FGF21 Contributes to Metabolic Improvements Elicited by Combination Therapy with Exenatide and Pioglitazone in Patients with Type 2 Diabetes

Ricardo J. Samms[1*], Christine C. Cheng[1], Marcel Fourcaudot[2], Sami Heikkinen[3], Ahmed Khattab[2], John Adams[2], Eugenio Cersosimo[2], Curtis Triplitt[2], Curtis Puckett[2], Kostas Tsintzas[4], Andrew. C. Adams[1], Muhammad A. Abdul-Ghani[2], Ralph A. DeFronzo[2] and Luke Norton[2*]

Affiliations: [1]Eli Lilly and Company; [2]Diabetes Division, University of Texas Health San Antonio, TX; [3]Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland; [4]School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK

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***Address all correspondence to:**

Ricardo Samms, Ph.D and Luke Norton, Ph.D

Email: samms_ricardo_j@lilly.com and nortonl@uthscsa.edu

Abstract

Background: Fibroblast growth factor 21 (FGF21) is increased acutely by carbohydrate ingestion and is elevated in patients with type 2 diabetes (T2D). However, the physiological significance of increased FGF21 in humans remains largely unknown.

Objectives and Approach: We examined whether FGF21 contributed to the metabolic improvements observed following treatment of patients with T2D with either triple (metformin/pioglitazone/exenatide) or conventional (metformin/insulin/glipizide) therapy for 3-years. Forty-six patients with T2D were randomized to receive either triple or conventional therapy to maintain HbA1c < 6.5%. A 2-hour 75 g oral glucose tolerance test (OGTT) was performed at baseline and following 3-years of treatment to assess glucose tolerance, insulin sensitivity and β-cell function. Plasma total and bioactive FGF21 was quantitated before and during the OGTT at both visits.

Results: Patients in both treatment arms experienced significant improvements in glucose control, but insulin sensitivity and β-cell function were markedly increased after triple therapy. At baseline, FGF21 levels were regulated acutely during the OGTT in both groups. After treatment, fasting total and bioactive FGF21 were significantly reduced in patients receiving triple therapy, but there was a relative increase in the proportion of bioactive FGF21 compared to conventionally treated subjects. Relative to baseline studies, triple therapy treatment also significantly modified FGF21 levels in response to a glucose load. These changes in circulating FGF21 were correlated with markers of improved glucose control and insulin sensitivity.

Conclusions: Alterations in the plasma FGF21 profile may contribute to the beneficial metabolic effects of pioglitazone and exenatide in human patients with T2D.

New and Noteworthy

In patients with T2D treated with a combination of metformin/pioglitazone/exenatide (triple therapy), we observed reduced total and bioactive plasma FGF21 levels, and a relative increase in the proportion of circulating bioactive FGF21 compared to patients treated with metformin and sequential addition of glipizide and basal insulin glargine (conventional therapy). These data suggest that FGF21 may contribute, at least in part, to the glycemic benefits observed following combination therapy in patients with T2D.

Introduction

As an atypical member of the Fibroblast Growth Factor (FGF) family of proteins, FGF21 is released primarily from the liver, and under certain physiological conditions, functions as a hormone with potent and pleiotropic metabolic effects. Interest in the therapeutic potential of FGF21 originates from early studies demonstrating that the protein stimulated glucose uptake into adipocytes *in vitro*, and improved glucose tolerance in diabetic rodents and primates (1, 2). The whole-body metabolic effects of FGF21 are likely the result of both neural and peripheral activity (3). In diet-induced obese (DIO) mice, central and peripheral administration of FGF21 increases energy expenditure by activating the sympathetic nervous system (4) and promoting white adipose tissue (WAT) browning (5). However, in human clinical trials, engineered FGF21 analogs have only partially recapitulated data generated in preclinical rodent studies. In patients with obesity and type 2 diabetes (T2D), FGF21 administration for 4-weeks improved dyslipidemia and bodyweight, but had no effect of glycemic control (6, 7). These discordant findings highlight the need for further studies aimed at understanding the basic biology of FGF21 in human subjects.

The mRNA expression and hepatic secretion of FGF21 is regulated by multiple nutritional signals, including fasting/refeeding and high carbohydrate diets (8), and may be directly regulated by the transcription factor Carbohydrate Responsive-Element Binding Protein (ChREBP) (9). We recently demonstrated in healthy human subjects that FGF21 is acutely regulated following oral ingestion of a

glucose load (10). The ingestion of fructose similarly increases FGF21 levels acutely in human subjects (11). We and others also have demonstrated that FGF21 is increased in patients with obesity, impaired glucose tolerance (IGT) and T2D (12-14), and correlates with muscle and hepatic insulin resistance (13). Circulating levels of FGF21 also are increased in patients with nonalcoholic fatty liver disease (NAFLD) and hypertriglyceridemia (15, 16). The paradox between increased FGF21 levels in patients with metabolic disease and the therapeutic benefits of exogenously administered FGF21 analogs has led to speculation that obesity and T2D are "FGF21-resistant" states (17). While this hypothesis has been disputed in rodents (18), questions remain about the significance of elevated FGF21 in metabolic diseases. An alternative explanation is that FGF21 activity is altered in obese and diabetic conditions. Like many peptide hormones the half-life of FGF21 is relatively short (1 to 2 hours), and the protein circulates in both inactive and bioactive forms in healthy participants (19). Inactive FGF21 is generated via proteolytic cleavage of the C-terminus by the serine dipeptidase fibroblast activation protein (FAP) (19-21). Interestingly, although we previously have shown that FAP is increased in patients with T2D (10) recent data suggest that FAP may be dispensable for glucose control in rodents (22).

In the present study we examined total and bioactive circulating FGF21 levels in patients with T2D before and after the initiation of therapeutic interventions designed to improve glucose homeostasis. We hypothesized that a treatment regimen that included the thiazolidinedione (TZD) pioglitazone and the Glucagon-like peptide-1 (GLP-1) receptor agonist exenatide would reduce FGF21 levels compared to patients treated with metformin followed by sequential addition of sulfonylurea and glargine insulin. We also explored whether the dynamic changes in FGF21 during oral glucose tolerance tests (OGTT) were differentially regulated by these therapeutic interventions. Our findings demonstrate that combination therapy with pioglitazone and exenatide significantly lowers fasting and postprandial circulating total FGF21, but increases the relative abundance of bioactive FGF21 in plasma. This contrasted with conventional therapy, which had no effect on FGF21 levels. Critically, these differences were not related to weight loss, and FGF21 levels were highly correlated with measures of improved insulin sensitivity in patients treated with triple therapy. These data reveal new insights into the bioactivity of FGF21 in human

subjects, and highlight the possible involvement of FGF21 signaling in the metabolic improvements elicited by therapeutic interventions in patients with T2D.

2. Materials and Methods

2.1. Study Participants: The humans subjects analyzed in the present study represent a subset of patients from the Efficacy and Durability of Initial Combination Therapy for Type 2 Diabetes (EDICT) study (23). This ongoing study is an open-label, single-center, randomized controlled trial (clinicaltrials.gov registration no.: NCT01107717) examining the efficacy, durability and safety of initial triple combination therapy with anti-diabetic agents (metformin/pioglitazone/exenatide). All patients in the present study completed 3-years of follow-up.

2.2. Study Protocol: A detailed description of the EDICT study is provided elsewhere (23). The participants were randomized based on age, sex, BMI, diabetes duration and HbA1c level to receive either initial triple combination therapy with metformin/pioglitazone/exenatide (triple therapy) or metformin with sequential addition of glipizide and then basal insulin glargine (conventional therapy) to maintain HbA1c levels at < 6.5%. Triple therapy participants were started on metformin (1000 mg/day), pioglitazone (15 mg/day) and exenatide (5 μg twice daily). These doses were increased at the 1-month follow-up to 2000 mg and 30 mg of metformin and pioglitazone, respectively, and 10 μg of exenatide twice daily. At the 3-month follow-up, pioglitazone was further increased to 45 mg if the target HbA1c of 6.5% was not reached.

In the conventional arm, patients were started on a metformin dose of 1000 mg/day, which was increased to 2000 mg/day if the fasting plasma glucose was above 110 mg/dl at the 1-month follow-up. At the same visit, participants not meeting this fasting plasma glucose target were started on a glipizide dose of 5 mg/day. If fasting plasma glucose remained above 110 mg/day at the 2-month follow-up, or if HbA1c was above 6.5%, the daily dose of glipizide was increased to 10 mg/day and then 20 mg/day. Glargine insulin was started at the 3-month timepoint if fasting plasma glucose or HbA1c remained above 110 mg/dl and 6.5%, respectively. The insulin dose began with 10 units taken before breakfast and was escalated weekly by 1 – 5 units to 60 units/day to maintain fasting plasma glucose below 110 mg/dl.

Beyond the first 3-months, study participants were seen every 3 months. At each visit the medication dose was adjusted to maintain fasting plasma glucose below 110 mg/dl and HbA1c below 6.5%.

2.3. Physiological Assessment: All participants underwent a 2-hour 75 g oral glucose tolerance test (OGTT) at baseline and at the 3-year follow-up visit. During the OGTT, plasma samples were obtained at −30, −15, and 0 minutes and every 15 minutes thereafter for analysis of plasma glucose, insulin, C-peptide and free fatty acids (FFA). At the baseline and 3-year follow-up visit, samples were also collected for the analysis of fasting plasma glucose, HbA1c, plasma triglycerides and cholesterol.

2.4. Determination of Insulin Sensitivity and β-cell Function: The incremental area under the plasma glucose, insulin and C-peptide curves during the OGTT was determined using the trapezoidal rule. The effect of each treatment on whole-body insulin sensitivity was estimated from the OGTT data using the Matsuda index (24). Adipose tissue insulin resistance (Adipo-IR) was estimated from the fasting plasma FFA and insulin concentration, as previously described by us (25, 26). Several studies have examined the relationship between Adipo-IR and metabolic diseases. Adipo-IR is elevated in obese subjects (27) and is closely correlated with worsening glucose tolerance and T2D (25, 28, 29). Confirming the utility of the Adipo-IR measurement, in validation studies Adipo-IR was strongly correlated with the suppression of adipocyte lipolysis during the multistep insulin clamp, which is considered the gold-standard approach of assessing adipose tissue insulin resistance (30). Beta-cell function was calculated as the insulin secretion/resistance (disposition) index, using the following formula $\Delta\text{C-peptide}_{0-120(\text{AUC})}/\Delta\text{Glucose}_{0-120(\text{AUC})} \div$ insulin resistance (calculated as the inverse of the Matsuda Index) (31).

2.5. Total and Bioactive FGF21 Quantification: The quantitation of FGF21 was carried out on plasma samples obtained before and during the OGTT at baseline and at the 3-year follow-up. To measure total FGF21 levels, we used a sandwich ELISA from Biovendor (catalog #: RD191108200R), and for bioactive FGF21 an ELISA from Eagle Bioscience (catalog #: F2131-K01) was employed. The fraction

of bioactive FGF21 to total FGF21 was calculated. All samples within each treatment arm (baseline and 3-year follow-up) were analyzed together in the same ELISA batch. To control for any batch effects between treatment arms, we used the ELISAtools analysis package (v0.1) in R (v3.5.1) (32).

2.6. Statistical Analysis: Data were analyzed using GraphPad Prism v7.4 or R (v3.5.1). Differences in fasting subject characteristics at baseline and at the 3-year follow-up were examined using T-tests with Welch's correction, where appropriate. To examine the effect of a glucose challenge on FGF21 during the OGTT at baseline, repeat-measures two-way analysis of variance (ANOVA) with treatment group (triple or conventional therapy) and OGTT time (0 – 120 minutes) as the main factors was performed. To examine the effect of each treatment on the dynamic change in FGF21 during the OGTT at the 3-year follow-up, repeat-measures two-way ANOVA with visit (pre/post) and OGTT time as the main factors was performed. Post-hoc analyses were performed using Holm-Sidak's multiple comparison tests. Because FGF21 levels during the OGTT deviate significantly from normality, all FGF21 statistical analyses were performed on Log10 transformed data.

3. Results

3.1. Effect of Triple and Conventional Therapy on Glucose Homeostasis: At baseline there were no significant differences in bodyweight, duration of diabetes, or fasting plasma glucose and lipid profiles in subjects randomly assigned to receive either conventional or triple therapy (**Table 1**). Participants in both treatment arms had improvements in their lipid profiles, including reduced total cholesterol, but only those receiving triple therapy experienced an increase in HDL cholesterol and a reduction in plasma triglycerides (**Table 1**). Both groups experienced significant improvements in glucose control as evidenced by the decrease in the HbA1c and fasting plasma glucose at the 3-year follow-up, as well as improved glucose tolerance during the OGTT (**Fig 1A and Fig 2A**). The mean HbA1c at 3 years achieved the recommended treatment goal (HbAc1 < 6.5%) in both treatment groups (**Table 1**).

In the triple therapy group, β-cell function estimated from C-peptide and plasma glucose excursions during the OGTT was increased (**Fig 2A** and **Fig 2C**; and **Fig 3B**), as was whole-body insulin sensitivity quantitated using the Matsuda Index (**Fig 3A**). The plasma FFA levels during the OGTT were significantly lower following triple therapy treatment (**Fig 2D**), suggesting improved adipose tissue insulin sensitivity. However, while Adipo-IR was reduced the by triple therapy, this did not reach statistical significance (**Fig 3C**). In the conventional arm, there was no detectable improvement in insulin resistance or β-cell function during the OGTT (**Fig 3A – 3C**).

3.2. Effect of Triple and Conventional Therapy on Total and Bioactive FGF21: At baseline, plasma FGF21 levels were significantly altered following a glucose load. Total FGF21 declined gradually during the OGTT to a nadir at the 90-minute time-point, before recovering to the fasting levels at 120 minutes during the OGTT (**Fig 4A**). This change in plasma FGF21 concentration during the OGTT was similar in both treatment groups. At the 3-year follow-up, however, there was a significant reduction in the fasting (**Table 1**) and postprandial (**Fig 4A**) total plasma FGF21 levels in patients treated with triple therapy, but not in those receiving conventional therapy. Moreover, triple therapy treatment altered the profile of

plasma FGF21 during the OGTT (treatment x OGTT interaction, $P < 0.001$), such that total plasma FGF21 concentrations at 120 minutes during the OGTT rose significantly above the fasting levels (**Fig 4A**).

We next investigated the contribution of bioactive FGF21 to the changes in total plasma FGF21 before and after each treatment, and during the OGTT. Compared to baseline, absolute fasting bioactive FGF21 levels were reduced in patients receiving triple therapy, but conventional therapy had no impact on bioactive FGF21 (**Table 1** and **Fig 4B**). The reduction in fasting bioactive FGF21 in the triple therapy treated patients was modest compared to the reduction in total FGF21, resulting in a relative increase in the proportion of bioactive FGF21 in these patients at the end of treatment compared to those receiving conventional therapy (**Fig 4C**). There was a significant effect of glucose load on plasma bioactive FGF21 during the OGTT at baseline (**Fig 4B**). Consistent with the total FGF21 data, triple therapy treatment affected the response of bioactive FGF21 to the glucose challenge (treatment x OGTT interaction, $P < 0.05$) such that bioactive FGF21 was significantly increased at the end of the OGTT (**Fig 4B**). There was no interaction between the treatment and OGTT in the conventional treatment arm of the study. The ratio of bioactive to total FGF21 in the plasma increased during the OGTT in both treatment groups at baseline and after 3 years of treatment (**Fig 4C**). However, the AUC of the bioactive fraction of FGF21 was significant higher during the OGTT after triple therapy treatment (**Fig 4C**). Thus, treatment strategies that target core physiological defects in patients with T2D are associated with distinct alterations in fasting and postprandial total and bioactive FGF21.

3.3. FGF21 is Associated with Improved Insulin Sensitivity: To explore potential causal relationships between FGF21 and improvements in glucose metabolism and insulin sensitivity, we performed linear regression analysis. At the 3-year follow-up the change in both total (**Fig 5A** and **5B**) and bioactive (**Fig 5C** and **5D**) FGF21 was significantly correlated with the improvement in HbA1c and fasting plasma glucose in the combined cohort. This effect was driven primarily by the triple therapy-treated group. Absolute levels of total and bioactive FGF21 were highly correlated with HbA1c and fasting plasma

glucose in patients treated with triple therapy (**Fig 6A – 6D**), but not in those treated with conventional therapy. Of particular interest, total FGF21 also was significantly and positively correlated with Adipo-IR (**Fig 6E**) and negatively associated with the Matsuda Index of insulin sensitivity (**Fig 6F**) in the triple therapy group. Confirming prior human studies (33-35), FGF21 levels after triple therapy treatment were positively associated with triglycerides ($r = 0.469$, $P < 0.05$; data not shown). No such correlations were observed in the conventional treatment group. Although clearly correlative in nature, these data highlight a potential role for FGF21 in the metabolic improvements observed in patients with T2D treated triple therapy.

4. Discussion

There are several novel findings in the present study that advance our understanding of FGF21 biology in humans with metabolic disease. First, we demonstrate that the increase in FGF21 levels in patients with T2D may reflect alterations in the stability of circulating FGF21. This is because in the present study, the triple therapy combination reduced total FGF21 levels but increased the ratio of bioactive to total FGF21 compared to conventional therapy. Second, FGF21 levels at the end of the study were correlated with measures of glucose control and insulin sensitivity, suggesting that restoration of FGF21 levels in patients with T2D may have a beneficial metabolic impact. Third, the current data extend previous observations that FGF21 levels are altered postprandially in humans, and indicate that alterations in the dynamic response of FGF21 to a glucose challenge may influence glucose disposal in patients with T2D.

It is important to emphasize that both treatment groups experienced excellent glucose control and, on average, reached the recommended treatment target HbA1c of ≤ 6.5%. The key difference between the two treatment groups was that only triple therapy improved both insulin resistance and β-cell function. This finding is consistent with the known effects of pioglitazone and exenatide in humans (36, 37), but also highlights the lesser-known impact of pioglitazone treatment on β-cell function (38, 39). Triple therapy treatment also improved adipose tissue insulin sensitivity to the suppression of lipolysis, as evidenced by the reductions in plasma FFA levels during the OGTT and fasting Adipo-IR, although the latter did not reach statistical significance. The improvements in insulin resistance were likely driven by pioglitazone, which has potent and well documented insulin sensitizing effects in patients with T2D (39).

The metabolic improvements observed in the triple therapy group were not due to greater weight loss in these patients. We did not detect any differences in bodyweight between the two treatment groups at the 3-year follow-up, and neither treatment led to significant changes in bodyweight or BMI. Compared to newer GLP-1 receptor agonists, weight loss with Exenatide is relatively modest. In two phase 3 clinical studies 5 μg Exenatide was associated with body weight changes of –0.9 ± 0.3 kg and −1.6 ± 0.4 kg. This is lower than that observed in our study (–3.1 kg), which is likely explained by the shorter duration of the

phase 3 studies and the higher baseline BMI of the patients enrolled in our study. It is possible that the weight loss in our 3-year study would have been more pronounced in the absence of pioglitazone, which has well documented effects on fluid retention and weight gain.

In healthy human subjects, we previously showed that FGF21 increases during an OGTT, but that this response is largely attenuated in patients with T2D and may reflect the attenuated insulin response during the OGTT in these patients (10). We broadly confirm these results here. In the T2D subjects at baseline, total FGF21 levels remained relatively flat but followed a bimodal pattern, reaching a nadir at 90 minutes before returning to fasting levels at 120 minutes. After triple therapy treatment, the response of total FGF21 during the OGTT more closely resembled that observed in healthy subjects (10). This effect was more marked for bioactive FGF21 levels, which increased significantly at the end of the OGTT only after treatment, although there was no difference between the two treatment groups. The differences in total FGF21 during the OGTT between the two groups post-treatment may reflect the increased β-cell function in patients receiving triple therapy treatment. In follow-up insulin clamp studies in healthy human subjects, we concluded that FGF21 was regulated by insulin (10). However, it's possible that other factors, including plasma glucose levels and hepatic insulin resistance, are important determinants of postprandial FGF21 levels.

The changes in total and bioactive FGF21 in the triple therapy treated group raise several interesting questions regarding the underlying mechanisms, and the physiological importance of FGF21 in facilitating improvements in glucose disposal, insulin resistance and β-cell function. It is well established that multiple metabolic disorders are characterized by increased FGF21 levels in humans, but few studies have examined the impact of different T2D treatment approaches on FGF21 in humans. While our study design cannot definitively establish causal relationships, recent findings in healthy humans suggest that hyperglycemia upregulates plasma FGF21 levels (40). Given the marked reduction in fasting and postprandial glucose levels in patients treated with triple therapy in our study, it is possible that this is responsible for the large reduction in total FGF21 in these patients. Changes in FGF21 may also partly reflect a direct effect of each drug or drug combination. Several studies have demonstrated that FGF21

regulates the antidiabetic action of TZDs (41-43). In white adipose tissue, rosiglitazone increases FGF21 expression via transcriptional activation of PPARγ (41, 44), and in liver FGF21 is regulated by PPARα (45). In FGF21 knockout mice the metabolic improvements elicited by rosiglitazone are attenuated (41), although this has been disputed (46). While it is possible that pioglitazone regulated FGF21 in our study through direct agonism of hepatic and/or adipocyte PPARs, this appears unlikely given that FGF21 levels were reduced in the triple therapy group. Similar results were obtained in a previous study that demonstrated that rosiglitazone alone reduced fasting FGF21 in human patients with T2D (47). An alternative explanation is that the GLP-1 receptor agonist exenatide may be responsible, in part, for the changes in FGF21 in our study. In support of this hypothesis, two previous human studies demonstrated that exenatide treatment decreased plasma FGF21 levels (48, 49). Whether FGF21 is required for the metabolic action of GLP-1-based therapies will require rigorous testing in liver-specific FGF21 knockout mice. However, it is important to emphasize that, as alluded to above, the effect of both exenatide and pioglitazone on FGF21 levels in humans and mice is contradictory, highlighting the need for additional human studies focusing on the role of FGF21 in mediating the metabolic effects of T2D therapies.

Regardless of the mechanisms involved, alterations in circulating FGF21 are likely physiologically significant and may have contributed to the metabolic improvements observed in patients treated with triple therapy. Fasting and postprandial FGF21 were significantly correlated with several parameters that were improved with triple therapy treatment, most notably plasma glucose levels and insulin sensitivity. In mice, FGF21 has been implicated in mediating α- to β-cell transdifferentiation following GLP-1 treatment (50), and protects against high-fat diet induced pancreatic islet hyperplasia (51). Interestingly, the negative correlation with the Matsuda Index, and positive correlation with Adipo-IR, following triple therapy strongly supports the notion that increased FGF21 is associated with worsening insulin resistance in humans. To the best of our knowledge, our study is the first to show that the ratio of bioactive to total FGF21 is differentially modified by distinct treatment strategies. Recent studies have highlighted the role of FGF21 cleavage in humans *in vivo* (8, 52), and we demonstrated that FAP is increased in patients with T2D (10), which is consistent with reduced FGF21 stability in insulin

resistant states. The metabolic significance of FGF21 cleavage has not been explored in humans, but based on our findings it is tempting to speculate that increased stability of FGF21 plays an important metabolic role in humans.

In conclusion, we show that treatment of T2D patients with a therapeutic combination that includes exenatide and pioglitazone improves glucose tolerance, insulin sensitivity and β-cell function, and is associated with reduced total FGF21 and a relative increase in bioactive FGF21 compared to patients treated with conventional therapies. Our findings suggest that the stability of FGF21 was increased by triple therapy treatment, and indicate that changes in total and bioactive FGF21 may play an important role in the treatment of T2D. More studies will be needed to determine the requirement of FGF21 for the metabolic improvements observed following treatment with GLP-1 receptor agonists and pioglitazone in human subjects.

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Table 1. Baseline and 3-year follow-up fasting characteristics of subjects randomized to receive either conventional or triple therapy.

	Conventional Therapy			Triple Therapy			Conventional vs. Triple
	Baseline	**3-Year**	**Pre-Post P value**	**Baseline**	**3-Year**	**Pre-Post P value**	**Change score P Value**
Number (n)	21	21	-	25	25	-	
Age (years)	50.7 (± 3.1)	53.7 (± 3.1)	-	50.2 (± 1.9)	53.2 (± 1.9)	-	
Sex (Male, %)	62	62	n.s.	44	44	n.s.	n.s.
Diabetes Duration (Months)	7.6 (± 1.9)	10.6 (± 1.9)	n.s.	4.7 (± 1.4)	7.7 (± 1.4)	n.s.	n.s.
Weight (Kg)	103.1 (± 6.7)	101.0 (± 7.1)	n.s.	101.1 (± 5.5)	98.0 (± 5.0)	n.s.	n.s.
BMI (Kg/m^2)	39.2 (± 2.0)	38.9 (± 2.0)	n.s.	40.1 (± 2.0)	40.1 (± 2.0)	n.s.	n.s.
HbA1c (%)	7.9 (± 0.3)	6.3 (± 0.2)	< 0.001	7.9 (± 0.2)	5.7 (± 0.1)	< 0.001	n.s.
FPG (mg/dL)	169.2 (± 10.4)	128.8 (± 12.6)**	< 0.01	181.1 (± 10.8)	109.6 (± 3.9)	< 0.001	< 0.05
Total Cholesterol (mg/dL)	182.4 (± 10.6)	155.0 (± 8.7)**	< 0.01	201.4 (± 7.8)	167.4 (± 6.6)	< 0.001	n.s.
HDL (mg/dL)	40.1 (± 1.9)	37.6 (± 1.6)	n.s.	42.7 (± 1.8)	50.3 (± 3.5)	< 0.05	< 0.05
TG (mg/dL)	168.5 (± 19.5)	173.9 (± 28.1)	n.s.	169.4 (±14.5)	114.8 (±11.8)	< 0.001	n.s.
Total FGF21 (pg/ml)	492.6 (± 80.0)	563.5 (± 78.0)	n.s.	538.9 (± 61.0)	331.9 (± 42.1)	< 0.001	< 0.01
Bioactive FGF21 (pg/ml)	188.5 (± 44.0)	194.6 (± 32.9)	n.s.	211.4 (± 27.0)	159.8 (± 22.9)	< 0.01	< 0.01

Data are mean ± SEM. BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein cholesterol; TG, triglycerides; n.s., non-significant.

Figure Legends

Fig 1. Plasma glucose (**A**), insulin (**B**), C-Peptide (**C**), and free fatty acids (FFA, **D**) during an oral glucose tolerance test (OGTT) at baseline and after 3-years of conventional treatment in patients with T2D. *P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline.

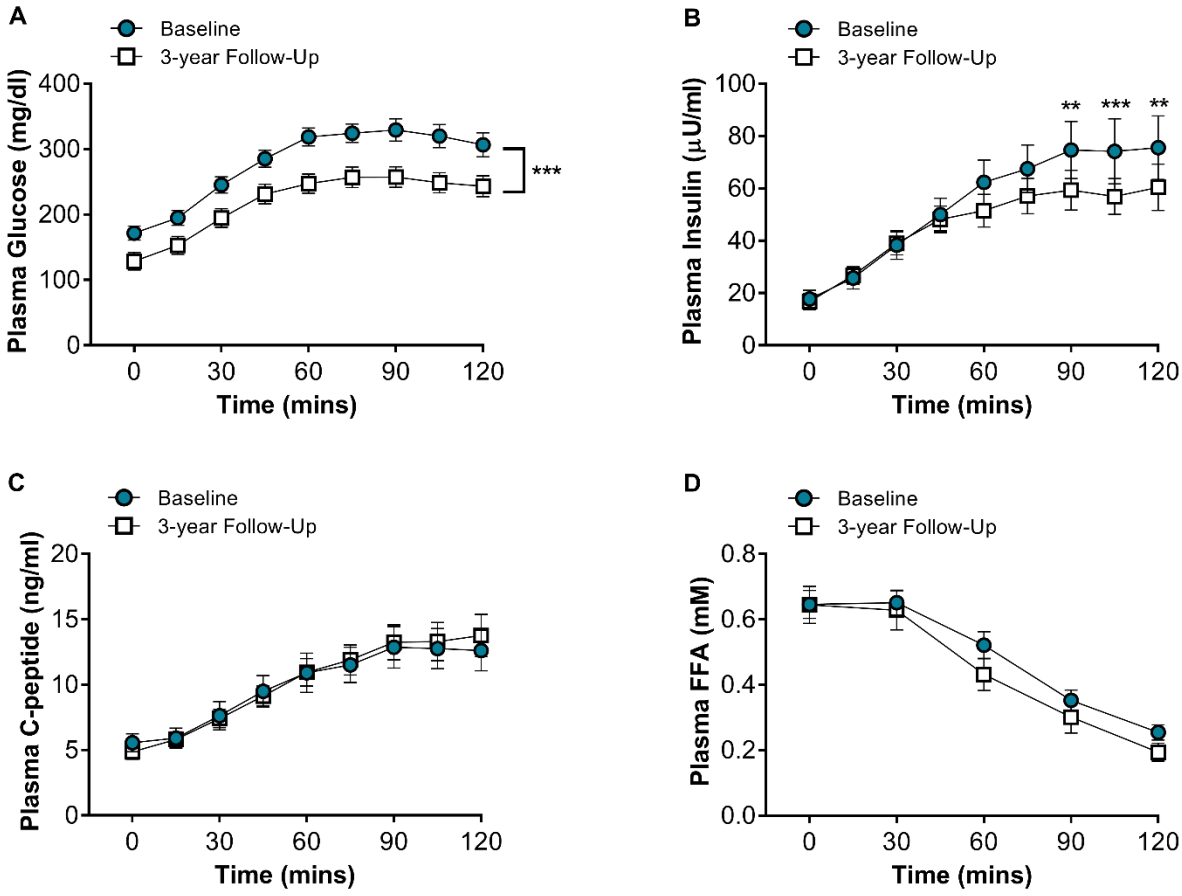
Fig 2. Plasma glucose (**A**), insulin (**B**), C-Peptide (**C**), and free fatty acids (FFA; **D**) during an oral glucose tolerance test (OGTT) at baseline and after 3-years of triple therapy treatment in patients with T2D. *P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline.

Fig. 3. Matsuda Index of insulin sensitivity (**A**), β-cell function (**B**) and adipose tissue insulin resistance (Adipo-IR) (**C**) at baseline and after 3-years of conventional or triple therapy treatment in patients with T2D. *P < 0.05, ***P < 0.001 vs. baseline.

Fig 4. Total (**A**), bioactive (**B**) and relative bioactive FGF21 (**C**) before (**left panel**) and after (**middle panel**) conventional or triple therapy treatment during an oral glucose tolerance test (OGTT) in patients with T2D. The area under the OGTT curve is shown (**right panel**). *P < 0.05, **P < 0.01, ***P < 0.001 indicates main effect of treatment on FGF21 levels. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 indicates significance at indicated time point vs. fasting in both treatment groups. ^^^P < 0.001 indicates significance at indicated time point vs. fasting in the triple therapy group after an interaction between the treatment and OGTT was detected using 2-way ANOVA. All FGF21 statistical analysis was carried out on Log transformed data.

Fig 5. Linear regression analysis of the change in fasting total or bioactive FGF21 and glycated hemoglobin (HbA1c; **A and C**) and fasting plasma glucose (**B and D**) following 3-years of either conventional or triple therapy treatment.

Fig 6. Linear regression analysis of fasting total or bioactive FGF21 and glycated hemoglobin (HbA1c; **A and C**), fasting plasma glucose (**B and D**), adipose tissue insulin resistance (Adipo-IR; **E**) and the Matsuda index of insulin sensitivity (**F**) following 3-years of triple therapy treatment.



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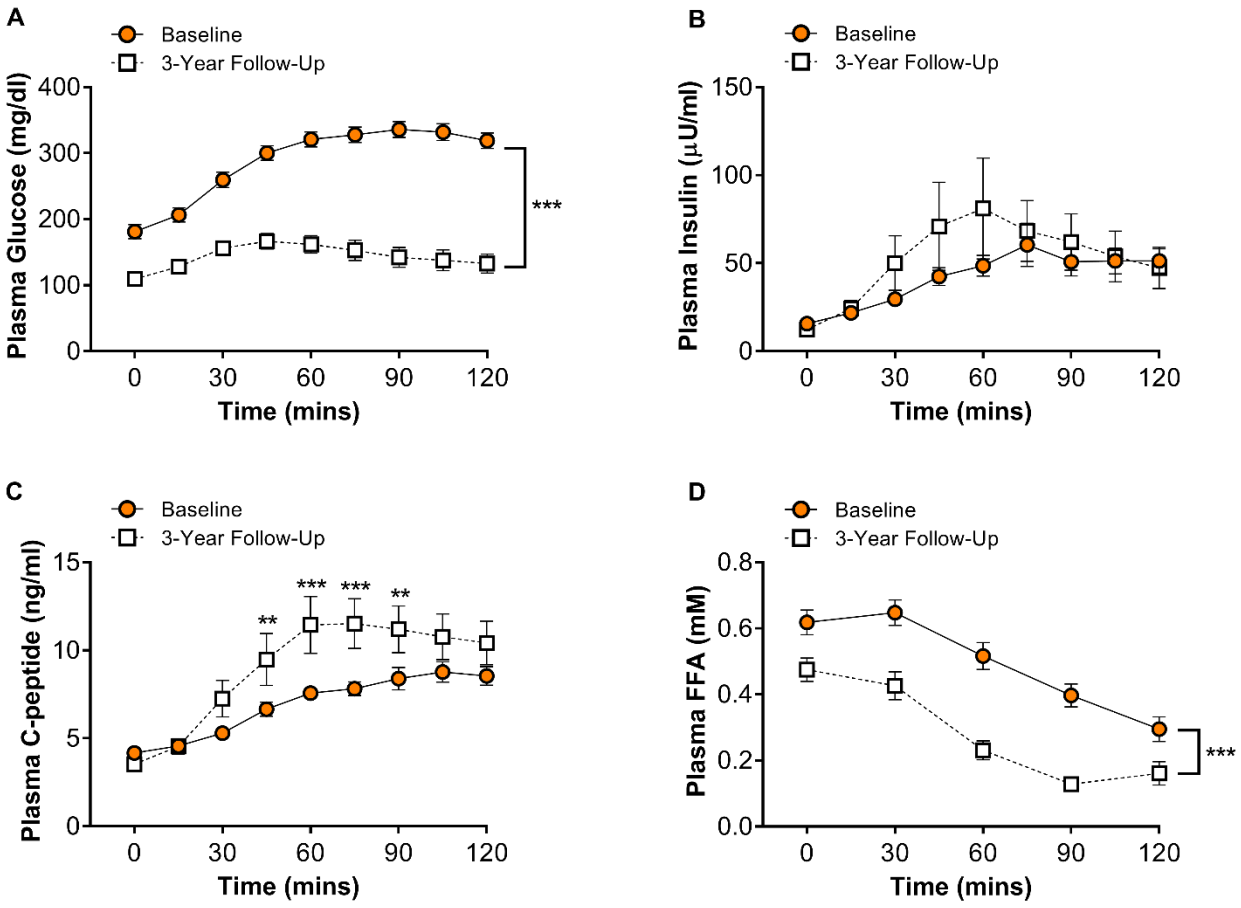
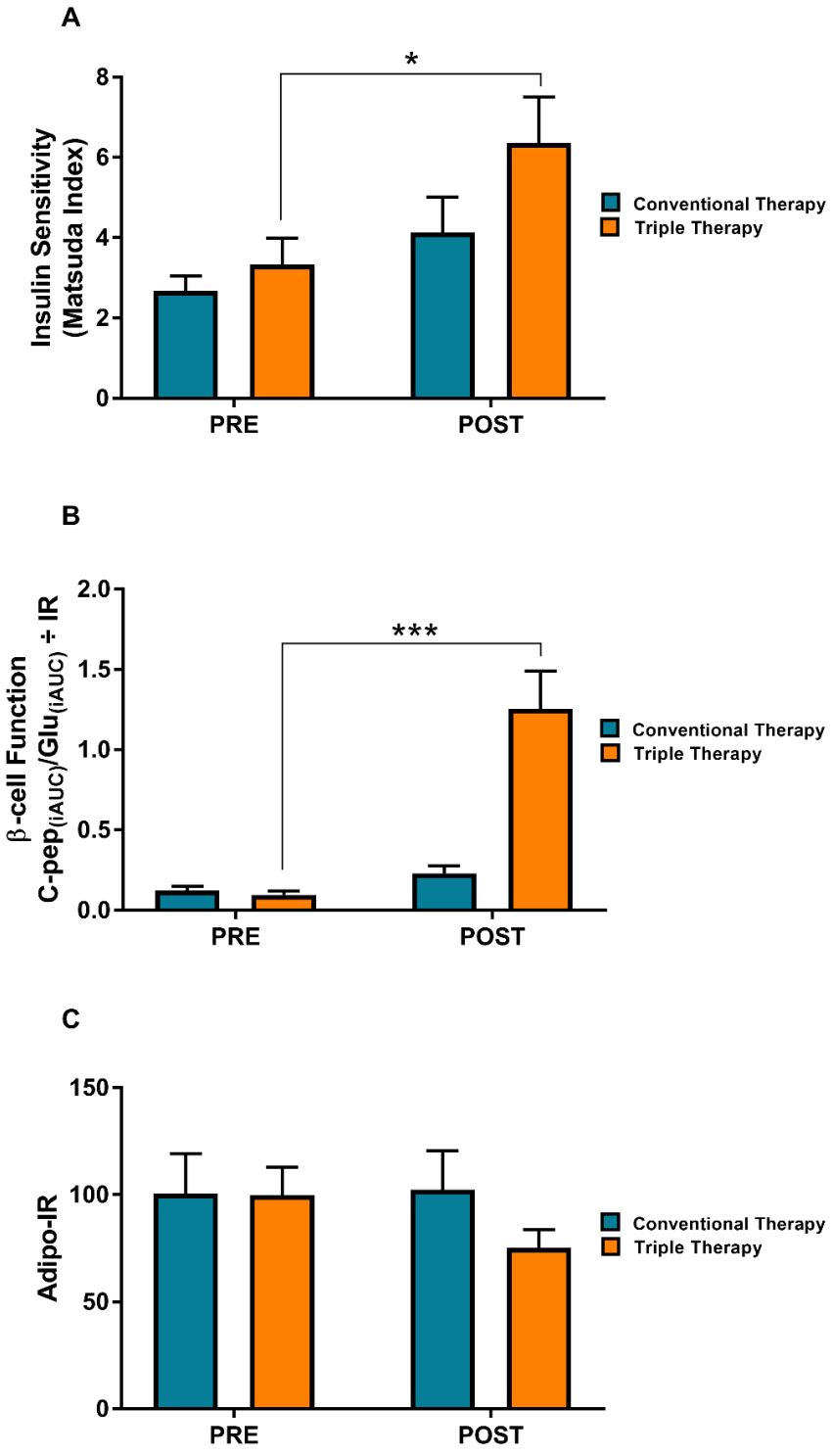


Fig 2. Plasma glucose (**A**), insulin (**B**), C-Peptide (**C**), and free fatty acids (FFA; **D**) during an oral glucose tolerance test (OGTT) at baseline and after 3-years of triple therapy treatment in patients with T2DM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline.



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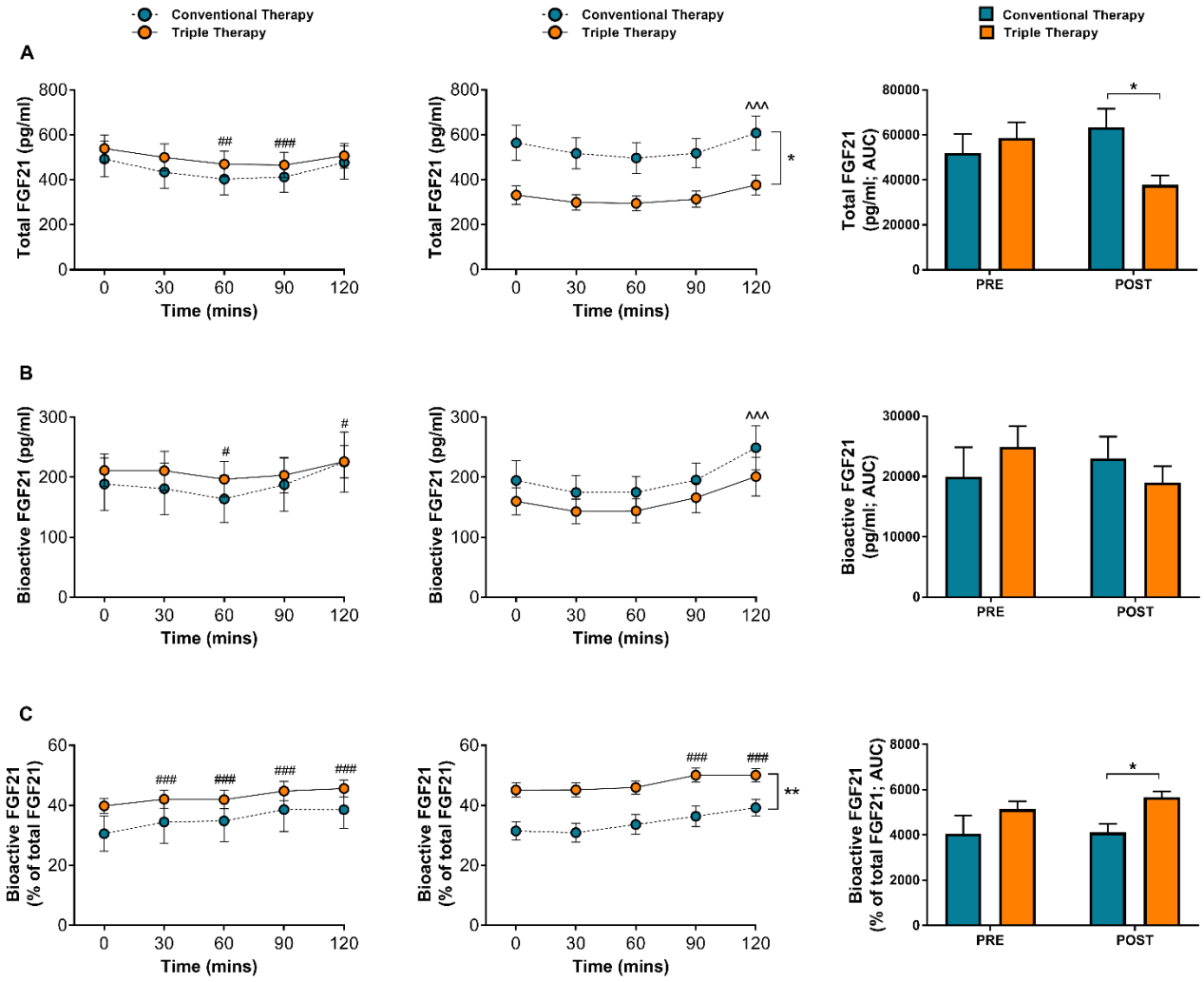

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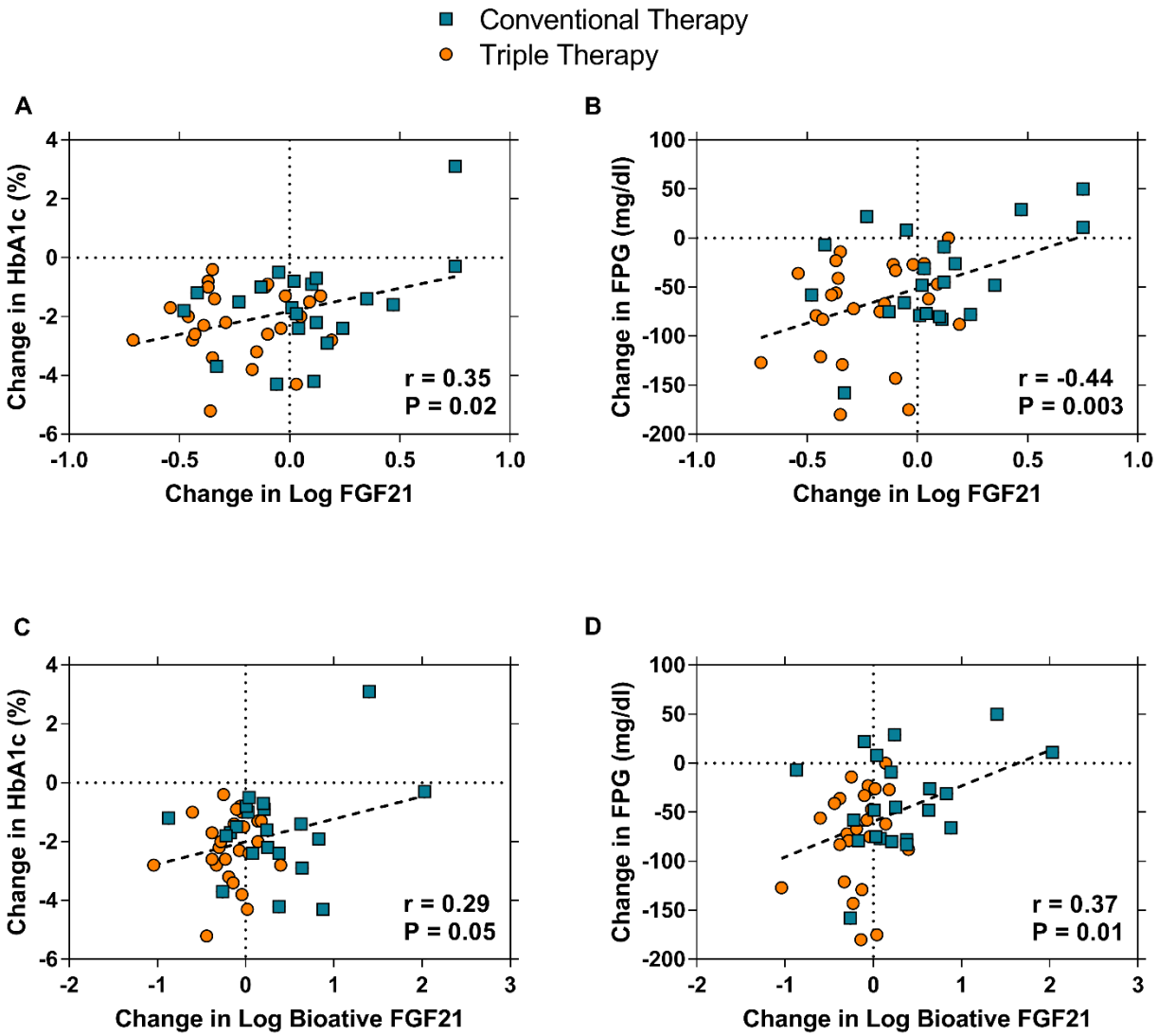


Fig 5. Linear regression analysis of the change in fasting total or bioactive FGF21 and glycated hemoglobin (HbA1c; **A and C**) and fasting plasma glucose (**B and D**) following 3-years of either conventional or triple therapy treatment.

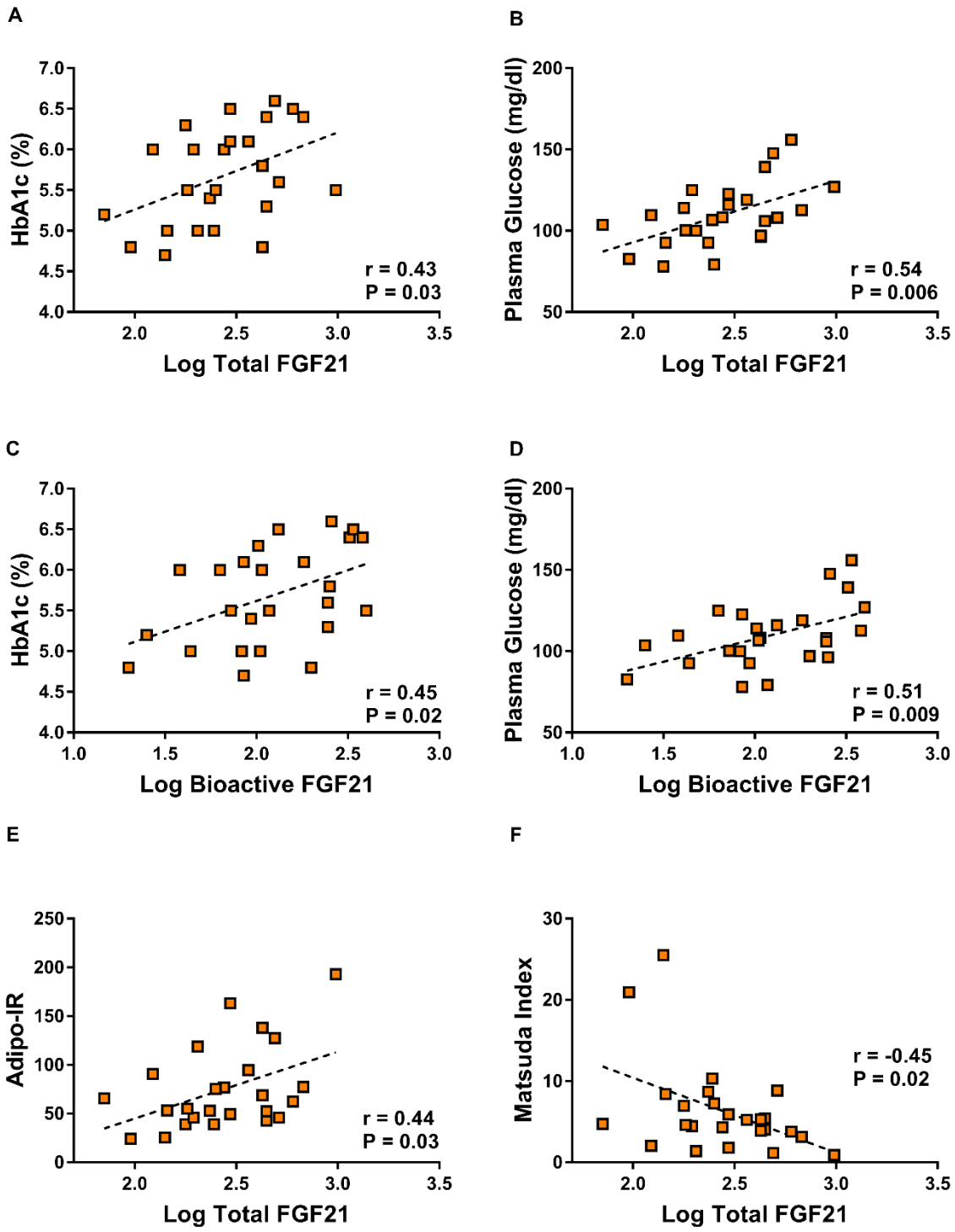


Fig 6. Linear regression analysis of fasting total or bioactive FGF21 and glycated hemoglobin (HbA1c; **A and C**), fasting plasma glucose (**B and D**), adipose tissue insulin resistance (Adipo-IR; **E**) and the Matsuda index of insulin sensitivity (**F**) following 3-years of triple therapy treatment.

Graphical Abstract

FGF21 Contributes to Metabolic Improvements Elicited by Combination Therapy with Exenatide and Pioglitazone in Patients with Type 2 Diabetes

STUDY DESIGN

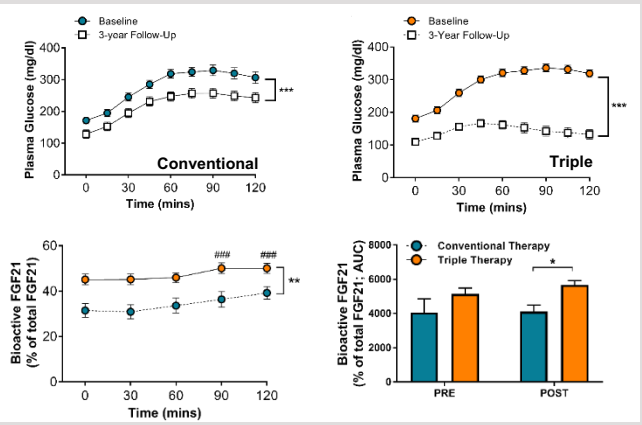
Patients with Type 2 Diabetes

Conventional Therapy		Triple Therapy
Metformin + Glipizide + Insulin	vs.	Metformin + Pioglitazone + Exenatide

3-year follow-up
(OGTT, total and bioactive FGF21)

OUTCOMES

Triple therapy increased bioactive FGF21 relative to total FGF21, and improved insulin sensitivity and β-cell function



CONCLUSION

Our findings suggest a potential role for FGF21 in mediating the glycemic benefits observed following triple therapy in patients with type 2 diabetes