Articles in PresS. Am J Physiol Endocrinol Metab (February 28, 2017). doi:10.1152/ajpendo.00464.2016

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3 # Insulin resistance and normal thyroid hormone levels:
4 # prospective study and metabolomic analysis.

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8

9 **Abbreviated title: Thyroid hormones and insulin resistance**

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26 Word Count Abstract: 250
27 Word Count Main Text: 3,591
28 Tables: 4
29 Figures: 4
30

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34 **Abstract**

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36 While hyper/hypothyroidism causes dysglycemia, the relationship between thyroid hormone
37 levels within the normal range and insulin resistance (IR) is unclear. In 940 participants with
38 strictly normal serum concentrations of free triiodothyronine (fT_3), free thyroxine (fT_4), and
39 thyroid-stimulating hormone (TSH)) followed up for 3 years, we measured insulin sensitivity (by
40 the insulin clamp technique) and a panel of 35 circulating metabolites. At baseline, across quartiles
41 of increasing fT_3 levels (or fT_3/fT_4 ratio) there emerged most features of IR (male sex, higher BMI,
42 waist circumference, heart rate, blood pressure, fatty liver index, free fatty acids, and triglycerides
43 levels, reduced insulin-mediated glucose disposal and ß-cell glucose sensitivity). In multiadjusted
44 analyses, fT_3 was reciprocally related to insulin sensitivity and, in a subset of 303 subjects, directly
45 related to endogenous glucose production. In multiple regression models adjusting for sex, age,
46 BMI and baseline value of insulin sensitivity, higher baseline fT_3 levels were significant predictors
47 of the decreases in insulin sensitivity. Moreover, baseline fT_3 predicted follow-up increases in
48 glycemia independently of sex, age, BMI, insulin sensitivity, ß-cell glucose sensitivity and baseline
49 glycemia. Serum tyrosine levels were higher in IR and were directly associated with fT_3; higher
50 α–hydroxybutyrate levels signaled enhanced oxidative stress impairing tyrosine degradation. In 25
51 morbidly obese patients, surgery-induced weight loss improved IR and consensually lowered fT3.
52 High-normal fT_3 levels are associated with IR both cross-sectionally and longitudinally, and predict
53 deterioration of glucose tolerance. This association is supported by a metabolite pattern that points
54 at increased oxidative stress as part of the IR syndrome.

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56

57 **Introduction**

58

59 Triiodothyronine (T_3), the biologically active thyroid hormone, has long been known to
60 influence metabolism by modulating cellular respiration (35). In the disease range, both
61 thyrotoxicosis and frank hypothyroidism are associated with glucose intolerance (3, 4, 9, 17, 18,
62 26). Mechanisms include effects of T_3 on insulin action, hepatic gluconeogenesis, lipolysis and
63 lipid oxidation, intracellular insulin signaling, and ß-cell function (reviewed in 8). Whether the
64 association between T_3 and insulin sensitivity extends to the normal serum T_3 concentration range
65 is, however, less clear. Thus, Roef and co-workers reported that in healthy euthyroid young men
66 higher levels of both free T_3 (fT_3) and free T_4 (fT_4) were associated with higher body fat mass and
67 insulin resistance (estimated as the homeostasis model assessment for insulin resistance, HOMA-
68 IR) (28). On the other hand, Roos et al. (29) found that low normal fT_4 – but not fT_3 – levels were
69 associated with HOMA-IR. Likewise, high normal fT_3 clustered with components of the metabolic
70 syndrome in euthyroid Japanese patients with type 2 diabetes (34), but a low T_3/T_4 ratio was
71 associated with hyperinsulinemia and insulin resistance in prediabetic Pakistani subjets (10).
72 Even less clear is the relation of euthyroid hormone levels to risk of incident metabolic
73 disturbances (8). Thus, while Roos et al. (29) reported a cross-sectional association between low
74 normal fT_4 and dyslipidemia (higher serum LDL-cholesterol and triglycerides), in a longitudinal
75 study of young mean and women in southern Spain (32) higher fT_4 or fT_3 at baseline predicted a
76 greater increment in body weight 6 years later.
77 There are multiple possible explanations for these discrepancies and uncertainties, such as
78 small size of study groups, mostly cross-sectional nature of the data, and use of crude proxies of
79 insulin resistance. Interestingly, for plasma glucose and arterial blood pressure higher baseline
80 levels are predictive of, respectively, diabetes and hypertension, as is expected of tracking variables
81 along a continuous distribution. In the case of thyroid hormones, however, dysglycemia and a
82 degree of insulin resistance (mostly hepatic in the case of thyrotoxicosis (23)) emerge at both

83 extremes of the distribution of circulating hormone levels, while the pattern of associations within
84 the normal range remains, as discussed above, uncertain.
85 We carried out a complete cross-sectional and longitudinal analysis of the relationships
86 between thyroid hormones and insulin resistance in an large cohort of strictly euthyroid individuals
87 in whom insulin sensitivity was measured directly by the hyperinsulinemic euglycemic clamp
88 technique. In addition, we used targeted metabolomics to gain a broader view of the associated
89 metabolic signatures.
90
91 **Materials and Methods**
92
93 *Study cohorts* RISC is a prospective, observational, multicenter cohort study whose rationale
94 and methodology have been published previously (16). In brief, participants were recruited
95 according to the following inclusion criteria: either sex, age 30–60 years, BMI 17–44 kg/m^2, and
96 clinically healthy. Initial exclusion criteria were treatment for obesity, hypertension, lipid disorders
97 or diabetes, pregnancy, cardiovascular or chronic lung disease, weight change of ≥5 kg in past
98 month, cancer (in past 5 years), and renal failure. Exclusion criteria after screening were arterial
99 blood pressure ≥140/90 mmHg, fasting plasma glucose ≥7.0 mmol/L, 2-hour plasma glucose (on a
100 standard 75-g OGTT performed in each subject) ≥11.0 mmol/L or known diabetes, total serum
101 cholesterol ≥7.8 mmol/L, serum triglycerides ≥4.6 mmol/L, and electrocardiographic abnormalities.
102 Baseline examinations included 1,538 subjects receiving an OGTT. Of these, 1,267 subjects also
103 received a euglycemic-hyperinsulinemic clamp; their baseline data have been published (11). At
104 baseline, subjects were classified as normal glucose tolerance (NGT), impaired fasting glycemia
105 (IFG), or impaired glucose tolerance (IGT) according to ADA definitions (2).
106 All 1,267 subjects of the baseline clamp cohort were recalled 3 years later and 1,040 (82%)
107 participated in the follow-up evaluation. The follow-up study included all the baseline
108 measurements except for the glucose clamp. Baseline measurements of circulating thyroid
109 hormones were obtained in 1,018 participants; of these, 1,000 had follow-up data. Local ethics

110 committee approval was obtained by each recruiting center; participants signed a written consent
111 form.

112 A second cohort included 25 patients (18 women and 7 men, aged 43 ± 8 years) undergoing
113 elective bariatric surgery (percutaneous Roux-en-Y gastric bypass, described in ref. 24) for morbid
114 obesity (BMI 45.9 ± 5.4 kg/m^2). In these patients, laboratory measurements and a standard 75-g
115 OGTT were obtained before surgery and 1 year later, when their body weight had stabilized.

116 *OGTT* Blood samples were taken before and at 30, 60, 90, and 120 min into the OGTT. Blood
117 samples were separated into plasma and serum, aliquotted, and stored at -80°C for glucose, insulin,
118 and C-peptide determination. Samples were transported on dry ice at prearranged intervals to
119 central laboratories.

120 *Insulin clamp* On a separate day within 1 week of the OGTT, a euglycemic hyperinsulinemic
121 clamp was performed in all subjects. Exogenous insulin was infused at a rate of 240 $pmol \cdot min^{-1} \cdot m^{-2}$
122 simultaneously with a variable 20% dextrose infusion adjusted every 5–10 min to maintain plasma
123 glucose level within 0.8 mmol/l (±15%) of the target glucose level (4.5–5.5 mmol/l). In a subset of
124 303 subjects, endogenous glucose production (EGP) was measured by the tracer glucose technique,
125 as described (16).

126 *Analytical procedures* Plasma glucose was measured by the glucose oxidase technique. Serum
127 insulin was measured by a specific time-resolved immunofluorometric assay (AutoDELFIA, Insulin
128 kit, Wallac Oy, Turku, Finland) with the following assay characteristics: detection limit >3 pmol/L,
129 intra- and interassay variation 1.7 and 3.5%, respectively. The intra- and interassay coefficient of
130 variation was <5 and <10%, respectively. Free fatty acids (FFA) were assayed by a fluorimetric
131 method (Wako, Neuss, Germany). Serum adiponectin was determined by an in-house time-
132 resolved immunofluorometric assay (TR-IFMA, R&D Systems, Abingdon, UK). Glucagon was
133 assayed using an antibody highly specific for the free C-terminus of the molecule (pancreatic
134 glucagon), with the following assay characteristics: normal range (5–20 pmol/L), sensitivity <1
135 pmol/L; within-assay CV <5% at 20 pmol/L; and between-assay CV <12%. Serum levels of fT_3

136 (reference range 2-4 pg/mL), fT_4 (reference range 7-17 pg/mL), TSH (reference range 0.25-4.5
137 μIU/mL), anti-peroxidase (reference value ≤10 IU/mL) and anti-thyroglobulin antibodies (reference
138 value ≤30 IU/mL) were measured on a Tosoh AIA 600/21/1800 automated immunoassay analyzer.

139 *Quantitative (targeted) assays (35 metabolite panel)* For absolute quantitation, metabolites
140 were analyzed by isotope dilution UHPLC-MS/MS assays using two similar methods (7, 13). In
141 brief, in the first method, 50 μl of EDTA plasma samples were spiked with internal standard
142 solution and subsequently subjected to protein precipitation by mixing with 250 μl of methanol.
143 Following centrifugation, aliquots of clear supernatant were injected onto an UHPLC-MS-MS
144 system consisting of a ThermoTSQ Quantum Ultra Mass Spectrometer and a Waters Acquity
145 UHPLC system equipped with a column manager module and two different columns. Each sample
146 was analyzed using two different chromatographic systems to cover the various analytes. Octanoyl
147 carnitine, decanoylcarnitine, glutamic acid, threonine, tryptophan, betaine, palmitoyl-
148 glycerophosphocholine (palmitoyl-GPC), and oleoyl-glycerophosphocholine (oleoyl-GPC) were
149 eluted with a 0.01% formic acid in water/acetonitrile-water-ammonium formate (700:300:2.7)
150 gradient on a Thermo, BioBasic SCX column. Ionization was achieved by positive HESI mode.
151 Palmitic acid, palmitoleic acid, margaric acid, stearic acid, linolenic acid, adrenic acid, and linoleic
152 acid, were eluted isocratically with 15% 5 mM ammonium bicarbonate in water and 85%
153 acetonitrile-methanol (1:1) on a Waters, Acquity BEH C18 column. Ionization was achieved by
154 negative HESI mode. Quantitation was performed based on the area ratios of analyte and internal
155 standard peaks using a weighted linear least squares regression analysis generated from fortified
156 calibration standards in an artificial matrix, prepared immediately prior to each run. The following
157 corresponding stable labeled compounds were used as internal standards: palmitic acid-$^{13}C_{16}$,
158 margaric acid-d_3, stearic acid-d_3, linoleic acid-$^{13}C_{18}$, linolenic acid-$^{13}C_{18}$, (also used for palmitoleic
159 acid), octanoyl carnitine-d_3, decanoyl-carnitine-d_3, glutamic acid-d_5, threonine-$^{13}C_4$-^{15}N,
160 tryptophan-d_5, and linoleoyl-glycerophosphocholine (L-GPC)-d_9 used for palmitoyl-GPC and
161 oleoylGPC).

162 In the second method, 50 µl of EDTA plasma were spiked with stable labeled internal
163 standards and subsequently subjected to protein precipitation by mixing with 200 µl of 1% formic
164 acid in methanol. Following centrifugation, aliquots of clear supernatant were injected onto an
165 Agilent 1290/AB Sciex QTrap 5500 mass spectrometer LC-MS/MS system equipped with a turbo
166 ion-spray source using three different chromatographic systems (mobile phase/column
167 combinations). α–hydroxybutyrate, β–hydroxybutyrate, 3-hydroxyisobutyric acid, 3-methyl-2-
168 oxobutyric acid (3MOB), 3-methyl-2-oxopentanoic acid (3MOP), 4-methyl-2-oxopentanoic acid
169 (4MOP), L-GPC, and oleic acid were eluted with a gradient (mobile phase A: 0.01% formic acid in
170 water; mobile phase B: acetonitrile/methanol 1:1) on a Waters Acquity C-18 BEH column (2.1 mm
171 x 100 mm, 1.7 µm particle size) and detected in negative mode. 2-aminoadipic acid (2-AAA),
172 creatine, glycine, isoleucine, leucine, phenylalanine, serine, and tyrosine were eluted with a gradient
173 (mobile phase A: 0.05% perfluoropentanoic acid in water; mobile phase B: 0.05%
174 perfluoropentanoic acid in acetonitrile) on a Waters Acquity C-18 BEH column (2.1 mm x 100 mm,
175 1.7 mm particle size) and detected in positive mode. To an additional 60 µl aliquot of clear
176 supernatant, 60 µl of a 2,4-dinitrophenylhydrazine solution (1% 2,4-dinitrophenylhydrazine in
177 acetonitrile/acetic acid 85:15) were added. An aliquot of the reaction mixture was injected onto a
178 Waters Acquity/Thermo Quantum Ultra LC-MS/MS system equipped with a HESI source. The
179 2,4-dinitrohydrazone derivatives of α–ketoglutaric acid and α–ketobutyric acid were eluted with a
180 gradient (mobile phase A: water/ammonium bicarbonate, 500:1; mobile phase B:
181 acetonitrile/methanol,1:1) on a Waters Acquity C-18 BEH column (2.1 mm x 50 mm, 1.7 mm
182 particle size) and detected in negative mode.

183 Quantitation was performed as in the first method. Stable isotope labeled compounds
184 α–hydroxybutyrate-d_3, β–hydroxybutyrate-d_4 (used for both β–hydroxybutyrate and 3-
185 hydroxyisobutyric acid), 2-AAA-d_3, 3-MOB-d_7, 4MOP-d_3 (used for both 3MOP and 4MOP),
186 α–ketobutyric acid-$^{13}C_4$, α–ketoglutaric acid-$^{13}C_4$-$^{15}N_2$, creatine-d_3, glycine-$^{13}C_2$-^{15}N, isoleucine-

187 $^{13}C_6$, leucine-d_3, L-GPC-d_9, oleic acid-$^{13}C_{18}$, phenylalanine-d_8, serine-d_3, tyrosine-d_4, and valine-
188 $^{13}C_5$-^{15}N, were used as internal standards.

189 *Data analysis* Insulin sensitivity of glucose disposal was calculated as the M value during the
190 final 40 min of the 2-hour clamp (normalized to the FFM and the mean plasma insulin
191 concentration measured during the same interval (M/I, in units of $\mu mol \cdot kg_{FFM}^{-1} \cdot min^{-1} \cdot [nmol/l]^{-1}$)
192 (11). Insulin sensitivity of lipolysis was indexed as the plasma FFA levels during the final 40 min
193 of the clamp (14). Insulin sensitivity was also estimated from OGTT-based plasma glucose and
194 insulin concentrations by the Oral Glucose Insulin Sensitivity Index (OGIS) (21), which has been
195 previously shown to be well correlated with the clamp-derived M/I value (20).

196 β-cell function was assessed from the OGTT using a model describing the relationship between
197 insulin secretion rate (ISR, expressed in $pmol \cdot min^{-1} \cdot m^{-2}$) and glucose concentration (22). The mean
198 slope of this dose-response function measures ß-cell glucose sensitivity (ß-GS). The integral of ISR
199 during the whole test (total IS) was also calculated. Insulin clearance was calculated as the ratio of
200 exogenous insulin infusion rate and steady-state plasma insulin concentrations during the clamp
201 (37).

202 An allometric index of hepatic steatosis (Fatty Liver Index, FLI) was calculated as follows:

203 $\exp\{0.953 \cdot \ln[TG] + 0.139 \cdot BMI + 0.718 \cdot \ln[\gamma GT] + 0.053 \cdot waist - 15.745\} \cdot 100/(1 + \exp$
204 $\{0.953 \cdot \ln[TG] + 0.139 \cdot BMI + 0.718 \cdot \ln[\gamma GT] + 0.053 \cdot waist - 15.745\})$ (5).

205 *Statistical analysis.* Data are reported as mean ± SD; variables with skewed distribution are
206 summarized as median and interquartile range and were logarithmically transformed for use in
207 parametric statistical testing. Group values were compared by the Mann-Whitney or the Kruskal-
208 Wallis test for continuous variables, or the χ^2 test for nominal variables; paired values were
209 compared by Wilcoxon sign-rank test. ANCOVA was used to adjust group comparisons for
210 potential confounders. Information about smoking habits and alcohol consumption was obtained
211 using validated questionnaires (16). However, these data were not included among the covariates of

212 multivariate analyses because of their reported rather than measured nature and to avoid
213 overadjustment. Simple associations were tested by Spearman's *rho* (ρ) and logistic regression was
214 used to predict outcome. Principal component analysis was run with the Varimax rotation method
215 using JMP®7.0. A p value ≤ 0.05 was considered statistically significant.
216
217 **Results**
218
219 *Baseline* Although none of the 1,018 participants with complete baseline data had clinical
220 thyroid disease, 940 were strictly euthyroid (*i.e.*, serum fT_4, fT_4 and TSH levels each within the
221 respective reference range); primary analyses were based on the data from this cohort, which
222 included subjects with impaired fasting glycemia (IFG, 15%) and subjects with impaired glucose
223 tolerance (IGT, 10%). Across glucose tolerance status (data not shown), clinical and metabolic
224 characteristics varied as expected; in addition, both serum fT_4 and fT_3 concentrations – as well as
225 their ratio – increased through NGT/IFG/IGT, whereas serum TSH levels did not differ. Serum fT_4,
226 but not serum fT_3, was reciprocally related to serum TSH levels (ρ = -0.14, $p<0.0001$). Grouping
227 subjects by quartile of fT_3/fT_4 ratio (data not shown) yielded a similar pattern of differences in
228 clinical and metabolic characteristics.
229 When the euthyroid cohort was stratified by quartile of fT_3 levels (**Table 1**), higher fT_3
230 concentrations were associated with a preponderance of male sex, younger age, higher BMI, WHR,
231 and FLI. In addition, heart rate and systolic and diastolic blood pressure values, and serum
232 triglycerides increased, while plasma HDL-cholesterol decreased, across fT_3 quartiles. Insulin
233 sensitivity declined with increasing fT_3 levels (as did ß-cell glucose sentitivity, and serum
234 adiponectin concentrations), whilst plasma insulin, proinsulin, and glucagon levels, and insulin
235 secretion rates all increased (**Table 2**). With regard to insulin sensitivity, fT_3 was reciprocally, and
236 fT_4 was positively, related to M/I; these associations were still present after adjusting for the
237 strongest correlates of insulin sensitivity, namely, sex, family history of diabetes, age, BMI, and
238 WHR (**Table 3**). Obesity and insulin resistance were independently associated with higher serum

239 fT_3 concentrations (data not shown). Furthermore, serum fT_3 concentrations, but not fT_4 or TSH,
240 were directly, though modestly, associated with fasting EGP after adjusting for sex, age, BMI,
241 WHR, and family history of diabetes (**Figure 1**).

242 *Follow up* At the 3-year follow up, the fasting plasma glucose concentration of the euthyroid
243 RISC cohort had increased significantly by 0.13±0.60 mmol/L (or 3.1±12.4%, p<0.0001) in
244 concomitance with a decrease in insulin sensitivity of 4.8±19.5% (p<0.0001). In multiple
245 regression models adjusting for sex, age, BMI, and baseline value of the dependent variable, higher
246 baseline fT_3 levels were significant predictors of the increase in fasting glucose and the decreases in
247 insulin sensitivity and ß-cell glucose sensitivity observed at follow up (**Table 3**); in these models,
248 baseline fT_4 levels were concordant with fT_3 in predicting the longitudinal changes in ß-cell glucose
249 sensitivity. In a fully adjusted logistic model, the probability of being in the top quartile of fasting
250 glucose changes (+0.71±0.45 mmol/L) was still predicted by baseline fT_3 levels (**Figure 2**).

251 *Metabolic signatures* Branched-chain and aromatic amino acids and their metabolites (3-
252 hydroxyisobutyrate, 3-methyl-2-oxobutyrate, 3-methyl-2-oxopentanoate, and 4-methyl-2-
253 oxopentanoate) were all increased across increasing baseline fT_3 concentrations (**Table 4**), while
254 glycine and, to a lesser extent, glutamate were decreased. In contrast, most circulating lipid
255 molecules were unrelated to fT_3 levels, and only palmitoyl-glycerophosphocholine and decanoyl-
256 carnitine were reduced. Products of amino acid metabolism such as α–hydroxybutyrate (and its
257 precursor α–ketobutyrate) and betaine were significantly increased, as was the intermediate of the
258 tricarboxylic cycle, α–ketoglutarate; creatine was significantly reduced.

259 Circulating tyrosine levels were directly related to fT_3 concentrations and inversely related to
260 insulin sensitivity (**Figure 3**). Moreover, when plasma tyrosine was included in this predictive
261 model, it replaced fT_3 with an odds ratio of 1.25 [95% C.I. 1.04-1.45]. By a principal component
262 analysis using 3 factors (explaining 41% of the total variance), insulin resistance and serum fT_3
263 levels were highly collinear, and loaded on a cluster including raised branched-chain (and their
264 metabolites) and aromatic amino acids, reduced glycine concentrations, and higher levels of all

265 measured amino acid metabolites (data not shown), while fatty acids and ß-hydroxybutyrate formed
266 a separate, tight cluster, and the remaining metabolites (acyl-carnitines, phospholipids and other
267 amino acids) clustered around an unidentified third factor. Both branched-chain amino acids and
268 FFA were positively associated with α–ketobutyrate.

269 In the surgical cohort, body weight had declined from 127 ± 18 to 85 ± 14 kg ($p<0.001$) at 1
270 year postsurgery; at this time, insulin sensitivity was enhanced by 37% (from 328 ± 78 to 449 ± 69
271 $ml \cdot min^{-1} \cdot m^{-2}$, $p<0.0001$) while serum fT_3 levels had concomitantly decreased (from 2.94 ± 0.64 to
272 2.58 ± 0.40 pg/mL, $p<0.0001$). Postsurgery, serum TSH (from 1.67 ± 0.69 to 1.06 ± 0.59, $p<0.01$)
273 and the fT_3/fT_4 ratio (from 0.29 ± 0.10 to 0.21 ± 0.09, $p<0.0001$) were significantly reduced.

274
275 **Discussion**

276
277 In our cohort of healthy European subjects, higher fT_3 levels within a strictly euthyroid range
278 were unequivocally related to insulin sensitivity in a reciprocal fashion even after controlling for the
279 main correlates of insulin resistance (including BMI, which made a separate contribution in the
280 overweight/obese range). The phenotype of the individuals with high-normal fT_3 included all the
281 main features of the insulin resistance syndrome, namely, central body fat accumulation, insulin
282 resistance of glucose uptake and lipolysis, dyslipidemia, relative excess of liver fat, higher resting
283 heart rate and blood pressure, higher circulating insulin, proinsulin and glucagon, and lower
284 adiponectin levels, higher insulin secretion rates and impaired ß-cell function (Table 2). An
285 important finding was the direct relationship between fT_3 and fasting endogenous glucose
286 production, which has been postulated from previous human studies in nondiabetic and diabetic
287 patients with experimental (18) or spontaneous hyperthyroidism (17), and in healthy volunteers
288 following pharmacologic T_3 administration (31). Further support for the association of fT_3 with
289 insulin resistance derives from the finding of higher fT_3 levels in subjects with dysglycemia (IFG or
290 IGT). Strong proof is provided by the follow-up data, showing that higher baseline fT_3 levels were
291 associated with worsening in the chief determinants of glucose tolerance, *i.e.,* insulin sensitivity and

292 ß-cell function, independently of conventional confounders. Correspondingly, in a multivariate
293 logistic model fT_3 was independently associated with incident increments in plasma glucose on top
294 of standard predictors (Figure 2). Finally, the dataset from the surgical cohort confirmed that with
295 weight loss the improvement in insulin resistance was consensual with a significant decrease in
296 serum fT_3 levels.

297 Of note is that TSH concentrations were essentially unrelated to metabolic variables.
298 Furthermore, the presence of anti-peroxidase or anti-thyroglobulin autoantibodies did not influence
299 any of the relationships between fT_3 and metabolic variables nor did they show any predictive value
300 in the longitudinal analyses (data not shown). The latter findings agree with those of a recent
301 retrospective study of obese (BMI>30 kg/m^2) euthyroid subjects (36). More importantly, the
302 fT_3/fT_4 ratio consistently mirrored serum fT_3 levels across quartiles of fT_3 (Table 1), in adjusted
303 relationship with insulin resistance, and in response to surgically-induced weight loss. To the extent
304 that the fT_3/fT_4 ratio in the serum reflects conversion of fT_4 into fT_3 through type 2 deiodinase
305 activity (19), insulin resistance and/or the attendant hyperinsulinemia appear to also stimulate this
306 process.

307 It is of interest that in the current data higher baseline fT_3 levels did not predict body weight
308 changes at follow up. This is likely due to the close relationship of the hormone with insulin
309 resistance, which has been shown not to be an independent predictor of weight changes in the RISC
310 cohort (27). Clearly, ouside the euthyroid range, low fT_3 and high fT_3 are associated with
311 overweight and leanness, respectively, and acute weight gain and weight loss are accompanied by
312 reciprocal changes in serum fT_3 (30).

313 The association between higher fT_3 and insulin resistance could be interpreted to indicate that a
314 primary, if mild, overproduction of thyroid hormones – thyroidal, extrathyroidal, or both – exerts a
315 negative impact on endogenous glucose release (Figure 1) as well as on insulin action on peripheral
316 tissues (skeletal muscle and adipose tissue). However, a reverse cause-effect relationship, whereby
317 insulin resistance induces a relative oversecretion of fT_3, cannot be ruled out.

318 Targeted metabolomics analysis revealed an interesting pattern of associations, among which
319 the direct relationship between serum fT_3 and tyrosine concentrations has not been previously
320 reported. Raised circulating tyrosine levels may result from reduced degradation (yielding
321 accumulation of its upstream precursor, phenylalanine). In fact, the first step in tyrosine
322 degradation is its deamination to 4-hydroxyphenylpyruvate catalized by an α–ketoglutarate-
323 dependent reaction with tyrosine amino transferase (TAT); this enzyme is inhibited by cytosolic
324 cystine (a component of gluthathione) (6, 15). In turn, increased cystine levels result from
325 increased flux of methionine *via* homocysteine/cystathionine for the synthesis of glutathione under
326 conditions of enhanced oxidative stress (such as type 2 diabetes, obesity, and insulin resistance,
327 reviewed in ref. 1). Cleavage of cystathionine to cysteine generates α–ketobutyrate, the precursor
328 of α–hydroxybutyrate (12). This metabolic cascade (**Figure 4**) is supported by our current findings
329 of (a) increased levels of both α–ketobutyrate and α–hydroxybutyrate and reduced levels of glycine
330 across quartiles of fT_3 (Table 4), (b) close clustering of these metabolites with both tyrosine levels
331 and insulin resistance/fT_3, and (c) ability of plasma tyrosine levels to replace fT_3 as an independent
332 predictor of worse insulin resistance and dysglycemia at follow up (Figure 2). In previous work, the
333 close association between α–hydroxybutyrate and insulin resistance in nondiabetic individuals has
334 been interpreted as a read-out of metabolic overload – by both free fatty acids and branched-chain
335 amino acids – and oxidative stress (increased intracellular NADH/NAD ratio) (13), thereby
336 explaining the ability of this metabolite to predict incident diabetes in two different prospective
337 observational cohorts (12). In the current model, higher serum fT_3 concentrations are linked with
338 insulin resistance by a feed back on endogenous glucose production and, potentially, on insulin
339 action itself (Figure 4). Of interest in this regard is the case reported by Tahara and co-workers of a
340 diabetic patient with hypothyroidism and protein malnutrition, in whom supplementation with a
341 mixture of phenylalanine and tyrosine induced a rapid increase in thyroid hormone concentrations
342 (33). Of note is also the clustering of insulin resistance/fT_3 with the branched-chain amino acids
343 and their degradation products, a consistent metabolic signature of insulin resistance and metabolic

344 overload (25).

345 In summary, with the limitations that the RISC cohort is a highly selected one and the bariatric
346 group was relatively small, we demonstrate that serum fT_3 concentrations within the euthyroid
347 range are independently associated with insulin resistance both cross-sectionally and longitudinally.
348 This association is supported by a metabolite pattern that points at increased oxidative stress as part
349 of the insulin resistance syndrome.

350
351
352
353

354 **Author Contributions**

355

356 Conceptualization, E.F.; Methodology, E.F., J.C., M.N.; Investigation, J.C., R.N., M.N.;
357 Writing – Original Draft, E.F.; Writing – Review & Editing, E.F., G.I., J.C., M.N.; Funding
358 Acquisition, E.F., G.I.; Resources, M.N., G.I., J.C.; Supervision, E.F.

359

360 **Acknowledgments**

361

362 The RISC Study was originally supported by EU Grant QLG1-CT-2001-01252. The current
363 project was supported by an EMIF grant (IMI JU GA 115372-2).

364 The authors have nothing to disclose in relation to the work reported here.

365

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480 **Legend to the figures**
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482 **Figure 1 –** Association of fasting endogenous glucose production with serum fT_3 concentrations.
483 Variables were log-transformed. Plotted data are residuals after adjustment for sex, age, BMI, waist-
484 to-hip ratio, and family history of diabetes; lines are best fit (*full line*) and 95% confidence intervals
485 (*dotted lines*) of the power function.
486 **Figure 2 –** Multivariate logistic model of the probability of subjects being in the top quartile of the
487 changes in fasting plasma glucose (FPG) at follow up. The 95% confidence interval (C.I.) of the
488 odds ratios is calculated for 1 SD of the predictor variable. The model is adjusted for waist-to-hip
489 ratio and family history of diabetes.
490 **Figure 3 –** Circulating levels of fT_3, branched-chain amino acids (BCAA), and tyrosine by quartile
491 of insulin sensitivity (as the M/I value from the clamp). Plots are mean ± SEM.
492 **Figure 4 –** Pattern of associations between fT_3, insulin resistance (IR), endogenous glucose
493 production (EGP), and circulating metabolites. FFA = free fatty acids; BCAA = branched-chain
494 aminio acids; α–KB = α–ketobutyrate; α–HB = α–hydroxybutyrate; Tyr = tyrosine; Phe-ala =
495 phenylalanine; cyst. = cystine; TAT = tyrosine aminotransferase. Full lines refer to associations
496 found in the current database (with Spearman's *rho* values, all $p<0.001$), dotted lines to previously
497 reported associations.
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Table 1 – Clinical and biochemical characteristics of biochemically euthyroid subjects (n=940) by fT3 quartile.*

	1	2	3	4	*p*
fT3 (pg/mL)	2.29 ± 0.13	2.60 ± 0.06	2.82 ± 0.07	3.20 ± 0.20	<0.0001
fT4 (pg/mL)	10.8 ± 1.6	10.9 ± 1.8	10.8 ± 1.6	11.5 ± 1.8	<0.0001
TSH (mU/L)	1.68 ± 0.85	1.81 ± 0.87	1.75 ± 0.83	1.82 ± 0.80	ns
fT3/fT4 ratio	0.22 ± 0.04	0.25 ± 0.04	0.27 ± 0.04	0.28 ± 0.05	<0.0001
Sex (% female)	70	64	43	33	<0.0001
Familial diabetes (%)	25	31	31	28	ns
NGT/IFG/IGT (%)	80/15/5	76/13/11	73/16/11	75/15/10	0.048
Never smokers (%)	50	49	45	47	ns
Age (years)	45 ± 8	45 ± 8	44 ± 8	43 ± 8	0.0018
BMI (kg/m^2)	24.4 ± 3.6	25.3 ± 4.0	25.7 ± 3.6	26.4 ± 3.9	<0.0001
WHR (cm/cm)	0.84 ± 0.11	0.85 ± 0.10	0.89 ± 0.11	0.89 ± 0.09	<0.0001
Heart rate (bpm)	66 ± 10	67 ±10	69 ± 11	70 ± 10	0.0006
Systolic BP (mmHg)	115 ± 12	118 ± 12	120 ± 13	119 ± 11	<0.0001
Diastolic BP (mmHg)	73 ± 8	74 ± 8	76 ± 8	75 ± 7	<0.0001
Fasting glucose (mmol/L)	5.06 ± 0.52	5.06 ± 0.54	5.13 ± 0.60	5.15 ± 0.58	ns
Fasting FFA (µmol/L)	520 [250]	510 [25]	500 [295]	490 [280]	ns
Steady-state FFA (µmol/L)	24 [25]	25 [26]	30 [25]	35 [40]	<0.0001
LDL-cholesterol (mmol/L)	2.8 ± 0.8	2.9 ± 0.8	3.0 ± 0.8	3.0 ± 0.8	ns
HDL-cholesterol (mmol/L) (F/M)	1.6±0.3/1.4±0.	1.6±0.4/1.3±0.3	1.5±0.4/1.2±0.3	1.5±0.4/1.2±0.	<0.0001
Triglycerides (mmol/L)	0.87 [0.48]	0.91 [0.54]	0.99 [0.68]	1.02 [0.78]	0.0002
Fatty Liver Index (%)	12 [23]	19 [32]	23 [38]	31 [44]	<0.0001

* entries are mean±SD or median [IQR] for normally or non-normally distributed parameters; correspondingly, *p* values are from ANOVA or Kruskal-Wallis test. Frequencies are compared by χ^2. F = female, M = male; fT_3 = free triiodothyronine; fT_4 = free thyroxine; TSH = thyroid stimulating hormone; NGT/IFG/IGT = Normal Glucose Tolerance/Impaired Fasting Glycemia/Impaired Glucose Tolerance; BP = arterial blood pressure; FFA = free fatty acids.

509 **Table 2 – Hormone concentrations and metabolic functions of biochemically euthyroid subjects**
510 **(n=940) by fT3 quartile.***

	1	**2**	**3**	**4**	***p***
Fasting insulin (pmol/L)	26 [21]	28 [19]	31 [23]	37 [25]	<0.0001
Mean OGTT insulin (pmol/L)	190 [134]	186 [138]	200 [145]	222 [196]	0.006
Glucagon (pmol/L)	7.0 [3.0]	8.0 [4.0]	8.0 [5.0]	9.0 [5.0]	<0.0001
Proinsulin (pmol/L)	5.0 [4.0]	5.0 [3.8]	6.0 [4.0]	7.0 [6.0]	<0.0001
Adiponectin (mg/L)	8.4 [4.8]	7.8 [4.6]	7.4 [4.5]	6.9 [4.3]	0.0016
M/I ($\mu mol \cdot kg_{FFM}^{-1} \cdot min^{-1} \cdot [nmol/L]^{-1}$)	150 [86]	133 [81]	122 [84]	116 [79]	<0.0001
ß-GS ($pmol \cdot min^{-1} \cdot m^{-2} \cdot [mmol/L]^{-1}$)	118 [99]	112 [80]	105 [78]	107 [72]	0.011
Insulin clearance (L/min)	0.60 [0.18]	0.61 [0.17]	0.62 [0.20]	0.58 [0.17]	<0.001
Fasting ISR ($pmol \cdot min^{-1} \cdot m^{-2}$)	63 [37]	67 [34]	71 [35]	80 [44]	<0.0001
Total IS ($nmol \cdot m^{-2}$)	38 [16]	39 [17]	39 [16]	42 [20]	0.028

511 *entries are median [IQR]; *p* values are from Kruskal-Wallis test. OGTT = Oral Glucose Tolerance
512 Test; M/I = whole-body insulin-mediated glucose metabolism; ß-GS = ß-cell glucose sensitivity; ISR
513 = insulin secretion rate; IS = insulin secretion.
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Table 3 – Multiple regression analysis of the association of thyroid hormones concentration with metabolic variables at baseline and follow up in biochemically euthyroid subjects (n=940)**.**

	fT_3	**fT_4**	**TSH**	**fT_3/fT_4**
Baseline*				
Insulin sensitivity [G]	-0.09 (0.006)	0.11 (<0.001)	(ns)	-0.16 (<0.0001)
Insulin sensitivity [FFA]	-0.14 (<0.0001)	-0.09 (0.007)	(ns)	(ns)
ß-GS	-0.07 (0.04)	(ns)	(ns)	(ns)
Follow up¶				
Change in FPG	0.11 (0.0010)	(ns)	(ns)	(ns)
Change in ins.sens.	-0.11 (0.0014)	(ns)	(ns)	(ns)
Change in ß-GS	-0.09 (0.0117)	-0.13 (0.0001)	(ns)	(ns)

518 * entries are partial correlation coefficients (*p* values) for thyroid hormone (as the dependent
519 variable) adjusted for sex, family history of diabetes, age, BMI, and WHR. Serum fT_3, fT_4, and
520 TSH concentrations, insulin sensitivity of glucose uptake ([G], as the M/I), insulin sensitivity of
521 lipolysis ([FFA]), and ß-cell glucose sensitivity (ß-GS) were log-transformed for this analysis.
522 ¶ entries are partial correlation coefficients (*p* values) for change (as the dependent variable)
523 against thyroid hormone adjusted for sex, age, BMI, WHR, family history of diabetes, and
524 baseline value of the dependent variable.

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551 **Table 4 – Circulating metabolites** in biochemically euthyroid subjects (n=940) **by fT3 quartile.***

	1	**2**	**3**	**4**	***p***
Amino acids					
Arginine (mg/L)	13.4 ± 3.1	13.2 ± 3.0	13.5 ± 3.1	13.2 ± 3.5	ns
Glutamate (mg/L)	20.0 ± 15.5	17.4 ± 11.1	17.3 ± 9.8	18.5 ± 10.4	0.05
Glycine (mg/L)	18.8 ± 5.3	17.3 ± 5.2	17.0 ± 5.00	16.1 ± 4.8	<0.0001
Serine (mg/L)	11.1 ± 2.2	10.9 ± 2.3	11.1 ± 2.2	10.6 ± 2.1	ns
Threonine (mg/L)	15.7 ± 4.1	16.3 ± 3.8	16.1 ± 4.0	15.7 ± 3.4	ns
Tryptophan (mg/L)	11.1 ± 1.8	11.2 ± 1.9	11.3 ± 1.7	11.4 ± 1.8	<0.03
Tyrosine (mg/L)	9.7 ± 1.9	10.1 ± 2.0	10.2 ± 2.3	10.5 ± 2.1	<0.0003
Phenylalanine (mg/L)	8.5 ± 0.1	8.7 ± 0.1	8.8 ± 0.1	8.8 ± 0.1	<0.04
Leucine (mg/L)	13.7 ± 2.5	14.1 ± 2.7	14.7 ± 3.3	15.0 ± 2.6	<0.0001
Isoleucine (mg/dL)	6.8 ± 1.4	6.9 ± 1.6	7.7 ± 4.0	7.6 ± 1.5	<0.0001
Valine (mg/L)	23.2 ± 3.9	23.8 ± 4.3	24.8 ± 4.9	25.1 ± 4.0	<0.0001
Amino acid derivatives					
α–ketobutyrate (mg/L)	0.33 ± 0.19	0.41 ± 0.21	0.40 ± 0.22	0.39 ± 0.22	<0.0001
α–hydroxybutyrate (mg/L)	4.3 ± 1.7	4.6 ± 1.7	4.6 ± 1.6	4.9 ± 2.00	<0.002
2-aminoadipate (mg/L)	0.08 ± 0.03	0.08 ± 0.03	0.09 ± 0.04	0.09 ± 0.03	<0.0001
3-hydroxyisobutyric (mg/L)	1.26 ± 0.38	1.33 ±0.42	1.36 ± 0.48	1.39 0.38	0.002
3-methyl-2-oxobutyric (mg/L)	1.54 ± 0.43	1.62 ± 0.43	1.67 ± 0.38	1.71 ± 0.37	0.0001
3-methyl-2-oxopentanoic (mg/L)	2.48 ± 0.72	2.46 ± 0.69	2.72 ± 0.87	2.73 ± 0.73	<0.0001
4-methyl-2-oxopentanoic (mg/L)	3.81 ± 0.97	3.84 ± 0.95	4.02 ± 1.09	4.12 ± 1.00	0.0029
Lipids					
Adrenate (mg/L)	0.20 ± 0.08	0.19 ± 0.07	0.20 ± 0.07	0.21 ± 0.11	ns
Margarate (mg/L)	0.38 ± 0.14	0.38 ± 0.13	0.40 ± 0.15	0.39 ± 0.16	ns
Linoleate (mg/L)	15.3 ± 05	15.5 ± 0.5	15.3 ± 0.4	16.0 ± 0.5	ns
Linolenate (mg/L)	2.7 ± 1.5	2.8 ± 1.4	2.7 ± 1.4	2.6 ± 1.8	ns
Oleate (mg/L)	84.8 ± 33.0	84.2 ± 29.0	87.2 ± 33.3	85.0 ± 45.9	ns
Palmitate (mg/L)	32.5 ± 12.0	32.7 ± 11.5	32.9 ± 11.6	32.8 ± 15.3	ns
Stearate (mg/L)	13.0 ± 4.0	13.3 ± 3.8	13.8 ± 3.7	13.6 ± 4.9	ns
Palmitoleate (mg/L)	3.5 ± 1.9	3.3 ± 2.0	3.1 ± 1.8	3.0 ± 2.1	<0.004
Octanoyl-carnitine (mg/L)	0.031 ± 0.026	0.028 ± 0.019	0.026 ± 0.026	0.027 ± 0.019	ns
Decanoyl-carnitine (mg/L)	0.053 ± 0.050	0.045 ± 0.034	0.039 ± 0.025	0.043 ± 0.032	0.001
Linoleoyl-LPC (mg/L)	15.3 ± 5.0	15.6 ± 5.3	15.8 ± 5.4	15.5 ± 4.8	ns
Oleoyl-GPC (mg/L)	10.0 ± 2.8	9.6 ± 3.0	9.7 ± 3.1	9.6 ± 2.7	ns
Palmitoyl-GPC (mg/L)	37.0 ± 10.2	35.3 ± 9.1	35.0 ± 11.3	35.0 ± 9.7	<0.04
Other					
Betaine (mg/L)	4.1 ± 1.4	4.4 ± 1.5	4.4 ± 1.3	4.4 1.4	0.01
α–ketoglutarate (mg/L)	1.00 ± 0.42	1.12 ± 0.34	1.12 ± 0.37	1.14 ± 0.41	<0.0001
Creatine (mg/mL)	4.3 ± 2.2	4.5 ± 2.3	3.9 ± 1.8	3.8 ± 1.8	<0.001
ß–hydroxybutyrate (mg/L)	8.5 ± 8.9	10.7 ± 19.9	9.4 ± 9.8	8.1 ± 8.9	ns

552 *entries are mean±SD; *p* values are from Kruskal-Wallis test.

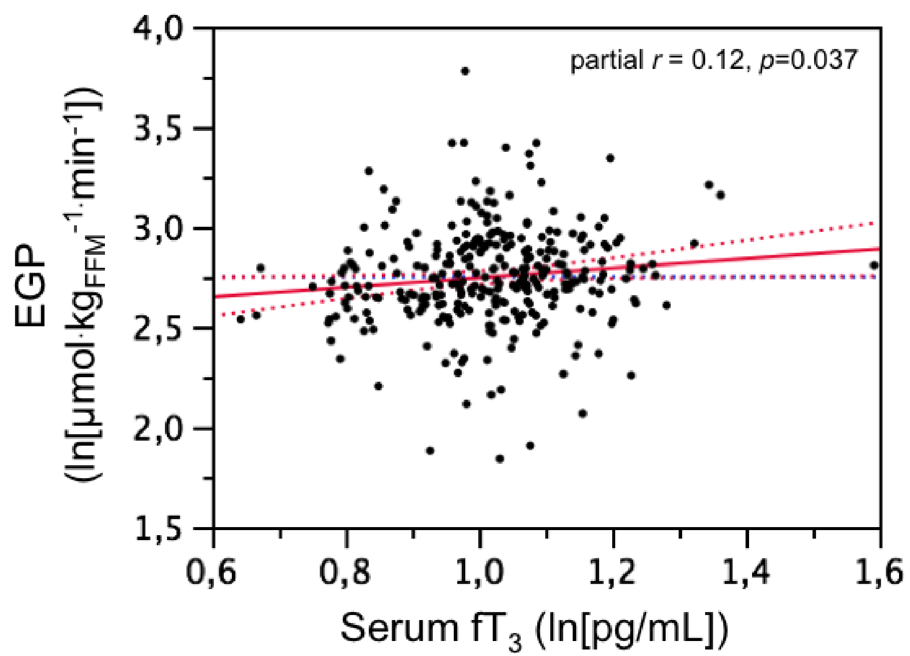

Figure 1

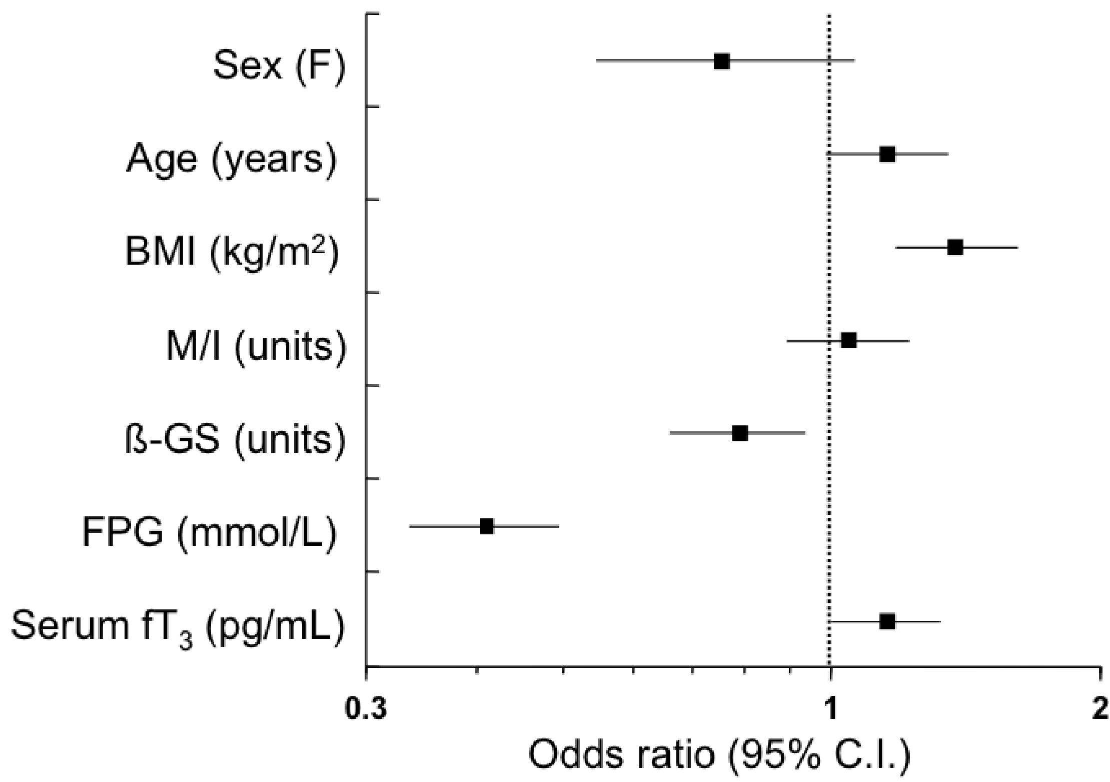

Figure 2

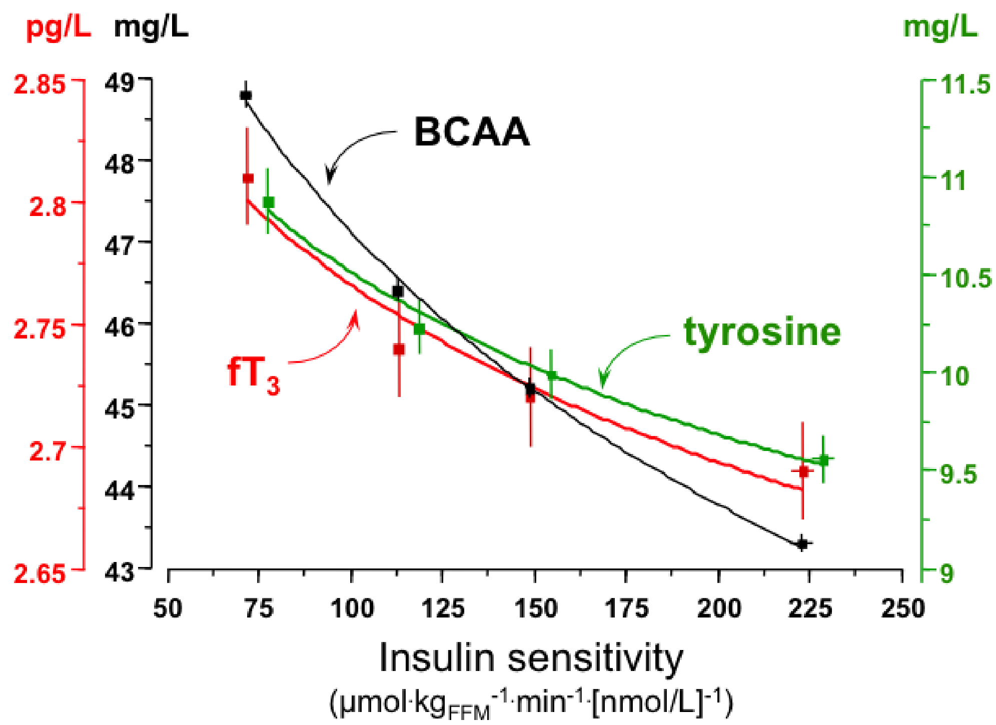

Figure 3

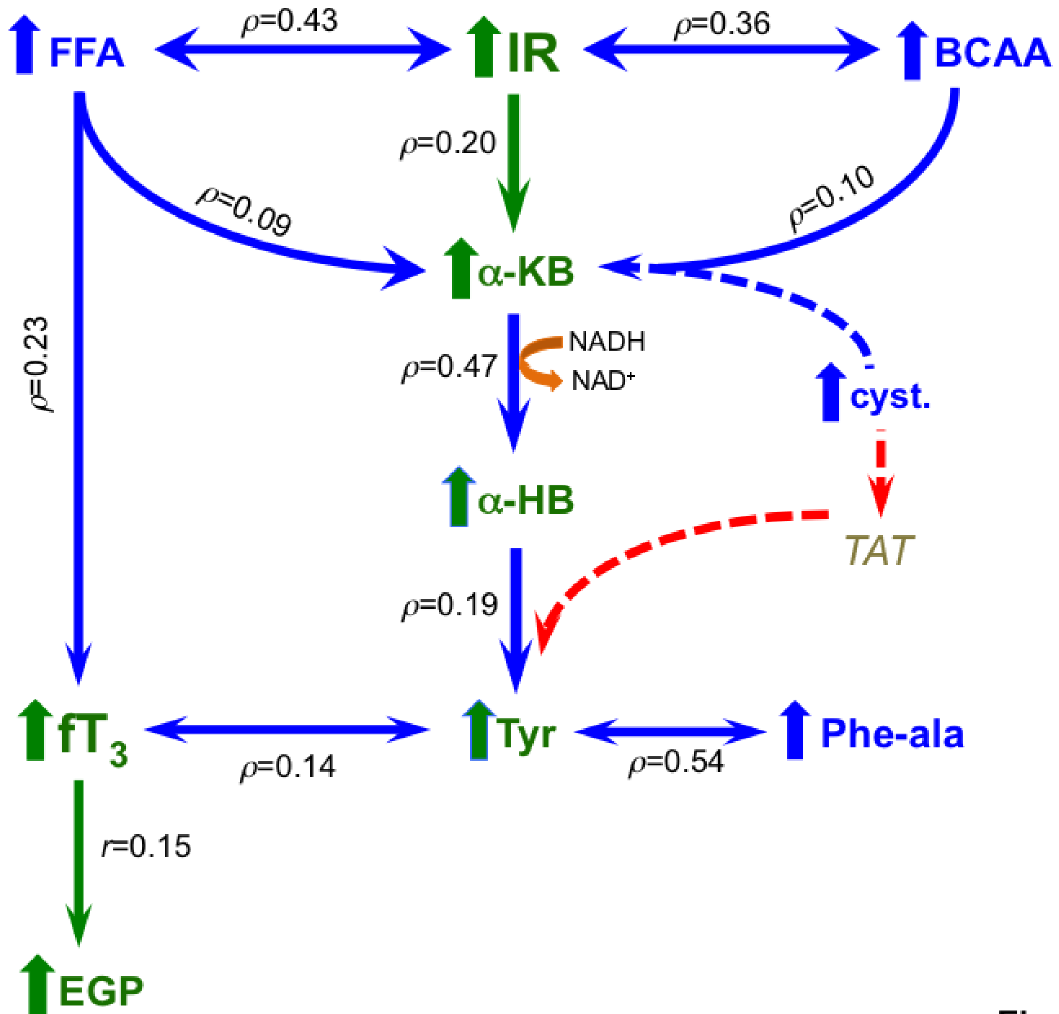

Figure 4