

File ID 111454  
Filename Chapter 3: Hyperglycemia prevents the suppressive effect of  
hyperinsulinemia on plasma adiponectin levels in healthy humans

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SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type Dissertation  
Title Adiponectin in glucose metabolism  
Author R.M.E. Blümer  
Faculty Faculty of Medicine  
Year 2008  
Pages 197

FULL BIBLIOGRAPHIC DETAILS:

<http://dare.uva.nl/record/280036>

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# 3

## Hyperglycemia prevents the suppressive effect of hyperinsulinemia on plasma adiponectin levels in healthy humans

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## Abstract

### Objective:

Adiponectin is a fat-derived hormone with insulin-sensitizing properties. In patients with type 2 diabetes plasma adiponectin levels are decreased. Since these patients are characterized by high plasma insulin and glucose concentrations, hyperinsulinemia and hyperglycemia could be responsible for the down-regulation of adiponectin. Insulin decreases adiponectin levels in humans. The effect of hyperglycemia is unknown.

### Methods:

To determine the selective effects of insulin, glucose or its combination on plasma adiponectin, clamps were performed in six healthy males on four occasions in a cross-over design: (1=reference clamp) lower insulinemic-euglycemic clamp (insulin 100 pmol/L, glucose 5 mmol/L); (2) hyperinsulinemic-euglycemic clamp (insulin 400 pmol/L, glucose 5 mmol/L); (3) lower insulinemic-hyperglycemic clamp (insulin 100 pmol/L, glucose 12 mmol/L); (4) hyperinsulinemic-hyperglycemic clamp (insulin 400 pmol/L, glucose 12 mmol/L). Adiponectin concentrations and HMW (high-molecular-weight) to total adiponectin ratio were measured at the start and end of the 6-hour clamps.

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### Results:

After the 6-hour study period, total plasma adiponectin levels were significantly ( $p=0.045$ ) decreased by 0.63  $\mu\text{g/ml}$  in the lower insulinemic-euglycemic clamp (clamp 1). In both euglycemic groups (clamp 1+2), adiponectin concentrations significantly declined ( $p=0.016$ ) over time by 0.56  $\mu\text{g/mL}$ , whereas there was no change in both hyperglycemic groups (clamp 3+4) ( $p=0.420$ ). In neither of the clamps, the ratio of HMW to total adiponectin changed.

### Conclusions:

Insulin suppresses plasma adiponectin levels already at a plasma insulin concentration of 100 pmol/L. Hyperglycemia prevents the suppressive effect of insulin. This suggests that, in contrast to glucose, insulin could be involved in the down-regulation of plasma adiponectin in insulin resistant patients.

## Introduction

Obesity is a major risk factor for insulin resistance, diabetes and cardiovascular diseases. In recent years, adipose tissue has been shown to synthesize and secrete a variety of biologically active molecules that influence systemic metabolism. These include among others adiponectin<sup>1-3</sup>. Adiponectin is a relatively abundant circulating plasma protein with insulin sensitizing properties. In animal experiments, administration of adiponectin ameliorates glucose metabolism by enhancing glucose uptake and suppressing hepatic glucose output<sup>4,6</sup>. In addition, adiponectin knock-out mice are more insulin resistant compared to wild-type mice<sup>7,8</sup>. In plasma, adiponectin circulates as several different entities, including a HMW, a hexameric (medium-molecular-weight (MMW)) and a trimeric (low-molecular-weight (LMW)) form. The HMW oligomer has been implicated as the major active form responsible for the insulin-sensitising effects of adiponectin in the liver and peripherally. In line with this, the ratio of HMW to total adiponectin has been described to correlate better with insulin sensitivity than total adiponectin levels<sup>9</sup>.

As adiponectin is mainly synthesized and released by white adipose tissue, it may be expected that its expression in adipocytes would increase in obesity. However, plasma levels of adiponectin<sup>10-12</sup> have been shown to be reduced in subjects with obesity as well as in patients with type 2 diabetes and HIV-lipodystrophy and this is considered to contribute to the degree of insulin resistance in these diseases. The factors responsible for this counterintuitive finding in these patients have not been fully determined yet. Since hyperinsulinemia and hyperglycemia are characteristic biochemical features of these patients, insulin as well as glucose could be responsible for the down-regulation of adiponectin. Insulin's inhibiting effect on adiponectin gene expression and plasma levels has been shown in several in vitro and in vivo studies<sup>13-15</sup>. However, additional factors have to be involved, as in the more advanced stages of type 2 diabetes, associated with decreased plasma insulin levels, adiponectin concentrations remain low<sup>16</sup>. Hypothetically, hyperglycemia could be involved in the low adiponectin levels under these circumstances. Indeed, several studies associated low adiponectin concentrations with poor glycemic control and high dietary glyceamic load in diabetic men<sup>17,18</sup>.

The effects of hyperglycemia per se and/or in combination with hyperinsulinemia on plasma adiponectin levels in human subjects have not yet been studied. We hypothesize that hyperglycemia, in addition to hyperinsulinemia, results in a (further) decline in plasma adiponectin levels. To test this hypothesis, we performed a controlled cross-over study in six healthy males on four occasions with plasma levels of insulin and glucose

aimed at respectively: (1) insulin 100 pmol/L, glucose 5 mmol/L; (2) insulin 400 pmol/L, glucose 5 mmol/L; (3) insulin 100 pmol/L, glucose 12 mmol/L and (4) insulin 400 pmol/L, glucose 12 mmol/L. Total and HMW oligomer plasma adiponectin levels were measured at baseline and at the end of the 6-hour clamps.

## Methods

### Subjects

We studied 6 healthy, non smoking, male volunteers (age  $21.7 \pm 1.2$  years; weight  $73.2 \pm 4.8$  kg; body mass index:  $21.8 \pm 0.9$  kg/m<sup>2</sup>). None of them used medication or had a positive family history of diabetes. All volunteers had normal plasma values of fasting glucose ( $4.6 \pm 0.2$  mmol/L), insulin ( $33 \pm 9$  pmol/L), erythrocyte sedimentation rate, complete blood count, lipid profile, renal and hepatic function and all had a normal oral glucose tolerance test, according to the American Diabetes Association criteria <sup>19</sup>. The study was approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam and all subjects gave written informed consent. The present study was part of a study on effects of insulin and hyperglycemia on different parameters <sup>20, 21</sup>. The data in the present study, except for the glucose and insulin levels, have not been published before.

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### Study Design

The study protocol had a crossover design with a wash-out period of 4 weeks. The sequence of the 4 different clamps was chosen at random in all subjects. Each volunteer served as his own control and was studied on four occasions with plasma levels of insulin and glucose aimed at respectively: (1=reference clamp) lower insulinemic-euglycemic clamp (insulin 100 pmol/L, glucose 5 mmol/L); (2) hyperinsulinemic-euglycemic clamp (insulin 400 pmol/L, glucose 5 mmol/L); (3) lower insulinemic-hyperglycemic clamp (insulin 100 pmol/L, glucose 12 mmol/L) and (4) hyperinsulinemic-hyperglycemic clamp (insulin 400 pmol/L, glucose 12 mmol/L).

For three days prior to the study, all volunteers consumed an isocaloric diet containing at least 250 g of carbohydrates with a maximum of 20% disaccharides. After an overnight fast subjects were admitted to the clinical research unit and confined to bed. The study started with placement of a catheter into an antecubital vein for infusion. Another catheter was inserted into a contra-lateral hand vein, kept in a thermo-regulated (60 °C) box for sampling of arterialized venous blood. Saline (NaCl 0.9 %) was infused with a slow drip

(30 ml/hr) to keep the catheters patent. At T=0 (9 AM) infusions of somatostatin (250 µg/h; Somatostatine-ucb; UCB Pharma B.V., Breda, the Netherlands) and glucagon (1 ng/kg • min; GlucaGen; Novo Nordisk, Alphen aan den Rijn, the Netherlands) were started to respectively suppress endogenous insulin and to replace the by somatostatin suppressed endogenous glucagon secretion. Concurrently infusions of insulin (Actrapid/L; Novo Nordisk) at a rate of 10 or 40 mU/m<sup>2</sup> body surface area (BSA) • min (aimed at plasma insulin levels of 100 and 400 pmol/L, respectively) and glucose 10 or 20 % at a variable rate to obtain eu- or hyperglycemia were started as well. All infusions were administered by calibrated syringe pumps (Perfusor fm, Braun, Melsungen AG, Germany). To maintain glucose concentrations at 5 or 12 mmol/L from T= 0 until T= 6, every 5 minutes plasma glucose concentration was measured on a Beckman glucose analyzer 2 (Beckman, Palo Alto, CA). At T=0 and every ten minutes from T= 5:40 till T= 6:00 blood samples were drawn for determination of insulin levels. Blood samples were drawn for measurement of concentrations of cortisol, catecholamines, glucagon, FFA (free fatty acid), growth hormone and total adiponectin as well as for the HMW to total adiponectin ratio immediately before and at the end of the infusions (T = 0 and T = 6, respectively). Blood samples were kept on ice immediately after collection and subsequently centrifuged for 10 minutes at 3000 rpm at 4 °C. All plasma samples were stored below -20 ° C.

### Assays

Plasma insulin, cortisol, glucagon, catecholamines and FFA levels were measured as described before <sup>22</sup>. Plasma adiponectin concentrations were measured in duplicate by RIA (Linco Research, St. Charles, MO): intra-assay CV 4-6 %; inter-assay CV 6-9 %; detection limit 0.5 µg/mL. The HMW to total adiponectin ratio was measured in duplicate by gel electrophoresis and western blot <sup>23</sup>. Human growth hormone (somatotropin) was determined with a chemiluminescent immunometric assay (Advantage, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA).

### Calculations and statistical analysis

Data were checked for normal distribution (Shapiro-Wilk test) and equal variances (Levene's test) using the residuals. Depending on the results of these tests, data were analyzed either parametrically or non-parametrically. Results are presented as mean ± SD. To analyse differences in basal plasma glucose and insulin concentrations between the 4 clamps, a repeated measures ANOVA was used. Glucose and insulin levels at the end of the 6h study period were compared between clamps using a repeated measures ANOVA with correction for baseline levels. To test whether plasma adiponectin levels

changed between baseline and after 6 hours of infusion in clamp 1, a paired t-test was used. The effects of hyperinsulinemia, hyperglycemia and their interaction on the change of adiponectin levels between T = 0 and 6 hours were analyzed by a repeated measures ANOVA with correction for baseline adiponectin concentrations. Changes in plasma adiponectin levels over time are presented as mean change (95% confidence interval (95% CI)). Correlations between changes in plasma adiponectin levels and changes in the other parameters between T=0 and T=6 hours were analyzed by Spearman's correlation coefficient. A sample size of six subjects would suffice to have 80% power to detect an absolute difference in adiponectin levels of 0.55  $\mu\text{g}/\text{mL}$  (~10%), assuming a standard deviation of the mean difference of 0.4  $\mu\text{g}/\text{mL}$  and using a paired t-test with a two-sided alpha of 0.05. SPSS statistical software version 12.0.1 (SPSS Inc, Chicago, IL, USA) was used to analyze the data.

## Results

### Glucose and insulin levels (table 1)

Basal plasma glucose and insulin concentrations were not significantly different between the 4 clamps. In all 4 clamps target levels of glucose and insulin were reached. During the first hour glucose levels rapidly increased towards target levels in both hyperglycemic groups and remained constant in both euglycemic groups. Glucose concentrations at the end of the 6h clamp were significantly higher in both clamps 3 and 4 ( $12.2 \pm 0.5$  and  $12.4 \pm 0.1$  mmol/L, respectively) compared to clamp 1 and clamp 2 ( $5.1 \pm 0.1$  and  $5.0 \pm 0.2$  mmol/L, respectively) ( $p < 0.0001$ ). The glucose levels in neither clamp 3 vs. clamp 4

**Table 1** Adiponectin, glucose and insulin

	Clamp 1		Clamp 2		Clamp 3		Clamp 4	
	T=0	T=6	T=0	T=6	T=0	T=6	T=0	T=6
Adiponectin ( $\mu\text{g}/\text{mL}$ )	5.4 $\pm$ 1.5	4.8 $\pm$ 1.0*	5.5 $\pm$ 1.9	5.0 $\pm$ 1.9†	5.7 $\pm$ 1.2	5.5 $\pm$ 1.3	5.5 $\pm$ 1.8	5.3 $\pm$ 1.3
HMW: total adiponectin ratio	0.13 $\pm$ 0.08	0.17 $\pm$ 0.12	0.16 $\pm$ 0.08	0.21 $\pm$ 0.12	0.12 $\pm$ 0.10	0.16 $\pm$ 0.09	0.16 $\pm$ 0.07	0.19 $\pm$ 0.12
Glucose (mmol/L)	5.2 $\pm$ 0.2	5.1 $\pm$ 0.1	5.2 $\pm$ 0.2	5.0 $\pm$ 0.2	5.2 $\pm$ 0.2	12.2 $\pm$ 0.5‡	5.0 $\pm$ 0.4	12.4 $\pm$ 0.1‡
Insulin (pmol/L)	39 $\pm$ 9	89 $\pm$ 6	39 $\pm$ 9	408 $\pm$ 61#	42 $\pm$ 18	127 $\pm$ 19##	32 $\pm$ 8	443 $\pm$ 34#

Values are given as mean  $\pm$  SD, \*  $P < 0.05$  vs. T=0 (clamp 1), †  $P < 0.02$  vs. T=0 (both clamp 1+2), ‡  $p < 0.0001$  vs. clamp 1 and 2, #  $p < 0.0001$  vs. clamp 1 and 3, ##  $p < 0.01$  vs. clamp 1

T=0: start of the clamp, T=6: end of the 6h clamp

- clamp 1: insulin 100 pmol/L, glucose 5 mmol/L

- clamp 2 : insulin 400 pmol/L, glucose 5 mmol/L

- clamp 3: insulin 100 pmol/L, glucose 12 mmol/L

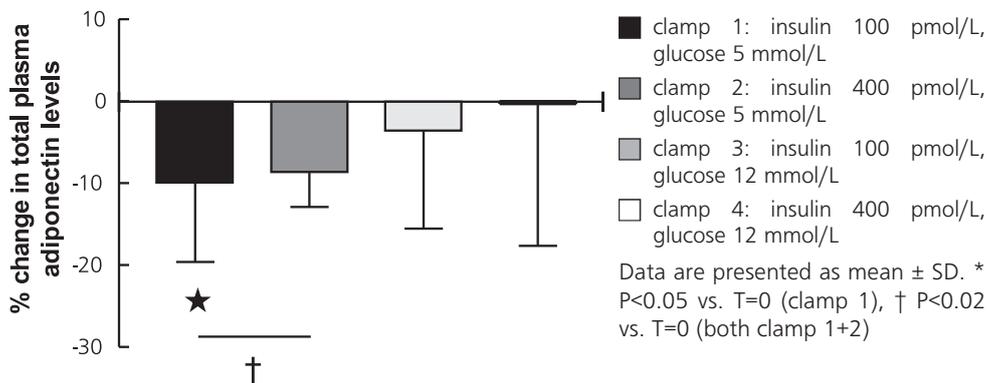
- clamp 4: insulin 400 pmol/L, glucose 12 mmol/L

nor in clamp 1 vs. clamp 2 differed at T=6 hours. Insulin levels were significantly higher at the end of the 6-hour study period in clamp 2 and clamp 4 ( $408 \pm 61$  and  $443 \pm 34$  pmol/L, respectively) compared to both clamp 1 and clamp 3 ( $89 \pm 6$  and  $127 \pm 19$  pmol/L, respectively) ( $p < 0.0001$ ). Between clamp 1 and clamp 3 at T=6 hours, there was a small difference in insulin levels, due to incomplete suppression of the hyperglycemia induced stimulation of endogenous insulin secretion ( $p < 0.05$ ).

### Adiponectin levels (table 1 and figure 1)

After 6 hours of infusion, total plasma adiponectin concentrations were significantly decreased compared to basal levels by  $0.63 \mu\text{g/mL}$  ( $p = 0.045$ , 95% CI: 0.02 - 1.24; 10% decrease) in the lower insulinemic-euglycemic clamp (reference clamp=clamp 1). The HMW to total adiponectin ratio did not change significantly during clamp 1 (0.04, 95% CI: -0.15 - 0.06). The mean decline in total plasma adiponectin levels over time was not different between the relatively low insulin and the high insulin groups ( $0.42 \mu\text{g/mL}$  vs.

Figure 1. Change in total plasma adiponectin levels (%) from the start till the end of the 6h-clamp in the 4 groups:



$0.32 \mu\text{g/mL}$ , mean difference of 0.10, 95% CI: -0.31 - 0.52). During the 6h clamp, there was a significant decrease ( $p = 0.016$ ) in total plasma adiponectin concentrations of  $0.56 \mu\text{g/mL}$  in both euglycemic groups (clamp 1+2) (95% CI: 0.10 - 1.01), whereas there was no difference ( $p = 0.420$ ) in both hyperglycemic groups (clamp 3+4) ( $0.19 \mu\text{g/mL}$ , 95% CI: -0.27 - 0.64). During the clamp, there was no significant change in the HMW to total adiponectin ratio in both euglycemic groups (0.05, 95% CI: -0.06 - 0.15), nor in both hyperglycemic groups (0.03, 95% CI: -0.07 - 0.14).

## Glucoregulatory hormones and FFA

In all clamps, there were no significant correlations between the changes over time in plasma adiponectin levels and the changes over time in plasma concentrations of glucagon ( $r = -0.13$ ,  $p=0.6$ ), growth hormone ( $r = -0.20$ ,  $p=0.4$ ), norepinephrine ( $r = 0.08$ ,  $p=0.7$ ), epinephrine ( $r = -0.11$ ,  $p=0.6$ ), cortisol ( $r = 0.09$ ,  $p=0.7$ ) or FFA ( $r = 0.21$ ,  $p=0.3$ ).

## Discussion

Plasma adiponectin levels are unexplained low in patients with type 2 diabetes and HIV-lipodystrophy. Since these patients often have hyperinsulinemia as well as hyperglycemia, insulin, glucose or the combination of both could be responsible for this decline. The present study for the first time describes the influence of hyperglycemia, hyperinsulinemia and its combination on plasma adiponectin concentrations in healthy male humans, in a study design in which each subject served as his own control. Insulin lowered plasma adiponectin levels, while hyperglycemia prevented this decline in healthy humans.

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Several studies investigated the effects of insulin on adiponectin production and secretion in vitro. Most, but not all <sup>13</sup>, in vitro studies found a stimulating effect on adiponectin gene expression <sup>24-26</sup> or secretion <sup>27, 28</sup> in 3T3-L1-adipocytes as well as in human adipocytes. Additionally, during hyperinsulinemic-euglycemic clamps in rats and healthy human subjects, the expression of adiponectin in respectively visceral and subcutaneous fat was moderately increased as well <sup>29, 30</sup>. In contrast to these in-vitro data on adiponectin expression and secretion, insulin lowered human adiponectin levels in plasma. Hyperinsulinemic-euglycemic clamp studies in lean subjects reduced total plasma adiponectin levels by 10-20% <sup>14, 15, 31</sup>. The increased total plasma adiponectin concentrations in patients with type 1 diabetes, specifically in those who are C-peptide-negative, could be in line with this inhibiting effect of insulin <sup>32, 33</sup>. Congruent with these human studies, we showed a 10% drop in total plasma adiponectin levels after six hours of infusion in clamp 1 with insulin levels aimed at 100 pmol/L. Since the mean decrease in plasma adiponectin levels over time was not different between the low and the high insulin groups, our data suggest that insulin has a maximal inhibiting effect on total adiponectin levels at concentrations of  $\geq 100$  pmol/L. A dose-independent negative effect on plasma adiponectin levels of insulin above 100 pmol/L has been described before <sup>15</sup>. Recently, a study reported that in nondiabetic subjects, hyperinsulinemia resulted in a reduction of HMW adiponectin levels as well as of the HMW to total adiponectin ratio <sup>34</sup>. However, a distinction between the selective effects of insulin and glucose

on adiponectin levels was impossible to make as only hyperinsulinemic-hyperglycemic clamps were performed in that study. In the present study, we did not find a difference in the HMW to total adiponectin ratio after six hours of infusion in the lower insulinemic-euglycemic clamp. This indicates that in healthy subjects, hyperinsulinemia per se does not primarily affect HMW adiponectin levels.

Besides insulin, other factors could have regulated adiponectin levels as well. Since there are no data on the effects of somatostatin, we can not exclude a role for somatostatin in the declined adiponectin levels. No significant correlations were found between changes over time in the levels of plasma adiponectin and changes over time in the plasma levels of glucagon, growth hormone, norepinephrine, epinephrine, cortisol nor FFA. Therefore these hormones do not seem to be involved in the decrease of adiponectin. As adiponectin does not have a circadian rhythm, it is unlikely that this phenomenon is responsible for our findings in the euglycemic clamps<sup>35</sup>. Therefore high insulin concentrations in patients with type 2 diabetes may be involved in down-regulating plasma adiponectin, although the effect of insulin on the long run remains unknown.

Between the initiation and the end of the 6h study period, plasma adiponectin concentrations significantly declined in both euglycemic groups, whereas there was no significant change in the hyperglycemic groups. These data suggest that the inhibiting effect on total plasma adiponectin levels of hyperinsulinemia is counteracted by hyperglycemia. The effect of hyperglycemia per se on adiponectin levels in human subjects has not been described previously. One study reported on the effects of hyperglycemia on adiponectin expression and plasma concentrations in rats<sup>29</sup>. A five hour clamp was performed with infusion of dextrose as well as somatostatin to achieve high glucose and low insulin levels, respectively. This clamp resulted in enhanced adiponectin expression in visceral adipose tissue, whereas plasma levels remained constant. In addition to the study in rats, we investigated the effect of hyperglycemia on plasma adiponectin levels during hyperinsulinemia, known to inhibit these levels. The common denominator in both studies is the absence of an absolute increase in plasma adiponectin levels. Therefore, it can be hypothesized that hyperglycemia per se does not increase plasma adiponectin, but only compensates in case of decreased adiponectin levels. Although the effects of hyperglycemia in absence of hyperinsulinemia remain unknown in human subjects, the relative increase in adiponectin levels during the hyperglycemic clamps could function as an adaptive mechanism to restrain glucose levels. In accordance with this hypothesis are the increased plasma adiponectin levels in human subjects with an acute severe infection, which is associated with insulin resistance and hyperglycemia<sup>36, 37</sup>. The mechanisms

by which acute hyperglycemia prevents the suppressive effect of hyperinsulinemia on plasma adiponectin levels remain to be elucidated.

There was no difference in the HMW to total adiponectin ratio neither during the euglycemic nor during the hyperglycemic clamps in the present study. This result is partly in contrast to the earlier described study, which reported a decline in the HMW to total adiponectin ratio during a hyperinsulinemic-hyperglycemic clamp in nondiabetic subjects<sup>34</sup>. The reason for this difference in study results may be related to the differences in study design as insulin levels (700 pmol/L in<sup>34</sup> vs. 400 pmol/L in the present study) and duration of the clamp (7h vs. 6h, respectively).

In the present study, we investigated the acute (6h) regulation of plasma adiponectin levels by insulin and glucose in healthy, insulin sensitive subjects. Our data can not fully explain the low adiponectin levels in diabetic patients, as those levels are usually much lower than can be explained by the degree of suppression by insulin found in our study. Additionally, in those subjects the concomitant hyperglycemia should have (partly) prevented the suppressive effect of insulin. Apparently, other regulatory mechanisms are involved as well in the low adiponectin levels in the chronic abnormalities of glucose regulation.

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In conclusion, insulin suppresses plasma adiponectin levels already maximally at a plasma insulin concentration of 100 pmol/L. Hyperglycemia prevents the suppressive effect of insulin on plasma adiponectin levels. This suggests that, in contrast to hyperglycemia, hyperinsulinemia could be involved in the down-regulation of plasma adiponectin in insulin resistant patients.

### Acknowledgments

We thank An Ruiter and Barbara Voermans from the department of Clinical Chemistry, Laboratory of Endocrinology and Radiochemistry for their excellent analytical assistance. This work was supported by a grant from the Dutch Diabetes Research Foundation (grant number 2002.00.008).

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