggravating factor for preclinical atherosclerosis in type 1 diabetes. The precise mechanisms explaining this association remain to be clarified.

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Even though many of the cardiovascular disease (CVD) risk factors recognized in type 2 diabetes are not present in type 1 diabetic subjects, the age-adjusted relative risk for CVD in type 1 diabetes is even higher than that in type 2 diabetes (1). Since the availability of data from Diabetes Control and Compli-

cations Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) studies, there is no doubt that intensive therapy positively affects the long-term incidence of micro- and macrovascular disease in subjects with type 1 diabetes (2,3). However, because the association between glycemic control and macrovascular disease is mainly obtained from epidemiological data, the role of glycemic control in macrovascular disease is still controversial. In contrast, intensive glucose control invariably increases the risk of hypoglycemia.

Iatrogenic hypoglycemia causes recurrent morbidity in most people with type 1 diabetes. Frequent and repeated episodes of hypoglycemia almost unfailingly result in a reduced ability or failure to recognize hypoglycemia symptoms and signs. This syndrome of hypoglycemia unawareness frequently occurs in type 1 diabetes, and patients without warning symptoms are then at a high risk for severe hypoglycemia (4). In addition, hypoglycemia is a major barrier to achieving normoglycemia over a lifetime of using intensive insulin therapy and thus precludes the long-term benefits of euglycemia (4). More recently, Gill et al. (5) reported QT prolongation and cardiac and rhythm disturbances in response to nocturnal hypoglycemia in ambulatory patients with type 1 diabetes, which may support the idea of an arrhythmic basis for “death in bed syndrome.”

Carotid intima-media thickness (cIMT) and the assessment of endothelial function have been shown to be markers of preclinical atherosclerosis and correlate with prevalent and incident cardiovascular disease (6). In the DCCT/EDIC, the progression of cIMT in the population of type 1 diabetic subjects was used as a measure of atherosclerosis (7).

It has also been reported that patients with type 1 diabetes presented higher cIMT and lower percentages of flow-mediated dilatation (FMD) with respect to healthy control subjects (8). Although hyperglycemia has been proven to increase the stiffness of intermediate-sized

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arteries and resistance of arteries, the analysis of discontinuous glucose profile datasets from the DCCT failed to find an association between glucose variability and the development of microvascular complications (9). Moreover, various measures for the assessment of glycemic variability have shown that there is no relationship between oxidative stress and glucose fluctuations in type 1 diabetes even though glucose variability was much higher than that in type 2 diabetes (10).

Acute hypoglycemia induces a rapid proinflammatory, platelet aggregatory, antifibrinolytic, and prothrombotic response (11,12). Recurrent hypoglycemic episodes may provoke changes in hemostatic factors and viscosity, which may reduce perfusion in diabetic microangiopathy (11,12). Rodrigues et al. (13) have recently reported that higher fibrinogen levels predict progression of coronary artery calcification in adults with type 1 diabetes. The SEARCH study has also described elevated inflammatory markers even in youth with type 1 diabetes and good metabolic control compared with control subjects, suggesting an explanation for accelerated atherosclerosis in type 1 diabetes (14). In addition, Feldman-Billard et al. (15) described hypoglycemia-induced hypertension in a group of diabetic patients. If hypoglycemia acutely provokes intense changes in hemodynamics and several hemorheological parameters, it could play a different role in atherosclerosis when chronically repeated.

Therefore, the aim of our study was to evaluate whether repeated episodes of hypoglycemia represent an aggravating factor for macrovascular disease in subjects with type 1 diabetes through early atherosclerosis-vascular assessment.

RESEARCH DESIGN AND METHODS

A total of 45 patients with type 1 diabetes were recruited for the study from 2007 to 2009. Subjects were invited to participate in the protocol if they fulfilled the following criteria: aged >18 years, type 1 diabetes duration >5 years, basal C-peptide <0.1 ng/ml, use of multiple doses of insulin in a basal-bolus schedule, and an absence of other major CVD risk factors, micro- or macrovascular complications (normal digital retinal photography results, absence of microalbuminuria, no neuropathy by clinical examination, normal ankle-brachial index, and normal stress echocardiography results), and no autonomic dysfunction

(Cardionomic system; Medimatica, Milan, Italy). Patients were not taking medication chronically (including statins, antihypertensive drugs, or anti-inflammatory drugs) except insulin.

Of the 45 type 1 diabetic patients, 25 were selected as a hypoglycemic group (H-group) presenting >4 nonsevere hypoglycemia episodes per week (last 8 weeks) and >2 severe hypoglycemia episodes in the past 2 years. All episodes of capillary glycemia <70 mg/dl were considered nonsevere hypoglycemia episodes based on four to six daily capillary blood determinations. Severe hypoglycemia events were defined as those associated with neuroglycopenia severe enough to require treatment from a third party. Of the 45 type 1 diabetic patients, 20 were chosen as age- and sex-matched diabetic control subjects (C-group) presenting <2 nonsevere hypoglycemia episodes per week (last 8 weeks) and with no previous episodes of severe hypoglycemia. Anthropometric measures, general biochemical parameters, A1C values (normal range 3.5–5.5%; Menarini Diagnostici, Firenze, Italy), and lipid profile were measured at the beginning of the study.

In addition, an age- and sex-matched healthy control group (22 subjects) was selected as a comparative group for ultrasound analysis. They satisfied the criteria of being nonsmokers, having a normal fasting glycemia and lipid profile, not having hypertension, diabetes, or dyslipidemia, and not having a family history of CVD or diabetes.

The protocol included an evaluation of the frequency/awareness of hypoglycemia, an assessment of glycemic profile/glucose variability, an evaluation of endothelial function (FMD), and a carotid and femoral IMT assessment. Inflammation and endothelial function markers were evaluated.

The study protocol, conducted according to the Declaration of Helsinki, was approved by the Hospital Clínic i Universitari Ethics Committee. Informed consent was obtained from all the patients and control subjects.

Evaluation of hypoglycemia awareness

Two different questionnaires (Clarke and Gold tests [16,17]) were used to evaluate hypoglycemia awareness. To assess signs and symptoms response to a standardized situation of hypoglycemia, an acute induction to hypoglycemia with intravenous insulin was performed (18).

Subjects with type 1 diabetes answered the Hypoglycemia symptoms score questionnaire (Edinburgh scale [19]) after 30 min of euglycemia first (80–120 mg/dl) and after 30 min of hypoglycemia (45–55 mg/dl) afterward. The tests scores for the two states were compared, and the results are expressed as a percentage of increase from the baseline.

Glycemic profile and glucose variability

Immediately before vascular studies, each patient with type 1 diabetes underwent continuous glucose monitoring (CGM) for 72 h using the Medtronic Gold system. Glucose variability was evaluated by calculating mean amplitude of glucose excursions (MAGE) from continuous sensor readings. MAGE over 24 h is the mean of the absolute differences between glucose peak and nadir values in excess of at least 1 SD of the mean glucose.

Ultrasound imaging

The carotid, femoral, and brachial artery ultrasound studies were performed with an Acuson Sequoia system (Acuson Corporation, Mountain View, CA), equipped with an 8-MHz linear array transducer. The FMD studies were performed by M.G., a trained endocrinologist with experience in >150 FMD studies. The cIMT and femoral IMT (fIMT) studies were done by R.G., a radiologist with >15 years of experience. M.G. and R.G. were masked to the patient groups when they performed the FMD and IMT studies.

FMD. All patients and healthy control subjects were evaluated after 6 h of abstinence from food and caffeinated drinks. Women were examined in the follicular phase of the menstrual cycle. Capillary glycemia was always between 80 and 120 mg/dl. The brachial artery was imaged longitudinally 5–10 cm above the antecubital fossa. Baseline images were recorded continuously for 1 min. Subsequently, a blood pressure cuff positioned 4 cm below the elbow was inflated up to 250 mmHg for 5 min. The artery was continuously imaged for 4 min during the hyperemia after release of the cuff pressure to determine endothelium-dependent vasodilatation. All images were analyzed using proprietary software (Brachial Analyzer; Medical Imaging Applications, Iowa City, IA). Dilatation was calculated as maximal lumen diameter after ischemia minus lumen diameter at baseline divided by lumen diameter at baseline. Results are expressed as a percentage.

cIMT. The common carotid artery, the carotid artery bulb, and the internal carotid artery near and far wall segments were scanned bilaterally. IMT was defined as the distance between the lumen-intima and the media-adventitia interfaces. Measurements were performed offline and consisted of six manual measurements at equal distances along 1 cm on the far wall of the common carotid artery (1 cm before the bifurcation), bulb, and internal carotid artery (1 cm after the bifurcation). The mean of the 36 values for right and left sides was considered a composite measurement (cIMTcomp). Atheroma plaques (focal intrusions into the lumen with a height >50% of the nearest IMT or diffuse IMT thickening >1.2 mm) were sought by using B-mode and color Doppler imaging in all the carotid segments.

fIMT. Mean fIMT was measured in the far arterial wall along the distal 1 cm before the bifurcation. Six measurements were done manually on each side. fIMT was expressed as the mean of the 12 values.

Intraobserver variability was evaluated by comparing results from repeated examinations of 15 subjects on 2 days a week apart. The correlation coefficients for cIMT, fIMT, and percent FMD were 0.91, 0.93, and 0.74, respectively. The correlation coefficient between two different readers was 0.91 for cIMT.

Inflammation and endothelial marker evaluation

After type 1 diabetic patients had rested 30 min in euglycemia, leukocytes, high-sensitivity C-reactive protein (hs-CRP) (Behring Nephelometer analyzer; Dade Behring, Marburg, Germany), von Willebrand factor (vWF) (ELISA-based commercial kit, DG-EIA vWF; Diagnostic Grifols, Parets del Valles, Spain), fibrinogen (Thromborel S; Dade-Behring), soluble intercellular adhesion molecule-1 (sICAM-1) (ELISA-based commercial kit; BKL Diagnostics, Barcelona, Spain), soluble E-selectin (ELISA-based commercial kit; BKL Diagnostics), and interleukin-1 β (1 β -IL) (ELISA-based commercial kit; BioSource Europe, Nivelles, Belgium) were measured to assess inflammation and endothelial function. All of these parameters were also measured after 30 min in hypoglycemia.

Statistical analysis

Results are presented as means \pm SD or percentages. Normal distribution was tested for each variable using the Kolmog-

orov-Smirnov test. The comparisons between groups were performed using a Student's *t* test for unpaired data for normally distributed variables or using a Mann-Whitney *U* test for nonnormally distributed variables. Proportions were compared with the use of a Fisher exact test. A multivariate linear regression analysis was performed to test whether repeated hypoglycemia was independently associated with cIMTcomp measurements. Covariates included age, sex, comorbidities (systolic blood pressure, BMI, and LDL cholesterol) and factors related to diabetes and glucose control (type 1 diabetes duration, A1C, and MAGE). *P* < 0.05 was considered statistically significant. All statistical calculations were performed with SPSS (version 14.0 for personal computers).

Sample size calculation

We planned a study of a continuous response variable from independent control subjects (C-group) and experimental subjects (H-group). Considering a true difference in the experimental and control means of 0.045 mm in cIMT, we needed to study at least 20 experimental subjects and 20 control subjects to be able to reject the null hypothesis that the population means of the experimental and control were equal with probability (power) 0.80. The type I error probability associated with this test of this null hypothesis was 0.05 (α).

RESULTS— The baseline characteristics of the H-group and C-group are shown in Table 1. There were no major differences in the whole set of clinical and laboratory parameters between type 1 di-

abetic subjects and healthy control subjects.

Hypoglycemia awareness and number of hypoglycemic episodes

As expected, H-group subjects had a significantly higher number of nonsevere hypoglycemia episodes per week and more severe hypoglycemia episodes than type 1 diabetic subjects in the C-group (nonsevere hypoglycemia: 5.22 ± 1.98 vs. 0.25 ± 0.50 episodes/week/subject during the previous 2 weeks, *P* < 0.01; severe hypoglycemia for 2 years before: 1.28 ± 0.45 vs. 0 episodes/patient/year).

Hypoglycemia awareness was evaluated using two different specific questionnaires. The Gold questionnaire classified 25 of 25 subjects in the H-group as having hypoglycemia unawareness but none in the C-group. On the other hand, the Clarke test classified 24 of 25 H-group subjects as having hypoglycemic unawareness and 1 of 25 as inconclusive. Again, all of the type 1 diabetic subjects from the C-group were classified as having normal awareness using the second test.

The mean score for the Edinburgh scale in euglycemia was not different between the groups (21.1 ± 2.7 vs. 20.5 ± 1.9 for the H-group and C-group, respectively). With respect to the signs/symptoms response during the acute induction of hypoglycemia, type 1 diabetic subjects in the H-group increased on average 46% on the Edinburgh scale between euglycemia and hypoglycemia, whereas those in the C-group increased 163% between both situations.

Table 1—Characteristics of study subjects

	H-group	C-group	<i>P</i> value
<i>n</i>	25	20	
Sex (male/female)	11/14	11/9	NS
Age (years)	34.6 ± 7.8	33.5 ± 8.7	NS
Type 1 diabetes duration (years)	16.1 ± 6.3	14.0 ± 6.5	NS
A1C (%)	6.6 ± 1.0	6.7 ± 0.7	NS
Total cholesterol (mg/dl)	171 ± 30	167 ± 34	NS
LDL cholesterol (mg/dl)	107 ± 26	101 ± 24	NS
HDL cholesterol (mg/dl)	52 ± 12	55 ± 11	NS
Triglycerides (mg/dl)	54 ± 26	47 ± 20	NS
Systolic blood pressure (mmHg)	107 ± 12	108 ± 13	NS
Diastolic blood pressure (mmHg)	71 ± 9	73 ± 10	NS
BMI (kg/m ²)	23.1 ± 2.9	23.5 ± 2.3	NS
Smokers (%)	0	0	NS

Data are means \pm SD.

Glycemic profile and glucose variability

With respect to the results obtained from the blinded CGM system data in type 1 diabetic patients, it was not surprising that the H-group subjects presented higher percentages of values and area under the curve <70 mg/dl with respect to the C-group (14.2 ± 8.9 vs. $6.3 \pm 7.0\%$ of values <70 mg/dl, $P < 0.02$ and 2.4 ± 1.8 vs. 0.6 ± 1.0 mg/dl area under the curve for low values, $P < 0.01$ for the H-group and C-group, respectively). Regarding glucose variability, MAGE was significantly higher in the H-group than in the C-group (136 ± 29 vs. 101 ± 28 mg/dl, $P < 0.01$).

FMD

Subjects from the H-group displayed lower percentages of FMD response to ischemia with respect to type 1 diabetic patients from the C-group (6.52 ± 2.92 vs. $8.62 \pm 3.13\%$, $P < 0.05$) (Table 2). Both type 1 diabetic groups were compared with the Healthy-Control Group (22 subjects, 12 women, aged 32.7 ± 6.8 years), and lower percentages of dilatation in the FMD test were found when compared with those obtained in the comparative group ($9.41 \pm 2.20\%$ for the Healthy-Control Group).

cIMT and fIMT

As shown in Table 2, all of the measures performed in carotid and femoral sites were higher in the H-group than in the C-group. With respect to carotid arteries, both cIMT and cIMTcomp were higher in the H-group than in the C-group (cIMT 0.53 ± 0.09 vs. 0.47 ± 0.08 mm; $P < 0.05$; cIMTcomp 0.59 ± 0.13 vs. 0.47 ± 0.07 mm, $P < 0.02$). In addition, fIMT was also lower in the C-group (0.51 ± 0.17 vs. 0.39 ± 0.09 mm, $P < 0.05$). Whereas atherosclerotic plaques were detected in either the carotid or femoral area in 10 of 25 subjects from the H-group, none were detected in the C-group.

As expected, the H-group also had thicker cIMT and fIMT with respect to the healthy control group, but there were no differences between the C-group and the healthy control group (cIMT 0.47 ± 0.05 mm and fIMT 0.39 ± 0.05 mm for the healthy control group) (Table 2).

Inflammation and endothelial function markers

vWF, fibrinogen, leukocytes, and sICAM-1 were significantly higher in the H-group. In contrast, no differences between the groups

Table 2—Mean values of carotid, femoral, and brachial ultrasound measures: FMD and IMT results and comparisons between groups

	H-group	C-group	P value
<i>n</i>	25	20	
Brachial artery measures			
Baseline brachial diameter (mm)	4.13 ± 0.76	3.80 ± 0.74	NS
Maximal FMD (%)	6.52 ± 2.92	8.62 ± 3.13	<0.05
Carotid artery measures			
Mean common carotid (cIMT, mm)	0.53 ± 0.09	0.47 ± 0.08	<0.05
Mean carotid bifurcation (mm)	0.67 ± 0.18	0.50 ± 0.07	<0.02
Mean internal carotid (mm)	0.58 ± 0.20	0.45 ± 0.09	<0.02
Mean carotid composite (cIMTcomp, mm)	0.59 ± 0.13	0.47 ± 0.07	<0.01
Subjects with carotid plaques	8/25	0	
Femoral artery measures			
Mean common femoral (fIMT, mm)	0.51 ± 0.17	0.39 ± 0.09	<0.05
Subjects with femoral plaques (%)	5/25	0	
Subjects with plaques in any carotid/femoral areas	10/25	0	

Data are means \pm SD.

were observed with respect to the basal determination of hs-CRP, soluble E-selectin, and 1β -IL (Table 3).

All previously mentioned parameters were also measured in the H-group and C-group after 30 min of hypoglycemia (nadir glucose concentrations: 39 ± 5 vs. 40 ± 4 mg/dl, NS for the H-group and C-group, respectively). There were no significant differences between the groups in changes evoked by hypoglycemia (Table 3).

In the multiple linear regression analysis, the allocation in the H-group determined cIMTcomp (β 0.082, $P < 0.02$) independently from the other covariates: age (β 0.008, $P < 0.001$), sex, disease

duration, BMI, systolic blood pressure, A1C, MAGE, and LDL cholesterol (β 0.001, $P < 0.03$). The complete model explained $\sim 73\%$ of cIMTcomp.

CONCLUSIONS— Our findings suggest that repeated hypoglycemic episodes in type 1 diabetic subjects are associated with a worse prognosis in terms of preclinical atherosclerosis profile represented not only by abnormalities in endothelial function but also by an increase in IMT in both carotid and femoral sites.

Type 1 diabetes is associated with premature arterial disease. There are some studies that have previously demonstrated endothelial dysfunction using

Table 3—Endothelial function and inflammation biochemical markers measured in both groups in euglycemia and hypoglycemia

	H-group	C-group	P value
<i>n</i>	25	20	
Euglycemia			
vWF euglycemia (%)	119 ± 29	93 ± 26	<0.02
Fibrinogen euglycemia (g/l)	2.82 ± 0.64	2.29 ± 0.44	<0.02
Leukocytes ($10^3/\mu$ l)	7.0 ± 1.8	5.6 ± 1.4	<0.05
sICAM-1 euglycemia (ng/ml)	408 ± 224	296 ± 95	<0.05
hs-CRP euglycemia (mg/dl)	0.23 ± 0.30	0.15 ± 0.17	NS
E-selectin euglycemia (ng/ml)	44 ± 21	49 ± 25	NS
1β -IL euglycemia (pg/ml)	2.92 ± 6.15	1.30 ± 2.65	NS
% increase in hypoglycemia*			
vWF	3.24 ± 12.85	16.97 ± 31.91	NS
Fibrinogen	2.84 ± 12.55	10.45 ± 23.27	NS
sICAM-1	4.15 ± 11.76	6.12 ± 13.31	NS
hs-CRP	-2.35 ± 9.02	20.35 ± 61.25	NS
Soluble E-selectin	4.74 ± 10.79	0.47 ± 11.30	NS
1β -IL	41.55 ± 143.30	33.30 ± 152.75	NS

Data are means \pm SD. *Expressed as percentage increased in each variable with respect to the baseline result.

FMD evaluation in type 1 diabetes (20). In addition, our article is not the first to demonstrate a higher mean cIMT in subjects with type 1 diabetes compared with a control group. In our study, the FMD response to ischemia was lower in subjects with type 1 diabetes compared with healthy control subjects, demonstrating early alteration of vascular function. In addition, in patients with repeated episodes of hypoglycemia, IMT (carotid and femoral) was higher than in the healthy control group. Hypertension, dyslipidemia, smoking, and urinary albumin excretion have been related to cIMT and atherosclerosis in type 1 diabetes (7). In our study, including patients with type 1 diabetes without CVD risk factors and microvascular and macrovascular complications, LDL cholesterol was also associated with the variation in cIMT.

The EDIC study showed that intensive insulin therapy slowed the increment of cIMT in type 1 diabetes (7). It has been found that acute hyperglycemia induces vascular changes and inflammatory response and alters myocardial ventricular repolarization in type 1 diabetes (21,22). Despite the assumption that glucose variability is greater than that in type 2 diabetes, data concerning whether glucose fluctuations are an independent risk factor for complications in type 1 diabetes are still controversial (23). However, it has been suggested that a high mean daily blood glucose, but not glucose variability, is related to arterial stiffness in patients with type 1 diabetes using CGM and a hyperglycemic clamp (24). In addition, Wentholt et al. (10) failed to demonstrate a relation between high glucose variability and elevated levels of a surrogate marker of vascular damage. In our study and considering all patients with type 1 diabetes as a whole, MAGE was not independently associated with cIMT.

Hypoglycemia is the most common and the most feared side effect of intensive insulin therapy and frequently is the major barrier to achieving glucose control as normal as possible (4). In the short term, the acute hemodynamic changes induced by hypoglycemia may precipitate and aggravate a vascular event during an acute episode (25). In the long term, especially if hypoglycemia is repeated, the abnormalities in coagulation, fibrinolysis, and inflammation associated with it could be related to the induction and progression of atherosclerosis. In our study, in addition to the induction of hypoglycemia unawareness, repeated episodes of

hypoglycemia were related to a worse prognosis in terms of preclinical atherosclerosis. Considering endothelial function, FMD was significantly reduced in type 1 diabetic subjects with repeated episodes of hypoglycemia compared with that in those patients without these episodes. Accordingly, coagulation markers of endothelial damage and acute-phase inflammation markers were significantly higher in the former group.

For structural changes, a comprehensive evaluation of carotid arteries gave significant higher values of IMT in subjects having repeated episodes of hypoglycemia. The multivariate regression analysis confirmed the association of repeated episodes of hypoglycemia and cIMTcomp independently of the other CVD risk factors considered. Likewise, data on IMT from femoral arteries confirmed that preclinical atherosclerosis was aggravated by repeated episodes of hypoglycemia not only in the carotid artery but also in peripheral vascular sites. It is noteworthy that in contrast to the findings with FMD, for IMT, differences with respect to the healthy control group were only significant in patients with type 1 diabetes and repeated episodes of hypoglycemia. This observation suggests that in addition to alterations in endothelial function that occur early in type 1 diabetes, recurring episodes of hypoglycemia could be considered an aggravating or accelerating factor. There is neither complete information concerning type 1 diabetes-specific determinants of vascular damage and their interrelationships nor the minimal time of exposure required for a preclinical cardiovascular alteration. Our results point to repeated episodes of hypoglycemia being considered as a new potential risk factor. The exposure to risk factor levels throughout the life span in young people promotes the accumulation of subclinical atherosclerosis, which will be transformed into CVD events, but typically not until much later in life.

As mentioned previously, there are recent studies specifically designed to address the effects of acute hypoglycemia, confirming its proinflammatory and prothrombotic effects (11,12). For some of the inflammatory markers (leukocytes and sICAM-1), we detected significantly higher values at baseline in subjects with repeated hypoglycemia. In both group of subjects with type 1 diabetes, insulin-induced hypoglycemia elicited a heterogeneous nonsignificant rise in endothelial and inflammatory markers without any

difference in the response observed with respect to the presence or absence of frequent hypoglycemia. This lack of response to provoked hypoglycemia in comparison with previous studies could be related, at least in part, to limitations of the experimental conditions of our protocol. In fact, the study of acute effects of hypoglycemia was not considered the main objective of our study, and the protocol was not designed to accurately assess mechanistic roles. As examples, we included a shorter period of hypoglycemia (30 min) before extraction and we did not control for insulin levels and the potential effect on vascular function. Moreover, and in contrast with some previous studies, we did not exclude those subjects with hypoglycemia unawareness because this clinical condition was seen in patients fulfilling our inclusion criteria. Further research is required to fully understand the link between repeated episodes of hypoglycemia and a worse prognosis in terms of preclinical atherosclerosis in type 1 diabetes. However, the putative role for endothelial and inflammatory factors in the mediation of hypoglycemia-induced vascular damage has to be taken into consideration.

In summary, in addition to the induction of hypoglycemia unawareness and an increased risk for severe hypoglycemia, repeated episodes of hypoglycemia could be related to and considered an aggravating factor for the preclinical atherosclerosis profile of type 1 diabetes.

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