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CICLO **XXXII**

Incretin Effect in Youths with Normal and Impaired Glucose Tolerance

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Introduction: candidate's research in the context

This project is part of a three years research activity conducted the candidate conducted at Yale University, aimed at investigating the physiology of youth onset prediabetes.

The candidate and his research group studied a large multiethnic cohort from the Yale Pediatric Diabetes and Obesity program to investigate the metabolic determinants of progression from normal to impaired glucose tolerance and overt diabetes and the mechanisms underlying changes in glucose tolerance over time.

*1. Physiology of prediabetes and determinants of glucose tolerance change over time. The study, conducted on multiethnic cohort of 526 obese youths (aged 8–21 years) describes that β -cell hyper-responsiveness, features those who reverted from normal to normal glucose tolerance, with ethnic background being one of the major determinants of change in glucose tolerance with non-Hispanic black ethnic origin (OR 5.06, 95% CI 1.86–13.76; $p=0.001$), with a two times greater annual increase in the oral disposition index (beta-cell function). The study has been published in 2018 (Galderisi et al; Trajectories of changes in glucose tolerance in a multiethnic cohort of obese youths: an observational prospective analysis. *Lancet Child Adolesc Health*.2018 Oct;2(10):726-735) and accompanied by an Editorial on the same issue.*

2. *Effect of dietary monosaccharides in the incretin response (gut derived hormones). First, in humans, the candidate's research group demonstrated that glucose and fructose can differentially induce insulin secretion in obese and lean youths, with obese youths exhibiting a hyper-insulinemic response to the fructose load mediated by the GLP-1 secretion. This could be one of the potential mechanisms supporting the hyper-secretive phenotype observed in obese youths. The study had been published in 2019. (Galderisi A et al, Fructose Consumption Contributes to Hyperinsulinemia in Adolescents With Obesity Through a GLP-1-Mediated Mechanism. J Clin Endocrinol Metab. 2019 Aug 1;104(8):3481-3490. doi: 10.1210/jc.2019-00161.)*

3. *Insulin clearance as a determinant of prediabetes in youths. A reduced insulin clearance has been associated with lower insulin sensitivity in obese youths with normal glucose tolerance and is a predictor for the longitudinal decline in beta cell function. (Galderisi A et al, Lower Insulin Clearance parallels a reduced insulin sensitivity in obese youths and is associated with a decline in β -cell function over time. Diabetes. 2019 Aug 9. pii: db190120. doi: 10.2337/db19-0120). The paper will be accompanied by an Editorial on Diabetes over the following months and has been presented at the last American Diabetes Association meeting (San Francisco, CA, 2019).*

4. *Additional observational studies on prediabetes in youths have been conducted from the candidate and the Yale research group at Yale, leaded by Prof Caprio, describing the role of one-hour post-load plasma glucose*

during the OGTT as predictor for the progression to prediabetes in obese youths (Tricò D, Galderisi A, Mari A, et al. One-hour post-load plasma glucose predicts progression to prediabetes in a multi-ethnic cohort of obese youths. Diabetes Obes Metab. 2019 May;21(5):1191-1198.); and the effect of rs7903146 Variant in the TCF7L2 on the b-cell function in obese youths (Cropano C, ... Dalla Man C, Cobelli C, Galderisi A, Kursawe R, Pierpont B, Goffredo M, Caprio S. The rs7903146 Variant in the TCF7L2 Gene Increases the Risk of Prediabetes/Type 2 Diabetes in Obese Adolescents by Impairing β -Cell Function and Hepatic Insulin Sensitivity. Diabetes Care. 2017 Aug;40(8):1082-1089. doi: 10.2337/dc17-0290.).

The above mentioned projects have been made possible thanks to the advanced training the candidate completed performing complex metabolic physiology studies, as the hyperglycemic clamp, the hyperinsulinemic hyperglycemic clamp and the iso-glycemic intravenous glucose tolerance test during his PhD years at Yale, mentored from Prof Caprio. Thus, the candidate was trained in the use of the minimal model and deconvolution to compute insulin secretion from a leading figure in the field, Prof Dalla Man. Prof Cobelli advised and mentored the candidate in the development of his PhD training.

Dr Galderisi has also devoted part of his time at Yale to type 1 diabetes research. He completed the following projects:

1. Role of hyperglycemia in the development of macrovascular complications. This project concludes a previous pivotal study conducted from Dr Galderisi as a resident at Harvard, where hyperglycemia was described as a potential driver of autoimmunity. Thus, after having accessed the DCCT cohort repository, the Harvard group, with the candidate, described, for the first time, the role of hyperglycemia in the development of cardiac specific autoimmunity that may, in turn, be responsible for the high rate of macrovascular complications observed in type 1 diabetes. The study was a groundbreaking discovery in the field, published in 2019 and presented at the American Diabetes Association meeting in 2019. (Sousa GR, Pober D, Galderisi A, et al Glycemic Control, Cardiac Autoimmunity, and Long-Term Risk of Cardiovascular Disease in Type 1 Diabetes Mellitus. Circulation. 2019 Feb 5;139(6):730-743.) An editorial, on the same issue, accompanies the paper.

2. The use of adjunctive therapies in type 1 diabetes, including liraglutide and pramlintide (Pramlintide but Not Liraglutide Suppresses Meal-Stimulated Glucagon Responses in Type 1 Diabetes. Galderisi A, Sherr J, VanName M, et al. J Clin Endocrinol Metab. 2018 Mar 1;103(3):1088-1094.) and SGLT2i (Reversal of Ketosis in Type 1 Diabetes Is Not Adversely Affected by SGLT2 Inhibitor Therapy. Siebel S, Galderisi A, Patel NS, et al Diabetes Technol Ther. 2019 Mar;21(3):101-104.)

3. *The first trial adopting inhaled insulin (Afrezza) during hybrid closed loop therapy in youths with type 1 diabetes has been recently submitted from the candidate with the Yale group and currently under review. (Galderisi A, Cohen N, ... Cengiz E, under revision).*

Additionally, the candidate, in the framework of a research thread involving the use of continuous glucose monitoring (CGM) in critical care (neonatal intensive care) conducted a collaborative work with the Yale researchers aimed at assess the reliability of a novel score, deriving from the Poincare' plot of CGM, for the risk of intraventricular brain hemorrhage in preterm neonates, published in 2019 as CLAIR-Score (Yale-Padova-Harvard approach to IVH). (Intraventricular Hemorrhage in Preterm Neonates. Galderisi A, Zammataro L, Losiouk E (...) Baraldi E, Cobelli C, Trevisanuto D, Steil GM. Diabetes Technol Ther. 2019 Mar;21(3):146-153.

During his PhD, the candidate has authored an invited editorial from The Lancet (Enlarging the loop: closed-loop insulin delivery for type 1 diabetes. Galderisi A, Sherr JL. Lancet. 2018 Oct 13;392(10155):1282-1284) and has been in charge of teaching activity at the Yale University for undergraduate courses.

Prof Carlo Giaquinto

Abstract

Background. Prediabetes includes a broad range of glucose metabolism alterations that increase the risk for diabetes in youths. Analogues of gut-

derived hormones (*incretins*) have been paved as a promising therapeutic option for youths with diabetes. Though, we lack *in vivo* studies assessing the *incretin effect* in prediabetes and early diabetes in youths as well as longitudinal assessment of the incretin response in this age.

We estimated the incretin effect in obese youths with normal and impaired glucose tolerance, by the use of the gold standard matched oral glucose tolerance test (OGTT) and iso-glycemic intravenous glucose tolerance test (iso-IVGTT).

Methods. We enrolled 30 overweight/obese youths with normal (NGT) and impaired (IGT) glucose tolerance. Each participant underwent a 180-minutes OGTT and an iso-IVGTT to quantify the *incretin effect*, followed by a hyperglycemic clamp to measure glucose and arginine induced insulin secretion. Seriated samples for plasma glucose, insulin, C-peptide and active GLP-1₍₇₋₃₆₎ were collected. The minimal model and deconvolution were adopted to estimate insulin secretion based on glucose and C-peptide. The hyperglycemic clamp-derived indices were A) M/I for insulin sensitivity, B) acute (0–10 min [first phase]) C-peptide response to glucose (ACPR_g), C) steady-state C-peptide concentrations at plasma glucose of 11.1 mmol/L, and D) arginine-stimulated maximum C-peptide response at plasma glucose >25 mmol/L (ACPR_{max}).

Results. We completed the three tests in 28 youths (15.9±2.4y, 14F, 13 NGT, 14 IGT). The NGT and IGT groups did not differ with respect to age, ethnicity, BMI, fasting glucose. No significant differences were observed between the two groups in either measure of β-cell function [ACPR_g, steady- state C-peptide, ACPR_{max}, (p=0.372, p=0.478 and p=0.230)] or in insulin sensitivity [M/I] (p=0.106). The *incretin effect* was ~30% higher in the NGT than IGT group (+28.3%[-4, 62] and -10.3%[-34.3, 14.2], p=0.022), in spite of a lower GLP-1 secretion rate during the OGTT in the NGT group (p<0.001).

Conclusion. Impairment of glucose tolerance in youths is associated with a reduced incretin effect in the absence of a significant impairment of β-cell function. The higher secretion rate of GLP-1 is suggestive for a primary incretin resistance. The incretin pathway is a potential target for therapeutic interventions in youth onset prediabetes.

Introduction

Prediabetes includes a wide range of dysglycemic phenotypes that, in obese youths, anticipate the onset of overt diabetes and is accompanied from a progressive decline of β -cell function, in the presence of reduced insulin sensitivity.^{1,2} The mechanisms involved in the progression to overt diabetes include primary defects of β -cell function in the context of an higher insulin resistance,³⁻⁵ reduced hepatic insulin clearance,⁶ impaired glucose effectiveness⁷ and gut-derived hormones alterations.⁸ The two incretin hormones, glucagon like peptide 1 (GLP-1) and glucose-dependent insulintropic peptide (GIP) play a key role in gut-mediated regulation of β -cell function, with GLP-1 analogues being one of the forefront drugs for the treatment of diabetes in adults.⁹ However, we still lack of evidence for the role of the gut- β -cell axis role in prediabetes as well as in progression to overt diabetes.¹⁰

The so called “*incretin effect*” represents the phenomenon featured by a higher insulin response after an oral glucose load compared with isoglycemic intravenous glucose infusion due to the gut derived incretin hormones.

The use of matched oral glucose tolerance test and intravenous glucose tolerance test is the gold standard measure to estimate the incretin effect.¹¹ A reduced incretin effect has been demonstrated in adults with type 2 diabetes^{12,13} and suggested from observations deriving from an exploratory pediatric study.⁸

The clinical relevance of exploiting the mechanisms role of the incretin in prediabetes and early diabetes in youth stands in its role as therapeutic target. *To date, there are no effective treatment able to slow down the progression from prediabetes to diabetes or to preserve the residual β -cell function in obese youth with diabetes.*

Early insulin treatment failed to protect β -cell function in the pediatric arm of the Restoring Insulin Secretion (RISE) Study,¹⁴ with youths exhibiting age-specific hallmarks with respect to insulin sensitivity, glucose responsiveness and insulin clearance over time and exhibiting an unabated decline in insulin secretion. The rapid worsening of glucose tolerance in obese youth, as well as the lack of effective therapeutic options in the pre-diabetic phase that anticipates the overt diabetes, makes compelling the necessity for dissecting the determinants of such a condition in pediatric.

One of the strongest genetic determinants for type 2 diabetes in both youths and adults, the variant *rs7903146* of the transcription factor 7-like 2 gene (*TCF7L2*),¹⁵ was associated with a blunted β -cell function during the oral glucose tolerance test, as described by our group¹⁶ *TCF7L2 may act by modulating incretin sensitivity of β -cell*, therefore being responsible for the *sensitivity of β -cell to the incretins and for diabetes risk progression over time.*¹⁷

Recently, the addition of liraglutide, a GLP-1 analogue, to metformin has been paved as a promising, effective and safe therapeutic option for the treatment of type 2 diabetes in youth.⁹ Indeed, to date, insulin remains

the only rescue drug after the therapeutic failure of metformin in youth with type 2 diabetes. ¹⁴

To this end, although GLP-1 analogues could be the most promising class of drug for the treatment of type 2 diabetes in youth ⁹, their efficacy has not been proved in pre-diabetes. ^{1,2,18}

Neither OGTT based observations nor metabolic test as the hyperglycemic clamp, can dissect the role of the incretin system in prediabetes as well as primary β -cell function decline due to a primary incretin defect. The differential insulin secretion after an oral and an intravenous load, mimicking the same glucose profile as the OGTT, is the only metabolic test that can reliably estimate the incretin effect.

Hitherto, we explored the glucose-induced insulin secretion and the incretin effect in obese youth with normal and impaired glucose tolerance by the use of an hyperglycemic clamp with arginine stimulation (*glucose-induced insulin secretion and non-glucose potentiation*) and an oral glucose tolerance test matched with an iso-glycemic intravenous glucose tolerance test (*incretin effect*).

This is the first pediatric study adopting the matched iso-glycemic protocol to assess the incretin effect in youths with the contemporary use of the hyperglycemic clamp.

Methods. We studied obese/overweight adolescents with normal and impaired glucose tolerance recruited from the Yale Pediatric Obesity Clinic (New Haven, CT). ¹⁹ The Yale Pediatric Obesity cohort is followed with periodic clinical and dietary evaluations, and a yearly OGTT.

The inclusion criteria for the study were a BMI >85th percentile, for age and sex, and an age between 8-21 years at the screening visit. Subjects using medications affecting glucose metabolism, diagnosed with syndromic obesity or participating in clinical trials including a structured dietary or exercise-based intervention ²⁰ were excluded from the study. Additionally, participants who resulted positive for at least one of the autoantibodies associated with type 1 diabetes ²¹ were excluded. The study protocol was approved by the Human Investigations Committee of the Yale School of Medicine. Participants provided assent and parents provided written informed consent to participate in the study.

Eligible participants underwent **A.** an *oral glucose tolerance test* (OGTT) as previously described ¹⁹ to assess glucose tolerance **B.** an *isoglycemic intravenous glucose tolerance test* (iso-IVGTT) matched with the glucose profile during the OGTT, to estimate the incretin effect and **C.** an *hyperglycemic clamp* with arginine stimulation to estimate glucose induced insulin secretion. ^{14,22}

Oral glucose tolerance test (OGTT). Prior to the OGTT, all subjects followed a weight-maintenance diet consisting of at least 250 g of carbohydrates per day for seven days before the study, as confirmed by the fact that body weight remained stable (measured to the nearest 0.5 kg). Subjects were studied in the Yale Center for Clinical Investigation (YCCI) at 8 a.m. after a 12-hour overnight fast. After the local application of a topical anesthetic cream containing (Emla, Astra Zeneca, Wilmington, Del.), one antecubital intravenous catheter was inserted for blood

sampling, and its patency was maintained by slow infusion of normal saline. Each child then rested watching a videotape throughout the test. Two baseline samples were then obtained for measurements of plasma glucose, insulin, c-peptide. Thereafter, flavored glucose in a dose of 1.75 g per kilogram of body weight (up to a maximum of 75 g) was given orally, and blood samples were obtained every 30 minutes for 180 minutes for the measurement of plasma glucose, insulin, c-peptide and GLP-1. Of note, to further reduce the intra-subject variability often associated with the OGTT,²³ all glucose samples were immediately spun and processed at the bed side using the a YSI2700-STAT-Analyzer (Yellow Springs Instruments, Yellow Springs, OH).

In accordance with the American Diabetes Association ²³, impaired glucose tolerance (IGT) was defined as a 2-hr plasma glucose level between 140 and 199 mg/dl; impaired fasting glucose (IFG) as a fasting glucose between 100 and 125 mg/dL; type 2 diabetes (T2D) as a fasting glucose level of 126 mg per deciliter or higher or a two-hour plasma glucose level of more than 200 mg/dl. (12)

Iso-glycemic intravenous glucose tolerance test (iso-IVGTT). Over the two months following the OGTT, participants were admitted to the Yale Center for Clinical Investigation (YCCI) at 8 a.m. after a 12-hour overnight fast for an intravenous infusions of dextrose (20%), designed to reproduce the plasma glucose profile observed during the OGTT. Matched glucose profiles were obtained by repeatedly measuring plasma glucose concentrations (every 5 minutes over 180 minutes test) with frequent

adjustments of the dextrose infusion rate according to the OGTT glucose target values at each time point, as previously described.^{24,25} and Blood samples were obtained every 5 minutes for 180 minutes for the measurement of plasma glucose, and every 30 minutes for insulin, and c-peptide.

Hyperglycemic clamp. Hyperglycemic clamp was performed to assess β -cell function in the context of whole body insulin sensitivity. The test was performed within 6 months from the OGTT after an overnight fasting and consisted of a two-steps procedure. At admission, one catheter was inserted for blood sampling, and the hand was warmed for blood arterialization, a second catheter was inserted in the contralateral arm for glucose infusion. During step 1, baseline glucose, insulin and c-peptide were measured at -20 and 0 minutes before glucose infusion and the average value was used to calculate baseline values (t=0). Blood for plasma glucose was drawn every 2 minutes during the first 10 minutes and then every 5 minutes and immediately centrifuged and analyzed using the glucose oxidase method (YSI 2300 STAT Plus Glucose Analyzer, YSI Life Sciences; Yellow Springs, IL). A standardized priming 20% dextrose (Hospira, Lake Forest, IL) infusion was administered during the first 10 minutes (200 mg/kg body weight), and then infusion rates were adjusted every 5 minutes to maintain plasma glucose at 11.1 mmol/L (200 mg/dL) for 120 minutes. (24) Blood samples for subsequent assays were drawn at 2, 4, 6, 8, 10, 20, 30, 40, 60, 80, 100, 100 and 120 minutes.

During the second step, the target blood glucose 25 mmol/L (450mg/dL) was achieved using a second bolus of 20% dextrose administered over 60 seconds (volume in mL calculated as $\text{weight [kg]} * [450 - \text{current blood glucose in mg/dL}] * 1.1 / 180$) as previously described.¹⁴ The dextrose infusion rate was adjusted according to bedside blood glucose monitoring every 5 min. Once the target blood glucose was attained for a minimum of 30 min, a bolus of 5g L-arginine was administered over 1 min. Blood samples for subsequent assays were drawn at -5, -1, 2, 3, 4, and 5 min relative to the arginine injection.¹⁴

Biochemical analysis. Plasma insulin was measured by radioimmunoassay (Linco, St. Charles, MO) that has <1% cross-reactivity with c-peptide and proinsulin. Plasma C-peptide levels were determined with an assay from Diagnostic Product (Los Angeles, CA). *Active* GLP-1 was measured by radioimmunoassay (EMD Millipore Corporation, Billerica, MA, USA). The assay used an antibody specific to GLP-1 (7-36) and (9-36) with no cross-reactivity with GLP-2, GIP, Glucagon or Oxyntomodulin. Intra-assay and inter-assay variability were <5% and <12% respectively.

Genotyping. Genomic DNA was extracted from peripheral blood leukocytes. Genotyping was performed with the use of a matrix-assisted-based laser desorption-ionization time-of-flight mass spectrometry on the MassARRAY platform (Sequenom) through the Yale Center for Genome Analysis. (22) Genotype for the SNP *rs7903146* at *TCFL2* was available for the entire cohort.

Calculations.

Clamp derived measures. Acute (first-phase) C-peptide (ACPR_g) to glucose was calculated as the mean incremental response above baseline (average of -10 and -5 min) from samples drawn at 2, 4, 6, 8, and 10 min after intravenous dextrose administration (17). Steady-state (second-phase) C-peptide concentrations were calculated as the mean of the respective measurements at 100, 110, and 120 min of the hyperglycemic clamp. (16) Acute C-peptide (ACPR_{max}) response to arginine at maximal glycemic potentiation (>25 mmol/L) was calculated as the mean concentrations in samples drawn 2, 3, 4, and 5 min after arginine injection minus the average concentration of the samples drawn 1 and 5 min prior to arginine. ¹⁴

Incretin effect and OGTT/Iso-IVGTT derived measures. β -Cell secretion was estimated from the C-peptide measured during the 180 minutes OGTT using the oral c-peptide minimal model. ²⁶⁻²⁸ The model assumes that glucose stimulated insulin secretion (φ_{total}) is made up of two components: 1. a dynamic component, named φ_d (10^{-9}) or dynamic responsivity index, representing secretion of promptly releasable insulin and proportional to the rate of glucose increase; 2. a static component, deriving from provision of new insulin to the releasable pool and characterized by a static index, φ_s (10^{-9} min^{-1}), and by a delay time constant, T (min). ²⁹ A similar approach was estimated using the C-peptide and glucose measures during the iso-IVGTT. ¹¹

Insulin secretion rate was estimated as previously described ^{26,30-32}

from C-peptide and glucose seriated measures:

$$\begin{cases} \dot{CP}_1(t) = -[k_{01} + k_{21}] \cdot CP_1(t) + k_{12} \cdot CP_2(t) + SR(t) & CP_1(0) = CP_{1b} \\ \dot{CP}_2(t) = k_{21} \cdot CP_1(t) - k_{12} \cdot CP_2(t) & CP_2(0) = CP_{2b} = CP_{1b} \frac{k_{21}}{k_{12}} \end{cases}$$

with CP1 and CP2 (pmol/l) being C-peptide concentrations in the accessible and peripheral compartments, respectively, and k01, k12, and k21 (min⁻¹) are C-peptide kinetic parameters fixed to standard values (30) to ensure numerical identification of the overall model. The SR is linked, by the model, to plasma C-peptide concentration by the two-compartment model of C-peptide kinetics proposed by Eaton et al.³³

The Incretin effect was estimated, as previously described ¹¹, as follow:

$$\text{Incretin effect (\%)}: 100 * \frac{\phi_{\text{total}}^{\text{OGTT}} - \phi_{\text{total}}^{\text{Iso-IVGTT}}}{\phi_{\text{total}}^{\text{Iso-IVGTT}}}$$

Where $\phi_{\text{total}}^{\text{OGTT}}$ and $\phi_{\text{total}}^{\text{Iso-IVGTT}}$ quantify insulin secretion based on C-peptide seriated measurements during the OGTT and the Iso-IVGTT. This approach has been described by Campioni et al. ¹¹

Therefore, the static and dynamic component were estimated by the same approach adopting ϕ_{static} and ϕ_{dynamic} as estimated during the two tests.

The GLP-1 secretion rate was estimated during the OGTT as described by Dalla Man et al ³²:

$$SR_{\text{GLP-1}} = SR * ((a * \text{GLP-1} + b * \ln(d\text{GLP-1})) + 1)$$

with a and b model parameters representing the percentage increase of Delta-SR due to the GLP-1 and GLP-1 rate of change, respectively and

dGLP-1 the derivative term for GLP-1 at each time point. SR_{GLP1} was estimated from the OGTT seriated GLP-1 measurements.

Statistical analysis. All the analyses were stratified by baseline NGT/IGT grouping variable. The primary outcome was the incretin effect estimated as percentage value from the ϕ term, as described above. The area under the curve for glucose, insulin, C-peptide, SR at 30 and 180 minutes, were reported for both the OGTT and the iso-IVGTT.

Distribution of continuous variables was examined for skewness and kurtosis. Non normally distributed continuous variables were analyzed by Kruskal-Wallis test, followed by post-hoc pair-wise Dunn-test, while normally distributed variables were compared by t-Student paired test. Categorical variables were compared using the Chi-square test. Data were summarized in tables using median (25th percentile, 75th percentile) for continuous variables and count (percent, %) for categorical variables.

Analyses were performed using STATA.13 software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) and Prism 8.0 software (GraphPad Software, La Jolla, CA). Model analyses were performed by the use of SAAMII 2.3 (The Epsilon Group) and Matlab R2018a.

Results.

Participants characteristics. We enrolled 30 participants, 28 completed the three tests. The two groups were matched for age,

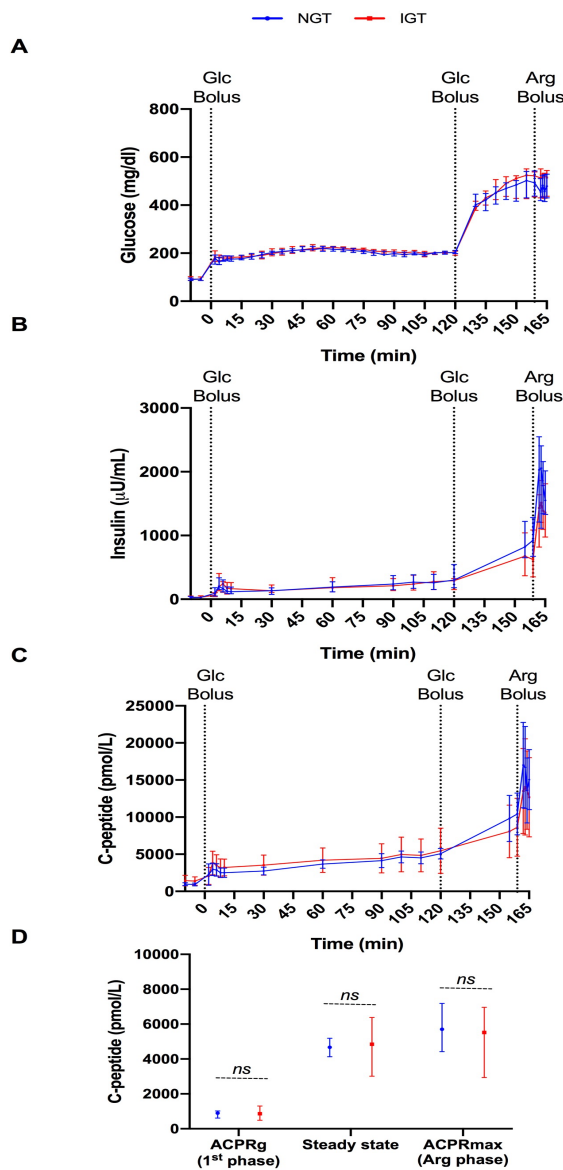
ethnicity, sex, BMI and pubertal developmental stage. Fasting glucose and insulin did not differ between the two groups, as well as the distribution of the T risk allele for *TCF7L2* (**Table 1**). Cohort's features are detailed in **Table 1**. All the participants had normal glucose tolerance within the 12 months preceding the screening OGTT. Glucose tolerance was defined according to the screening OGTT.

Participants underwent an oral glucose tolerance test, an iso-glycemic intravenous glucose tolerance test and a hyperglycemic clamp.

Table 1. Participants characteristics			
	NGT (n=13)	IGT (n=15)	p
Age (y)	16 (14, 18)	15 (14, 17)	0.588
BMI (kg/m ²)	37 (32.7, 41.0)	39.8 (32.7, 44.9)	0.257
Tanner Stage n (%) II-III IV-V	6 (46) 7 (54)	9 (60) 6 (40)	0.464
Ethnicity NHW/NHB/H	3/5/5	2/8/5	0.686
Sex	7(50)	7(50)	0.999
Fasting Glucose (mg/dl)	93 (89, 96)	96.5 (87.5, 100)	0.383
IFG n (%)	2 (13)	3 (23)	0.750
2h-Glucose (mg/dl)	125 (119, 129)	152 (145, 167)	<0.001
<i>TCF7L2</i> genotype CC/CT/TT	7/0/6	8/3/4	0.264
Fasting Insulin (microU/mL)	29.5 (20, 41)	41.5 (24, 62.5)	0.097
Fasting C-peptide (pmol/L)	1100.5 (871, 1286)	1307.5 (956, 1919.5)	0.053
Fasting GLP-1 ₍₇₋₃₆₎ (pmol/L)	3.10 (1.69, 13.30)	1.11 (0.96, 2.06)	0.058
NGT, normal glucose tolerance; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; BMI, body mass index; NHW, non-Hispanic White; NHB, non-Hispanic Black; H, Hispanic.			

β-cell glucose induced insulin secretion (hyperglycemic clamp).

As illustrated in **Figure 1A**, glucose profiles during the hyperglycemic clamp did not differ, with similar glucose values at baseline ($p=0.285$) and steady state ($p=0.883$), proving the robustness of the test.



No significant differences were observed between the two groups in either measure of β -cell function [ACPR_g, steady-state C-peptide and ACPR_{max}] ($p=0.372$, $p=0.478$ and $p=0.230$) or in insulin sensitivity [M/I] ($p=0.106$). (**Figure 1B-D**) The ACPR_{max} was higher in the NGT group without reaching statistical significance.

Figure 1. Hyperglycemic Clamp. Glucose (A), Insulin (B), C-peptide (C) profiles and C-peptide derived metrics (D). ACPR_g, acute-C-peptide glucose response; ACPR_{max}, Acute-C-peptide response to Arginine and maximal glucose load; Glc bolus, glucose bolus; Arg bolus, arginine bolus. NGT, normal glucose tolerance; IGT, impaired glucose tolerance. Data are reported as median, interquartile range.

Incretin effect. (OGTT and iso-IVGTT)

Glucose profiles of the oral and iso-glycemic intravenous glucose tolerance test were matched between the two groups, with a difference between the AUC of the two curves <5%, and similar baseline glucose values (p=0.442). (**Figure 2A**)

The insulin and C-peptide profiles were significantly higher during the oral than the intravenous glucose tolerance test (**Table 2, Figure 2B, 2C, 2F, 2G**), as expected, in both NGT and IGT. Therefore, the incretin effect, describing the difference in insulin secretion, based on C-peptide measurements, during the two tests, was quantified by the φ_{total} and its components ($\varphi_{dynamic}$ and φ_{static}) as described above.

The median incretin effect (φ_{total} incretin) was ~30% lower in the IGT groups (-10% [-34, 14]) than the NGT (28%[-4, 62]) (p=0.022). The overall incretin effect was, therefore, dissected into its static (φ_{static}) and dynamic ($\varphi_{dynamic}$) components. The static incretin effect was lower in the IGT group (p=0.014), with similar dynamic component between the two groups (p=0.210).

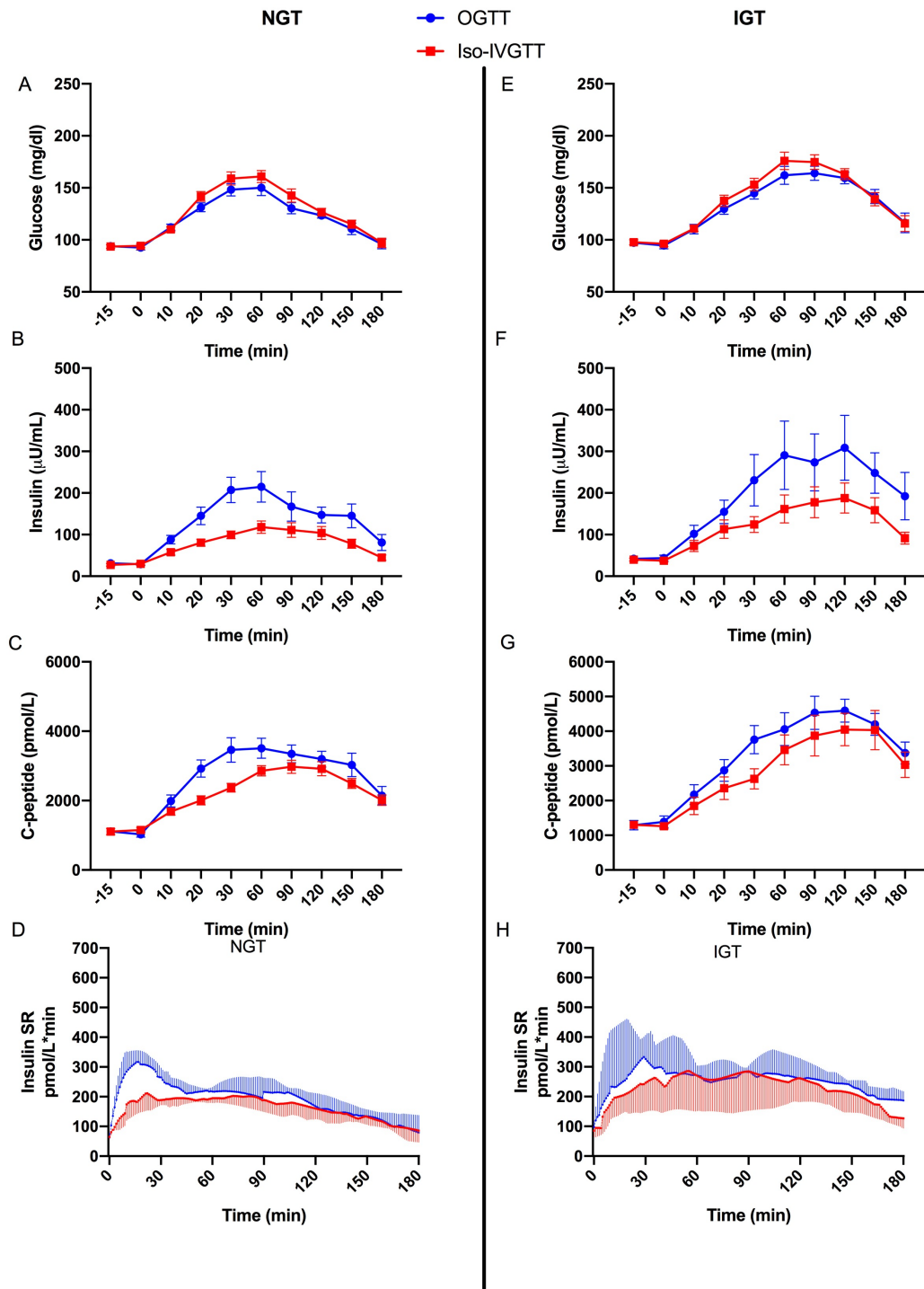


Figure 2. Glucose (A, E), Insulin (B, F), C-peptide (C, G) and insulin Secretion Rate (D, H) for normal (NGT) and impaired (IGT) glucose tolerance groups. OGTT, oral glucose tolerance test; iso-IVGTT, iso-glycemic glucose tolerance test. Data are expressed as median and interquartile range. In panel D and H the lower and upper quartile for OGTT and iso-IVGTT respectively have been omitted to highlight the incretin effect that can be quantified as the white area between the median values of the two tests.

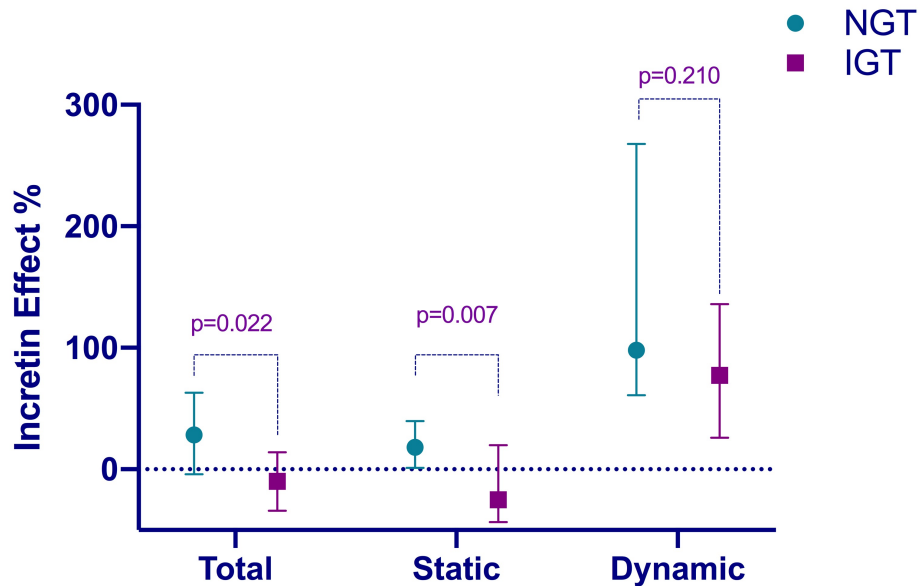


Figure 3. Incretin effect based on the minimal model estimate of insulin secretion (ϕ) during OGTT and iso-IVGTT for the total, static and dynamic components of the insulin secretion. Data are expressed as median, interquartile range.

Table 2. Glucose, Insulin and C-peptide profiles during the OGTT and the iso-IVGTT		
	NGT (n=13)	IGT (n=15)
Glucose AUC (mg/dL*min)		
OGTT	24134±954.2	27769±1243
Iso-IVGTT	25387±959.6	28799±1315
p	0.0013	0.0036
Insulin AUC (microU/L*min)		
OGTT	28518±5151	44534±11788
Iso-IVGTT	16949±2378	27050±5454
p	<0.0001	<0.001
C-peptide AUC (pmol/L*min)		
OGTT	560843±5199	724668±6952
Iso-IVGTT	474653±2883	628242±8350
p	<0.001	<0.001
OGTT, oral glucose tolerance test; Iso-IVGTT, isoglycemic intravenous glucose tolerance test; AUC, area under the curve; SR, secretion rate.		

As shown in **Figure 2D** and **2H**, adopting the area under the curve of the insulin secretion rate as a surrogate measure for incretin effect (that can be visualized as the white space between the secretion rate for NGT and IGT group) was greater during the first 30 minutes of the test, with an higher incretin effect in the NGT group ($AUC_{SR[0-30]}$ 122.2%(73, 140) vs 45%(30.4, 55.5) for the NGT and the IGT groups respectively ($p=0.010$)).

The incretin effect over the 180 minutes test did not differ between the two groups ($p=0.765$).

The GLP-1 secretion rate was higher in the IGT than the NGT group during both the first phase (0-30 minutes) and the whole test (0-180 minutes) ($AUC_{SR[0-180]}$ 35621pmol/L*min(34205, 37037) vs 48992pmol/L*min(47478, 50506), $p<0.001$; $AUC_{SR[0-30]}$ 7893pmol/L*min(7142,8643) vs 9004pmol/L*min(8079, 9928), $p<0.001$).

(Figure 4)

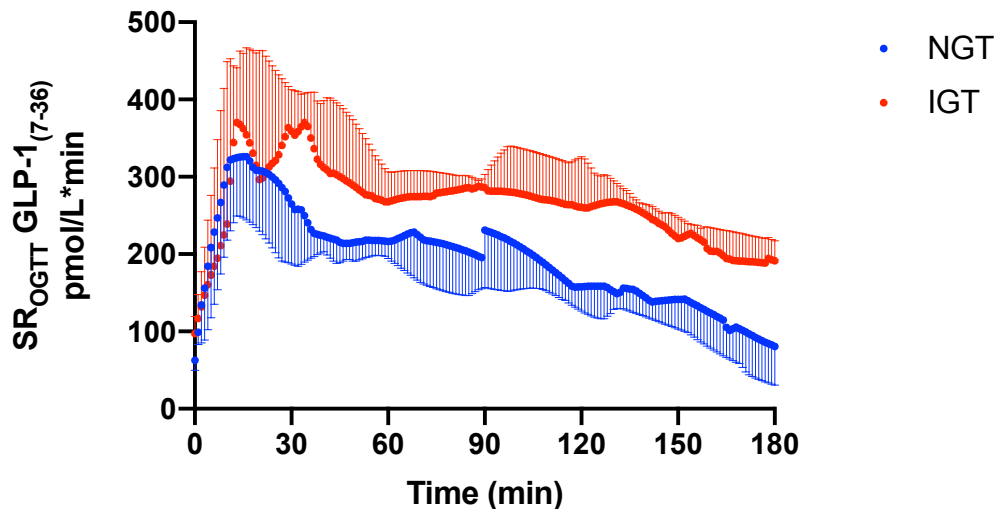


Figure 4. GLP-1(7-36) secretion rate. Data are expressed as median, interquartile range. Upper and Lower quartiles have been reported, respectively, for IGT and IGT.

Discussion

The impairment of glucose tolerance in overweight/obese youths is featured by a reduced incretin effect, as estimated through the matched OGTT and iso-glycemic intravenous glucose tolerance test (iso-IVGTT), in the absence of an impairment in glucose-stimulated β -cell secretion, as described by the hyperglycemic clamp.

The use of the gold standard iso-glycemic protocol to estimate incretin effect is a major point of strength of this study. Indeed, the adoption of minimal model based estimate of insulin secretion, relying on the seriated C-peptide measures, is unbiased from insulin differential clearance and from variability in matching the OGTT and iso-IVGTT that might affect, at some extent the estimated secretion using the secretion rate (SR) or the AUC approaches, elsewhere described.²⁴ In spite of the robustness of the approach, the incretin effect estimates describe a remarkable variability within the studied population, that has to be taken into account while interpreting the study's results. One of the determinants of intrasubject and inter-subjects variability resides in the dietary content, that differentially prime the incretin response prior to the test.^{34,35} Indeed, to minimize this potential confounder, participants were instructed to follow dietary recommendation one week prior to the three admissions as well as to avoid physical activity.

The φ_{total} based estimate of the incretin effect was, therefore, dissected into the static (φ_{static}) and dynamic (φ_{dynamic}) components of insulin

secretion as described.²⁶ The static component, indeed, represents the insulin release proportional to the difference between the actual and the baseline glucose. (5; 19) It has been referred to as β -cell “glucose responsiveness” (20) and is influenced by the time of gastric emptying and the incretin response (33). In contrast, the dynamic insulin secretion describes the early release of insulin. (19) We observed a reduced total incretin effect, consistently with a prevailing reduction in the static component, that is influenced by the incretin response. Similarly, when the incretin effect was estimate by the use of insulin secretion rate (SR) the first phase (SR_{0-30}) was reduced in the IGT group, while we couldn't observe any significant difference during the late phase of the tests.

The ~30% reduction in incretin effect we observed has been described in adults with type 2 diabetes using the iso-glycemic protocol^{12,36} and a similar decrease has been estimated in youth with IGT adopting surrogate estimates derived from the sole OGTT and the hyperglycemic clamp⁸ in the absence of the matched iso-IVGTT.

We observed a primary defect in the incretin response in the IGT group, in the absence of a reduced glucose-induced insulin secretion as described from the hyperglycemic derived clamp indices. Indeed, neither the ACPRg (acute c-peptide response to glucose), nor the steady state c-peptide as well as the $ACPR_{max}$ differed between the two groups, suggesting that the incretin deficit might be an early feature of prediabetes in youths. This finding can be justified, in spite of the different glucose tolerance of the two groups, with the recent onset of IGT of this cohort.

Indeed, participants from the Yale Pediatric Obesity Clinic are followed up with yearly OGTT and the 15 youths we enrolled received the diagnosis of IGT at the screening OGTT. In spite of the absence of a longitudinal observation, our findings are suggestive for an early role of the incretin effect in the development of IGT that anticipates the β -cell failure estimated by the use of the hyperglycemic clamp.

The reduced incretin effect in the IGT was not paralleled, in our cohort, by a contemporary decrease in GLP-1 response during the OGTT. Indeed, we observed an almost doubled GLP-1 response in the IGT group after the oral glucose load. These two findings are suggestive for an incretin resistance, instead of an incretin deficiency, as a pivotal mechanism in determining the reduced insulin secretion in the IGT group. GLP-1 has been described as normal,³⁷ increased^{8,38} or decreased¹³ in adults with IGT in adults and youths. The reliability of the current assays for the active form, the lack of the contemporary measurements of both active and total GLP-1 could have influenced the observed results. Though, recently, one of the strongest genetic risk determinant for T2D in both adults and youths, the *rs7903146* T allele in the transcription factor 7-like 2 gene (*TCF7L2*) has been demonstrated to cause an incretin resistance in adults, suggesting that incretin resistance, more than incretin deficiency, could play a key role in the early phase of dysglycemia.¹⁷ We genotyped our cohort, to this purpose, for the *rs7903146* SNP of *TCF7L2* *but* the distribution of the genotype did not change between the two

groups, although a gene-oriented study design, could address this question.

Though, the current study has several limitations. The use of oral glucose tolerance test instead of a mixed meal test prevented us from dissecting the role of other nutrients, including carbohydrates other than glucose, that may in turn influence the incretin secretion. Indeed, it has been described that fructose and glucose can differentially trigger GLP-1 secretion, with the former enhancing insulin secretion more than glucose by itself.^{35,39,40} The lack of measurements of total GLP-1 cannot exclude that GLP-1 conversion rate to the active form can contribute to the observed difference in the GLP-1 secretion rates.⁴¹ Additionally, the incretin effect, as estimated with the use of matched OGTT and isoglycemic intravenous test, accounts for the effect of both GLP-1 and GIP, that we did not measure in the current study. The matched anthropometric and metabolic features of the two groups, in the absence of a longitudinal assessment, do not rule out that the reduced incretin effect could be a consequence of hyperglycemia and not its cause,⁴² even though the recent-onset hyperglycemia, as above mentioned, is suggestive for a causative role of the incretin effect in determining the impaired glucose phenotype.

Our findings are supports a role for the incretins in the development of impaired glucose tolerance in obese youths. The reduced incretin effect, thereby, paralleled by an enhanced GLP-1 secretion, are suggestive for a primary β -cell resistance to the incretin stimulation, that could be a

potential pharmacological target for the early treatment of prediabetes, that, to date, still lack approved pharmacological interventions in youths.

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“Lastly, she pictured to herself how this same little sister of hers would, in the after-time, be herself a grown woman; and how she would keep, through all her riper years, the simple and loving heart of her childhood: and how she would gather about her other little children, and make their eyes bright and eager with many a strange tale, perhaps even with the dream of Wonderland of long ago: and how she would feel with all their simple sorrows, and find a pleasure in all their simple joys, remembering her own child-life, and the happy summer days.” (Lewis Carroll, 1865)

Although the many consider youth onset diabetes as a disease of “*poverty*”, as in US and Europe it prevails in certain ethnic groups and low-income families, it does not make it a disease we can overlook, leaving its primary care to “*politics*”. Indeed, if “*politics*” can be defined as action, commitment, protection of rights, we, as pediatricians and researchers, do a “*political*” job as we are committed to protect and care for those who cannot do it on their own, our kids. Therefore, we are committed to the “*care*” and the “*cure*” of their conditions.

Understanding the mechanism of a disease, as this thesis attempts to do means moving one step toward the most effective and “affordable” care, for this condition.

We cannot really cure the “*poverty*” as a risk factor for youth onset diabetes, but we can provide the best *care* for this condition that is, probably, the first step toward the “*care*” for the disease itself and, probably, for “*poverty*” itself. Therefore, “*care*” becomes a political task.

No matter how much we will struggle, we may fail in finding “*the cure*”, but we cannot stop us from seeking for it and we cannot prevent us from providing “*the care*” that our patients are in need for.

The care can protect the childhood memory of our patients, make it joyful, and let them to project their adult life, remembering, as Lewis Carroll wrote of his Alice, their “*own child-life and the happy summer days*” as a treasure.

This is one of our tasks as pediatricians, giving our patients a happy memory of their childhood and a hopeful projection of the adulthood, they want to live in.

And even if we can actually “*cure*” only a little amount of pediatric onset disease, we can “*care*” of all of them. *We have to.*

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