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Research Article

# Impact of Short-Acting Insulin Analogs on Biomarkers of Oxidative Stress and Chronic Systemic Inflammation in Patients with Type 2 Diabetes - Results from a Pilot Study for Hypothesis Generation

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## Abstract

Primary objective of this pilot study was to collect data for hypothesis generation regarding the impact of short-acting insulin analogs (insulin aspart/IA and insulin glulisine/IG in comparison to regular human insulin/RHI) on biomarkers of inflammation and oxidative stress in patients with type 2 diabetes. Twelve patients on basal insulin and oral drug treatment (11 male, age: 64 ± 9 yrs., HbA1c: 7.1 ± 0.6%, BMI: 31.5 ± 4.9 kg/m2) were randomized to intensive insulin therapy with insulin glargine and either IA, IG, or RHI. An OGGT was performed at baseline and after six months with assessment of nitrotyrosine, hsCRP, microcirculation (laser doppler) and mRNA macrophage activation markers (IL6, TNFalpha, eNOS, MAPK) after 0/1/2h. Reduction of HbA1c to near normal levels, and similar glucose excursions and microcirculation results were seen in all three groups. At endpoint, reductions in nitrotyrosine were observed with both analogs (-33 %) but not with RHI (+31 %, $p < 0.05$ vs. the combined analogs. IL-6 expression decreased with IG (-5 %) but increased with IA (+142%) and RHI (+64 %). TNFalpha expression decreased in all three groups (IG: -35%, IA: -71%, RHI: -30%). Vasoprotective eNOS expression increased with IG (+25 %) and decreased with IA (-28 %; RHI: +1 %). MAPK1-expression was reduced in all three groups (-16%, -23%, -33%). hsCRP was reduced by -78 % with IG only (IA: -2%, RHI: - 11%, $p < 0.05$ vs. IG in both groups). In this pilot study, improvement of the inflammatory vascular situation could be observed consistently with IG, partly with IA and barely with RHI.

***Keywords:*** *Insulin Analogs; Biomarkers; Oxidative Stress; Chronic Systemic Inflammation; Type 2 Diabetes*

## Introduction

Current treatment guidelines for type 2 diabetes recommend individualized treatment targets with HbA1c levels of < 6.5%, if complicating factors, such as age, existing complications, risk of future complications or risk of hypoglycaemia, are not suggesting different goals [1,2]. Insulin treatment to supplement the relative insulin deficiency is one of the key therapeutic approaches to achieve near normal glycemic control and different treatment strategies are available with and without combination with oral anti-diabetic drugs. The pharmacokinetic profile of subcutaneously injected regular human insulin, which is initially present as a hexameric macromolecule, with a slow onset of action and prolonged duration of action is suboptimal for efficient treatment of postprandial glucose excursions [3]. Several

modified rapid-acting insulin analogues have been introduced to address these deficiencies [4-7]. The amino acid sequence modifications in all three commercialized analogues (insulin aspart, insulin lispro, and insulin glulisine) lead to faster formation or the stable presence of dimers and monomers in the subcutaneous tissue resulting in faster absorption and faster onset of action in healthy subjects and people with diabetes [8-10]. In contrast to regular human insulin, insulin aspart, and insulin lispro, insulin glulisine is more stable and can be provided in a zinc-free formulation [6,11].

Some studies have addressed the question, whether there are differences between these analogs with respect to the pharmacokinetic profiles after s.c. injection in different patient populations. In comparison to insulin lispro, it has been shown that insulin glulisine shows a faster absorption in obese people with and without type 2 diabetes [12,13], most probably because of the lack of Zn-ions in the formulation.

It is currently assumed that all short-acting insulin analogs are more or less comparable with respect to their clinical effectiveness and safety, however, a comparative pharmacodynamic and pharmacokinetic test meal study in insulin-naive obese subjects with type 2 diabetes treated with similar doses in a cross-over protocol resulted in lower postprandial glucose levels (within the first hour) and higher insulin levels for insulin glulisine in comparison to insulin aspart [13]. These results are supported by previous euglycemic clamp studies indicating a faster onset of action for insulin glulisine in healthy volunteers in direct comparison with insulin aspart [14] and insulin lispro [12].

While all these trials have focussed on the glucose lowering insulin activity, it is well known that insulin also exerts vascular effects when binding to its specific endothelial receptors that are independent from its metabolic effects [15]. After binding to its vascular receptors under physiological conditions, insulin induces activation of endothelial nitric oxide synthase (eNOS). The consecutive secretion of nitric oxide (NO) results in vasodilatation, increases microcirculatory blood flow, enhanced erythrocyte deformability, and protection of the endothelial cells against oxidative reactants in the blood stream [15,16]. We have been able to demonstrate that a faster onset of action after s.c. administration is associated with a more pronounced vasodilatory effect in insulin-naive patients with type 2 diabetes [17]. Some clinical and laboratory biomarkers are associated with nitric-oxide formation in the endothelium, including but not limited to nitrotyrosine, asymmetric dimethyl-arginine (ADMA), and proinflammatory cytokines [18,19]. Under physiological conditions, insulin is secreted by the ß-cell in a pulsatile fashion with 8 - 12 pulses per hour, and it has been identified that the fast increases and decreases of the plasma insulin concentrations are the key trigger mechanism to maintain the sensitivity of the insulin receptors on the endothelial cells [16,20]. It is therefore tempting to speculate that a faster increase of insulin absorption after administration of short acting insulin analogs may have a more beneficial impact on microcirculation and chronic systemic inflammation than treatment with regular human insulin.

## Purpose of the Study

The purpose of this exploratory pilot study was to investigate whether the faster onset of action of insulin glulisine and insulin aspart in comparison to regular human insulin may translate into measurable differences in one or more of previously identified clinical or laboratory biomarkers indicative for vascular insulin action in patients with type 2 diabetes with stable basal insulin and oral drug treatment. The ultimate goal was to identify potentially suitable biomarkers and to perform an appropriate statistical power analysis for a future confirmatory trial.

## Patients and Methods

This prospective parallel randomized pilot study was performed in accordance with the ethical and regulatory standards as set forth by the Declaration of Helsinki and Good Clinical Practice guidelines. The study was approved by the responsible Ethical Review Board and all participants signed informed consent before any study procedure was performed. Subjects could be included if they had a known type 2 diabetes treated with a stable basal insulin therapy (with or without an additional antidiabetic drug) for a minimum of three months, an

HbA1c < 8.5% [69 mmol/mol], were between 30 to 75 years of age, and had a BMI < 40 kg/m$^2$. Patients were excluded if they had a hsCRP value > 10 mg/dL (indicating an acute inflammatory process), treatment with thiazolidinediones in the three months prior to the study, retinopathy, renal or hepatic failure, or any other serious or life-threatening disease, history of allergies or hypersensitivity to the study drugs, and other conditions preventing them from a successful trial participation as decided by the investigator.

This was a prospective, randomized, parallel mono-centric trial with three treatment arms and a duration of six months. After screening, patients were randomized to intensive insulin therapy with insulin glargine as basal insulin and with regular human insulin, insulin aspart or insulin glulisine as prandial insulin. Regular study visits occurred in 4-week intervals until the final visit at week 24. Safety variables were hypoglycemic events (values < 3.5 mmol/L, severe hypoglycemia: requiring external help), adverse events and laboratory safety parameters.

### Laboratory methods

The aim of the study was to investigate and compare the effects of insulin glulisine, insulin aspart and regular human insulin on postprandial nitrotyrosine concentrations (ELISA, Millipore, Cologne) after an oral glucose challenge. Several other clinical and laboratory markers of postprandial endothelial cell function, sub-clinical inflammation and cardiovascular risk were assessed in parallel: HbA1c (HPLC-method, Adams, Stadt), fasting glucose (Hitado Super GL, Stadt), insulin (CLIA, Millipore, Cologne), hsCRP (turbidimetry, Falcor, Menarini, Neuss), Matrix-Metalloproteinase-9 (MMP9, ELISA, RandD Systems, Hamburg), adiponectin and intact proinsulin (both ELISA, TecoMedical, Heidelberg). In addition, blood for monocyte/macrophage isolation was drawn and monocyte/macrophages were isolated by magnetic bead protocols directly after sampling. Macrophage activation was determined by isolation and quantification of mRNA expression markers (Interleukin-6, tumor necrosis factor-α), MMP9, NFkB (p105 and relA),NFkB-inhibitors (IkB-alpha and IkB-beta), eNOS, MAPK1 and MAPK3) as described previously [21].

### Microcirculation assessment

Skin blood flow was measured with laser-doppler fluxmetry. This technique allowed for simultaneous, non-invasive measurement of microvascular blood flow by laser-doppler fluxmetry (LDF) and for the measurement of hemoglobin content and oxygenation in the skin and the superficial muscle tissue by spectrometric determination (O2C, Lea Medizintechnik, Giessen, Germany). The skin probe transmits continuous wave laser light and white light to the skin tissue where it is scattered and collected at the skin surface at fibres in the probe. In this study, the measurements were performed at the lateral aspect of metacarpale II of one hand. After attaching the LDF probe to the skin, the position of the probe was kept constant for the overall glucose challenge procedure.

### Statistics

The statistical analysis and generation of tables and individual data listings were performed using the Microsoft Office 2007® software package and SPSS® software package under the Microsoft Windows 7® operating system using SPSS® software version 19.0 (Hersteller, Stadt). Data were analyzed in an exploratory sense. Exploratory interferential statistical evaluation of the efficacy variables was based on standard statistical procedures. P-values were calculated using the Mann-Whitney-U test (comparisons between the treatment groups) or the Wilcoxon signed-rank test (comparisons within each treatment group). A p-value less than or equal to 5% (significance level = 0.05) was considered to indicate a statistically significant difference. As vascular assessment results from studies investigating insulin glulisine vs. insulin aspart are missing, it was not possible to calculate sample size and statistical power. Therefore, the goal of the HERMES-Pilot-Study was to generate preliminary data for statistical considerations and estimations on the probability of success of a future larger confirmatory trial.

## Results

Twelve patients participated into the study and eleven completed the protocol. The drop-out happened by patient decision of one subject in the regular human insulin arm because of unsatisfactory glycemic control and a pulmonary embolism leading to hospitalization. All remaining eleven patients completed the study per protocol and were included into the per-protocol analysis (10 male, 1 female, age: 64 ± 9 yrs., BMI: 31.5 ± 4.9 kg/$m^2$, disease duration: 11 ± 4 yrs., HbA1c: 7.0 ± 0.6 % [53 ± 5 mol/mol]).

The primary objective was the difference in the percent increase of the oxidative stress biomarker nitrotyrosine after stimulation with a standardised meal. At baseline, comparable postprandial nitrotyrosine levels were seen at all groups. After 6 months, differences were seen between the groups with respect to the postprandial time-course of nitrotyrosine levels. The AUCs for the postprandial nitrotyrosine concentrations in the three treatment arms in the course of the study are provided in figure. Mean absolute relative change of $AUC_{0\text{-}120}$ from baseline to endpoint was -4.4 % with glulisine, -59.2% with aspart, and +31.3% with regular human insulin. The absolute relative change in AUC did not differ statistically significant because of the small size of the treatment groups. However, analyzing the two insulin analogs jointly in comparison to the small regular human insulin group lead to a statistically significant difference even under these pilot conditions $p < 0.05$).

The concentrations of other secondary laboratory markers at baseline and endpoint in the three groups is provided in table 1. Most likely because of the tight titration regimen, the mean HbA1c values improved in the course of the study and normal to near-normal HbA1c levels were attained at endpoint in all treatment groups. Glucose excursions during the meal tolerance tests were comparable between the three treatment arms, while insulin levels were highest with regular human insulin and lowest with insulin glulisine. Patients with insulin glulisine presented also with the lowest AUCs for the intact proinsulin concentrations. Mean adiponectin levels increased in all treatment groups and approximated normal values, especially in the two rapid-acting analog treatment arms. The mean values of hsCRP, an accepted biomarker for cardiovascular risk, were substantially reduced by insulin glulisine (-77.1%), were stable with insulin aspart (-2.3%) and decreased slightly with regular human insulin (-11%). These changes were statistically significant between insulin glulisine and both insulin aspart ($p < 0.05$) and regular human insulin ($p < 0.05$). No significant differences were detected between the three groups for the absolute MMP-9 concentrations or the changes in this biomarker.

| Biomarker | Insulin | Baseline Month 0 | Endpoint Month 6 |
|---|---|---|---|
| HbA1c [%]/[mmol/mol] | IG | 6.7 ± 0.7/50 ± 6 | 6.0 ± 0.4/42 ± 4 |
| | IA | 7.0 ± 0.7/53 ± 6 | 6.8 ± 0.9/51 ± 8 |
| | RHI | 7.3 ± 0.3/56 ± 3 | 6.7 ± 0.3/50 ± 3 |
| Glucose [mmol/L] | IG | 7.3 ± 1.5 | 7.5 ± 1.9 |
| | IA | 8.1 ± 2.2 | 7.9 ± 2.1 |
| | RHI | 7.3 ± 1.4 | 6.3 ± 0.4 |
| Insulin [pmol/L] | IG | 23 ± 9 | 33 ± 16 |
| | IA | 68 ± 70 | 36 ± 5 |
| | RHI | 72 ± 3 | 170 ± 172 |
| Intact Proinsulin [pmol/L] | IG | 2.7 ± 2.8 | 2.3 ± 2.9 |
| | IA | 8.6 ± 5.6 | 5.2 ± 1.5 |
| | RHI | 5.8 ± 4.3 | 4.9 ± 2.5 |

| | | | |
|---|---|---|---|
| Nitrotyrosine [nM/L] | IG | 70 ± 26 | 52 ± 37 |
| | IA | 68 ± 24 | 22 ± 16 |
| | RHI | 55 ± 25 | 90 ± 42 |
| hsCRP [mg/L] | IG | 6.3 ± 3.9 | 1.3 ± 0.8 |
| | IA | 2.6 ± 2.7 | 2.4 ± 2.7 |
| | RHI | 3.1 ± 1.8 | 3.1 ± 2.8 |
| MMP-9 [µg/L] | IG | 366 ± 95 | 386 ± 45 |
| | IA | 485 ± 192 | 607 ± 245 |
| | RHI | 371 ± 123 | 314 ± 65 |
| Adiponectin [mg] | IG | 4.8 ± 1.9 | 6.1 ± 3.2 |
| | IA | 3.5 ± 0.9 | 5.0 ± 2.0 |
| | RHI | 3.2 ± 1.0 | 3.7 ± 2.1 |

***Table 1:*** *Results of the protein biomarker assessments at baseline and endpoint (IG: Insulin Glulisine; IA: Insulin Aspart; RHI: Regular Human Insulin.*

The mRNA expression analysis revealed reductions in the expression of the proinflammatory cytokine TNF-alpha and the atherogenic signal transduction proteins MAPK1 and MAPK3 in all three groups. The percent changes of all measured mRNA biomarkers are provided in figure. While there seems to be an indication of more pronounced effects with insulin glulisine, none of these parameters differed significantly between the groups.

Skin blood flow was determined using Laser-doppler fluxmetry, which allowed for simultaneous, non-invasive measurement of microvascular blood flow and for the measurement of haemoglobin content and oxygenation in the skin and the superficial muscle tissue by spectrometry with analysis of microvascular haemoglobin oxygenation ($SO_2$ in %) and skin microvascular blood flow (Flow in arbitrary units, AU). The measurements were performed at 0 and 120 minutes during the oGTT and baseline and post-ischemic values were determined. All measurements were performed with a shallow and deep layer assessment of the skin. The results are provided in table 2. Tissue oxygen pressure remained unchanged. There was a tendency for improved blood flow both in the shallow and the deep skin layers with the two insulin analogs after 6 months, while the flow rates decreased with regular human insulin.

| | Visit [month] | Time [min] | IG | IA | RHI |
|---|---|---|---|---|---|
| $SO_2$ shallow (post-ischemic-baseline) [%] | 0 | 0 | 22 | 27 | 25 |
| | | 120 | 17 | 36 | 28 |
| | 6 | 0 | 14 | 32 | 0 |
| | | 120 | 16 | 22 | 24 |
| Flow shallow (post-ischemic-baseline) [AU] | 0 | 0 | 73 | 49 | 54 |
| | | 120 | 72 | 50 | 83 |
| | 6 | 0 | 71 | 42 | 34 |
| | | 120 | 83 | 58 | 59 |
| $SO_2$ deep (postischemic-baseline) [%] | 0 | 0 | 5 | 6 | 2 |
| | | 120 | 3 | 6 | 6 |
| | 6 | 0 | 3 | 6 | 0 |
| | | 120 | 2 | 1 | 1 |
| Flow deep (postischemic-baseline) [AU] | 0 | 0 | 83 | 72 | 74 |
| | | 120 | 83 | 67 | 100 |
| | 6 | 0 | 87 | 77 | 32 |
| | | 120 | 90 | 76 | 58 |

***Table 2:*** *Results of the microcirculation assessments in shallow and deep skin layers after ischemic challenge during the oral glucose tolerance tests. Values are corrected for baseline without ischemia (IG: Insulin Glulisine; IA: Insulin Aspart; RHI: Regular Human Insulin).*

The reported numbers of hypoglycemic events were very small in this study. There were 0.29 events per patient/month with glulisine (insulin aspart: 0.17, regular human insulin: 0.22). A total of 50 adverse events occurred in this study, including one serious adverse event (insulin glulisine: 14 events, insulin aspart: 17 events, regular human insulin 19 events). The serious event was a pulmonary embolism with hospitalization leading to early study termination for this patient in the regular human insulin arm. This event was classified as "not related" to the study drug. Most common adverse events were common cold (17 events, 34% of all adverse events) and hypoglycemia (15 events, 30%).

## Discussion and Conclusion

Recent publications have indicated that next to improving postprandial glycemia, short-acting insulin analogs may almost normalize postprandial microcirculation as assessed by laser-doppler fluxmetry [22,23]. In addition, insulin Glargine has been shown to also improve endothelial function in patients with type 2 diabetes mellitus [24]. However, data comparing the clinical and molecular endothelial effects of a combination treatment of Insulin Glulisine and Insulin Glargine in comparison to Insulin Aspart and Insulin Glargine or regular human insulin and Insulin Glargine is missing. In our pilot study, the intensive insulin treatment lead to changes in a variety of vascular and inflammatory biomarkers, which were more or less pronounced in the three treatment groups. There were more indications with respect to vasoprotective changes with the two rapid-acting insulin analogs than with regular human insulin. In addition, there appears to be a more favorable vascular risk reduction profile with insulin glulisine in comparison to insulin aspart. It is known that insulin binding to its endothelial receptor may induce positive (eNOS activation) or negative (MAPK activation) effects in the endothelial cells [15,16]. Timing of insulin increase may play an important role in this process, which might explain the differences observed with the two rapid-acting analogs in comparison to regular human insulin [16,17,20].

Also, a link has been seen between anti-oxidative biochemical effects of insulin analogs and their impact on microcirculation [17,22,23]. A faster absorption and a faster onset of action has been reported for insulin glulisine vs. insuln aspart in recent studies [12,13]. It is tempting to speculate that these pharmacokinetic differences may be the reason for the differences between the two rapid-acting insulin analogs in our study. However, most likely because of the small number of patients, no clear association could be detected between any of the biomarkers and the clinical microcirculation assessments in our pilot study.

The analysis of macrophage activation by assessment of mRNA expression of NFkB and other signal transduction proteins as well as of proinflammatory cytokines is a method to investigate the immediate impact of pharmacological interventions on chronic systemic inflammation. Ghanim and coworkers have demonstrated by a similar method that obese patients are presenting with an elevated state of chronic systemic inflammation, which can be slightly reduced with metformin treatment [22]. In addition, we have been able to demonstrate the impact of treatment with the anti-inflammatory drug pioglitazone on the chronic inflammatory state of circulating macrophages, which was fully pronounced already after three days of treatment, while the impact on blood glucose usually requires 4 - 6 weeks to develop to the maximal hypoglycemic effect [21]. In our pilot study, reductions in some proinflammatory mRNA expression markers were seen after the switch to any of the prandial insulins. This is in line with findings by Dandona., *et al*. who have reported about the anti-inflammatory effects of insulin in different patient populations [26,27].

Key limitation of this investigation is clearly the small sample size in each treatment arm and the gender distribution. Also, several of the observation parameters are known to have a large inter-individual variability. All results can only be described in a descriptive and exploratory sense and no relevant conclusions can be drawn from the results. This pilot study can therefore only be very carefully interpreted and may only serve as preliminary data pool for hypothesis generation and to perform an appropriate power analysis for potential future confirmatory studies.

Still, when switching type 2 patients from basal insulin treatment with or without additional oral anti-diabetic therapy to intensive insulin therapy with insulin glargine and a rapid-acting prandial insulin, modifications in the cardiovascular risk profile could be observed.

Assessment of multiple biochemical and clinical biomarkers for vascular function and chronic systemic inflammation resulted in more favourable outcomes for the two rapid-acting insulin analogs (glulisine and aspart) in comparison to regular human insulin. The majority of the indications for improvement of cardiovascular risk markers were seen for insulin glulisine (hsCRP, mRNA IL-6). These results would be supported by the potentially faster onset of action of insulin glulisine in comparison to insulin aspart, insulin lispro and regular human insulin [8,12-14]. However, the patient population was far too small, and the observation parameters had a far too high variability to justify any clinical conclusions from these results. Therefore, larger clinical studies with sufficient power and a confirmatory protocol approach are required before any clinically relevant conclusion can be drawn from our results.

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