Appendix 2

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PART B

FINAL REPORT

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Maarten Soeters

Draft manuscript objective 2

Critical assessment of plasma acylcarnitines before and after weight loss in obese subjects.

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Abstract

Acylcarnitines, fatty acid oxidation (FAO) intermediates, have been implicated in diet-induced insulin resistance and type 2 diabetes mellitus. Moreover plasma acylcarnitines have been associated with clinical parameters related to glucose metabolism such as fasting glucose levels and HbA1c. We investigated plasma acylcarnitines in relation to energy metabolism (including energy expenditure (EE)) and insulin sensitivity measurements in 60 obese subjects before and after a 12 week weight loss intervention. Since the acylcarnitine profile reflects FAO at the mitochondrial level, we expected plasma acylcarnitines to correlate with energy expenditure, insulin sensitivity and other clinical parameters before and during a weight loss intervention. In contrast, despite the amelioration of HOMA-IR, plasma acylcarnitines levels increased during weight loss. This is most probably attributable to higher lipolysis and lipid oxidation rates due to the caloric deficit and not by mitochondrial overload and incomplete FAO, as insulin signalling was not impaired. HOMA-IR, energy expenditure and respiratory exchange ratio were not related to plasma acylcarnitines. However a strong correlation between FFA and several acylcarnitines was found at baseline and during the weight loss intervention, again potentially reflecting lipolysis. Our data show that acylcarnitines do not relate to clinical parameters of glucose metabolism during weight loss. We therefore question the role of acylcarnitines in the etiology of insulin resistance and subsequent type 2 diabetes mellitus.

Introduction

With the increased incidence of obesity and type 2 diabetes mellitus, many studies focus on the interaction between lipid and glucose metabolism and the ensuing insulin resistance. The concept of lipotoxicity has been proposed as a mechanism by which increased lipid levels interfere with insulin signalling and eventually lead to hyperglycemia. However, the exact mechanisms and the individual lipids that induce insulin resistance have not been characterised definitively. From a cellular point of view, lipotoxicity is thought to occur on a cytosolic level via lipid overload (e.g. increased levels of ceramides, gangliosides or diacylglycerol) (1-4). Reduced mitochondrial content or capacity may result in elevated intracellular lipids (3, 5). Alternatively, increased fatty acid oxidation (FAO) rates that are not followed by an increase in tricarboxylic acid cycle (TCA) activity have been proposed to induce insulin resistance via accumulation of different mitochondrial metabolites such as acylcarnitines (1, 6, 7). This incomplete FAO is proposed to be reflected by altered plasma acylcarnitine profiles, with increased levels of mainly long chain acylcarnitines (1, 7, 8).

Acylcarnitines are fatty acids esterified to carnitine. They are synthetized by the outer membrane enzyme carnitine palmitoyl tranferase 1 (CPT1) to enable transmitochondrial membrane transport of long-chain acyl-CoAs. Inside the mitochondrion, carnitine is exchanged for CoA via the inner mitochondrial membrane enzyme CPT2. Here the released acyl-CoA can be further oxidized via beta-oxidation, in which every round the acyl-CoA is shortened by one acetyl-CoA. CPT-2 can convert acyl-CoAs into acylcarnitines again, which can be shuttled back into the cytosol and exported to the plasma compartment, ultimately composing a typical profile of acylcarnitines. Consequently, acylcarnitines are excellent indicators of altered FAO as demonstrated by conditions in which lipid oxidation rates are elevated or when lipid oxidation is impaired (e.g. short-term fasting and FAO disorders). More recently, metabolomic studies have shown that acylcarnitines may be implicated in insulin resistance (7-9), as elevated acylcarnitine levels are found in both rodent models of dietary insulin resistance (7) and in obese, insulin resistant humans (8). Also several acylcarnitine species correlate moderately with clinical

markers such as BMI, plasma glucose levels and insulin sensitivity in humans with obesity and type 2 diabetes mellitus (DM2) (8, 9).

Here we report on plasma acylcarnitine concentrations before and during weight loss in obese human subjects. Based on the association of long- and short-chain acylcarnitines with DM2, we hypothesized that these acylcarnitine levels would decrease with concomitant improvements in insulin sensitivity. In contrast, we showed that decreased body weight and improvements in insulin sensitivity were accompanied by increased plasma acylcarnitine levels. Moreover, we found that plasma acylcarnitines correlate strongly with plasma free fatty acids (FFA).

Research design and methods

Design of the study

60 obese subjects were recruited to take part in an outpatient study for weight loss prediction that has been published elsewhere (10). In brief, subjects aged 20-55 years and BMI 30-40 kg/m^2 were included. Exclusion criteria were as follows: type 2 diabetes, history of childhood obesity and previous bariatric surgery. After taking informed consent, subjects were randomized to one of three 12-week weight loss interventions: 1- diet (-600 kcal) alone, 2- diet with moderate exercise (~10% of daily expenditure) and 3- diet with the centrally acting serotonin-norepinephrine reuptake inhibitor sibutramine. During the study, subjects visited the clinical unit at 0, 4 and 12 weeks at 07:00 hours a.m. after an overnight fast for the measurement of body weight, anthropometry, indirect calorimetry and blood sampling (e.g. plasma acylcarnitine, glucose, fatty acids and insulin levels). The study was approved by the protocol review panel of GlaxoSmithKline, the Cambridge Local Research Ethics Committee (08/H0308/10) and the Wellcome Trust Clinical Research Facility Scientific Advisory Board. Subjects gave written informed consent before participation.

Body weight measurement and anthropometry by DXA

Body weight was measured with a SECA/PHILIPS scale (ANTONELLA PLEASE) in light clothing. Body composition was analysed by DXA (GE Lunar Prodigy, software version 12.2 (GE Healthcare, Madison, WI) and quantitative magnetic resonance (Echo MRI-AH; Echo Medical Systems, Houston TX). Indirect calorimetry was performed using a ventilated canopy calorimetry instrument (GEM Nutrition, Daresbury, UK) with the subject lying supine for 20 minutes before the measurement. Samples were analyzed every 30 seconds for 20 minutes. Gas exchanges of O_2 and CO_2 were computed to calculate respiratory exchange ratio (RER) and basal metabolic rate (BMR; kJ/min using the following formula: 15.9131 x O_2 consumption + 5.2069 x CO_2 production x 0.9950) (11).

Laboratory analyses

For plasma acylcarnitine analysis, 50 µl of plasma was mixed with 100 µl of internal standard mixture (50 µl of 5 µM [3,3,3-2H3]C3-carnitine and 2 µM [6,6,6-2H3]C6-, [8,8,8-2H3]C8-, [10,10,10-2H3]C10- and [16,16,16-2H3]C16-carnitine in acetonitril (ACN), and 50 µl of 26 µM [methyl-2H3]-L-carnitine in 10% ACN) [38]. The plasma samples were deproteinized by addition of 500 µl ACN and subsequent vortex mixing. Next, samples were centrifuged for 10 minutes at 4°C at a speed of 20.000 g. The supernatant was transferred into 4 ml glass vials and evaporated under a stream of nitrogen at 40°C. After evaporation, 100 µl butylation reagent (4:1 mixture of 1-butanol and acetylchloride) was added and incubated for 15 minutes at 60°C. Again evaporation was performed at 40°C. The residue was dissolved in 100 µl ACN, vortex mixed and transferred to Gilson vials for tandem mass spectrometric analysis (Waters/Micromass Quattro Premier XE). Acylcarnitine measurements were processed with Masslynx software version 4.1.

Acylcarnitines are depicted as C followed by chain length and degree of saturation. The acylcarnitines which we analysed were as follows: Free carnitine (C0), acetylcarnitine (C2; derived from both lipid and carbohydrate oxidation (CHO)), hydroxybutyrylcarnitine (C4OH; the sum of the *L* and *D* stereoisomers derived from FAO and ketone bodies respectively (12)), decanoylcarnitine and tetradecenoylcarnitine (C10 and C14:1 respectively; intermediates that

are only produced by FAO and thus indicative of FAO rate) and finally palmitoylcarnitine and oleoylcarnitine (C16 and C18:1 respectively; intermediates that originate from the diet).

Plasma glucose levels were measured by an in house glucose oxidase method (Antonella needs to check). Plasma insulin levels were analysed by an in house RIA (Antonella needs to check). Plasma free fatty acids were analysed with an NEFA-HR(2) in vitro enzymatic colorimetric method (Wako Diagnostics, Richmond VA).

Statistical analysis

For differences between subgroups at baseline, one-way ANOVA with Bonferroni correction was performed. To detect if plasma acylcarnitine levels at baseline predicted clinical parameters, Pearson correlation analyses were performed followed by Bonferroni correction. In case of significant results, multiple regression analysis was done to establish which acylcarnitine had the greatest effect on a single clinical parameter. Differences between days 0, 28 and 84 for whole group and within subgroup data were analysed using repetitive ANOVA analysis with Bonferroni correction. Statistical analysis was done with SPSS statistical software program version 20.0. Data are depicted as mean and standard deviation.

To analyse if changes in plasma acylcarnitine levels over time coincided with changes in clinical parameters we used a Bayesian hierarchical model with fixed and random effects. Individual variables were modelled by linear regression over time. For instance, changes in weight were modelled as: $W_i(t) = \alpha_i^{\omega} + \beta_i^{\omega} t + \varepsilon_{it}^{\omega}$. Here $W_i(t)$ is the is the weight of subject *i* at time *t*, α_i^{ω} is the mean weight of subject *i*, $\beta_i^{\omega} t$ is the is rate of change of the subject's weight over time *t* and $\varepsilon_{it}^{\omega}$ is a zero mean Gaussian error term: $\varepsilon_{it}^{\omega} \sim N(0, \sigma^2)$. Similarly, each of the acylcarnitines of interest was modelled for each individual patient by linear regression ($C_i(t) = \alpha_i^{c} + \beta_i^{c} t + \varepsilon_{it}^{c}$, where C is any acylcarnitine of interest). To analyse the correlation over time between individual clinical parameters (again given weight as example) and acylcarnitines we modelled β_i^{ω} as a linear regression over β_i^{c} : $\beta_i^{\omega} = \alpha + \beta\beta_i^{c} + \varepsilon_i$ where ε_i is again a zero mean Gaussian error term. We then applied Gibbs sampling to simulate from the

posterior distribution using standard software (Just Another Gibbs Sampler (JAGS, version 3.4.0, http://mcmc-jags.sourceforge.net/)). We used a single Markov chain with a burn-in of 100000 sweeps and then output the values α and β for a further 1000000 sweeps. The proportion of sweeps when β is greater than zero is then the posterior probability that variables are positively correlated, and the proportion of sweeps when $\beta < 0$ is the posterior probability that variables are negatively correlated. This procedure was repeated for all clinical parameters and acylcarnitines of interest followed by the Bonferoni-Holm method to allow for multiple testing (13).

Results

Whole group clinical data and plasma acylcarnitines at baseline (day 0)

Table 1 shows the clinical characteristics of the subjects at day 0 of the study. More women were included then men and a substantial part of the subjects showed slight impaired fasting plasma glucose levels (FPG) (14), as well as an elevated HOMA-IR index (15). Table 2 shows the levels of the plasma acylcarnitines of main interest (i.e. C0, C2, C4OH, C10, C14:1, C16 and C18:1). The levels of other acylcarnitines detected within the spectrum are given in the online supplemental data (supplemental figure).

To detect relationships between plasma acylcarnitines and clinical parameters at baseline, we performed Spearman correlation analyses. Our analysis showed that plasma FFA correlated strongly with C2, C14:1, C16 and C18:1-carnitine (see table 3, figure 4 and 5). No other correlations were found (see online supplemental data table x). Multiple regression identified C14:1 and C16-carnitine as contributors to the variation in plasma FFA (table 4). When C4OH-carnitine was omitted from the regression analysis (since this is an acylcarnitine that emerges predominantly during short-term starvation (12)), only C16-carnitine remained significant. Multiple regression analysis for HOMA-IR and plasma acylcarnitines yielded no significant results (data not shown).

Whole group clinical data and plasma acylcarnitines during the weight loss intervention

Figure 1 shows the effects of the weight loss interventions for the whole group. Weight decreased significantly between days 0 and 84 (~4.5 kg). As a consequence, BMI decreased between 0 and 28 days with no additional significant reduction between days 28 and 84. HOMA-IR, FPG and plasma insulin levels improved significantly between days 0 and 28, with no further improvement hereafter (figure 1). Plasma FFA levels were unaffected by the intervention. Body composition analysis revealed that fat mass continued to decrease during the entire weight loss intervention period although this effect was most pronounced during the first 28 days. Lean body mass decreased significantly (~0.6 kg) until 28 days only (figure 1). Substrate oxidation measurements by indirect calorimetry showed that both BMR and RER were significantly lower after 28 days with no further reduction afterwards (figure 1). FAO rates did not change upon weight loss. However protein oxidation rates and CHO rates both significantly decreased after 28 days, and remained at this level at 84 days (Fig x supplemental data). Therefore the decrease in energy expenditure was mainly resulting from decreased carbohydrate and protein oxidation, and a relative increase of FAO as energy source. Overall clinical improvements due to the weight loss intervention were greatest in the first 28 days, and stabilized at 84 days.

Whole group plasma acylcarnitine levels for days 0, 28 and 82 are shown in figure 2. The weight loss intervention resulted in an increase in free carnitine levels after 84 days. C2-carnitine and C4OH-carnitine were higher after 28 days with no further increase at 84 days. C14:1- and C18:1-carnitine showed a similar pattern over time with an initial increase in plasma levels after 28 days followed by a decrease at 84 days although at this point these acylcarnitine levels were still higher compared with day 0. C16-carnitine was significantly higher after 28 days followed again by a decrease to baseline levels (figure 2).

We used a Bayesian hierarchical model to analyse if changes in plasma acylcarnitine levels overtime coincided with changes in weight, fat mass, lean mass, HOMA-IR, FPG, FFA, RER and BMR. We found that the increase over time in C4OH-, C16- and C18:1-carnitine correlated significantly with a reduction in both total and lean body weight over time (Table 5).

Additionally changes in FFA over time correlated positively with changes in C16-carnitine (Table 5). We found no significant correlations for the other clinical parameters (data not shown).

Subgroup clinical data and plasma acylcarnitines at baseline (day 0)

Table 1 shows the clinical data results at baseline for the subgroups. Although in general there were no differences between the groups, there were a few exceptions that were primarily driven by the exercise and sibutramine group. Plasma glucose and RER were both higher in the sibutramine group when compared to the exercise group. The difference in RER was also found for FAO and CHO rates (see online supplemental data). Plasma FFA levels were lower in the exercise group when compared to the other two groups (Table 1).

We repeated Spearman correlation analyses for the subgroups at baseline for HOMA-IR and FFA (Figure 4 and 5). Plasma acylcarnitines did not correlate with HOMA-IR. FFA and acylcarnitines showed multiple correlations at the subgroup level (supplemental data): in the exercise group C2-, C14:1-, C16- and C18:1-carnitine correlated with plasma FFA but only C14:1-carnitine retained its significance after Bonferoni correction. In the placebo group C16-carnitine remained significantly correlated with plasma FFA after correction where as C2-carnitine lost its significance after Bonferoni. Finally, C2-, C16- and C18:1-carnitine correlated with plasma FFA in the sibutramine group, here C16- and C18:1-carnitine remained significant after correction (see online supplemental data).

Subgroup clinical data and plasma acylcarnitines during the weight loss intervention

Here we report on changes in weight, homeostatic model assessment of insulin resistance (HOMA-IR), FFA and RER in relation to changes in acylcarnitine levels (Figure 3, 4, 5 and 6). The other clinical parameters (FPG, insulin, fat mass, FAO and CHO) are presented in the online supplemental data. The sibutramine group showed continued weight loss until day 84 in contrast to the other two groups that significantly had lost weight at day 28 where after they remained stable. Following this, the sibutramine group had a significant reduction in HOMA-IR

at day 28 from where levels plateaued, but this was not the case in the exercise and placebo groups where HOMA-IR did not change. Plasma FFA did not change in any of the three groups. In the exercise and placebo groups, RER was not affected by the weight loss intervention, but the sibutramine group had a lower RER after 28 days that remained stable thereafter.

Plasma acylcarnitine levels showed quite differential responses to the weight loss intervention where overall the greatest effect was seen in the sibutramine group and a modest effect was seen for the exercise group. In the placebo group, C0-carnitine did not change whereas the exercise group showed a modest increase at day 84 compared to day 28. In the sibutramine group, C0-carnitine increased steadily during the intervention becoming significant at day 84. In contrast to the placebo and exercise group, C2- and C4OH-carnitine levels in the sibutramine group showed an increase at 28 days and remained high at 84 days. C10- and C14:1-carnitine were not significantly affected in the exercise group. In the placebo and sibutramine group, C10- and C14:1-carnitine initially increased at day 28. Finally, C16- and C18:1-carnitine were higher at day 28 compared to day 0 (with the exception of C16-carnitine). At day 84, C10-, C14:1-, C16- and C18:1-carnitine showed a significant or non-significant decrease compared to day 28 (see figure 2).

We repeated the Bayesian hierarchical modeling to analyse if changes in plasma acylcarnitine levels overtime coincided with changes in clinical parameters and found that the change in plasma C4OH- and C18:1-carnitine remained significantly correlated with weight change in the sibutramine group but not in the other groups (data not shown).

Discussion

Acylcarnitines have been suggested to play a role in insulin resistance (1, 16). In this study we have shown in obese humans that upon weight loss the plasma levels of acylcarnitines increase, and that acylcarnitines correlated positively with FFA at baseline and over time.

Several studies have reported that increased plasma acylcarnitine levels associate with obesity and insulin resistance (7-9, 17). Adams et al showed that C2-carnitine, derived from both lipid and carbohydrate oxidation, correlated positively with the HbA1c in diabetic subjects (9). Mihalik et al showed multiple correlations of short and longer chain acylcarnitines with glucose metabolism (8). Although they did not report on C2-carnitine, they found strong correlations with C4-dicarboxylcarnitine (C4-DC-CN) with fasting plasma glucose levels and HbA1c. In contrast, plasma FFA correlated positively with plasma C16-carnitine and other species being C2-, C14:1- and C18:1-carnitine. With respect to this strong correlation of acylcarnitines with plasma FFA, which are known to induce insulin resistance, the overall absence of correlations between acylcarnitines and markers of insulin sensitivity is remarkable (18, 19). Since plasma FFA are indicative of lipolysis, acylcarnitines may reflect white adipose tissue (WAT) breakdown (20). Here, the released FFA from WAT could drive FAO rates generating acylcarnitines.

The origin of the different acylcarnitines that correlate with plasma FFA is intriguing as well. As discussed above, C2-carnitine can be derived from both lipid and carbohydrate oxidation and the present correlation of C2-carnitine with plasma FFA suggests that lipid is the main source (21, 22). C16- and C18:1-carnitine are derived from palmitate and oleate, which are our main dietary fatty acids. Their correlation with plasma FFA may support lipolysis as a responsible mechanism since these dietary fatty acids are stored in WAT. Finally, C14:1-carnitine is an interesting acylcarnitine since it is only produced after two cycles of beta-oxidation of C18:1-CoA (23). Therefore C14:1-carnitine is a good marker of FAO. It remains unclear what the tissue origin of this acylcarnitine is, but as FAO also takes place in WAT, C14:1-carnitine can still be derived from WAT (24, 25). Alternatively, plasma FFA may reflect the load of fatty acids in general, thereby correlating with the metabolically most relevant acylcarnitines.

Following these observations at baseline, we studied the effects of a weight loss intervention on acylcarnitines and insulin resistance. Effects of a weight loss intervention on acylcarnitine profiles were described in only two studies that both studied lean subjects (26, 27). Redman et al compared caloric restriction with and without exercise in non-obese men and women, and showed no changes in acylcarnitines in the former group but increased acylcarnitines in the latter, accompanied by a greater improvement in insulin sensitivity analogous to our study (27). Here, caloric restriction combined with exercise possibly improves the coupling of FAO and TCA flux, preventing acylcarnitines to accumulate. Falk-Petersen et al demonstrated in lean sedentary insulin resistant offspring of parents with type 2 diabetes (26) that plasma acylcarnitine levels do not change after a 9 week hypocaloric diet, despite weight reduction and improved insulin sensitivity. Here, the unchanged plasma acylcarnitines are in contrast with our results and this is difficult to explain since the subjects were different from ours with respect to both body composition and insulin sensitivity. Whether differences in mitochondrial lipid flux handling or lipolysis explain the differential effects of the diet on plasma acylcarnitines remains elusive. Also, in these particular subjects, the initial insulin resistance may have been a result of increased intramyocellular lipids (IMCL) rather than acylcarnitines. Finally, the genetic susceptibility of insulin resistant offspring potentially blurs the etiological mechanism of insulin resistance (28). For both studies, it should be emphasized that plasma acylcarnitines do not reliably reflect individual tissue metabolite levels as we have shown recently (25, 29).

Although we did not find a convincing correlation at baseline between plasma acylcarnitine levels and HOMA-IR, changes over time show that the clear improvement in insulin sensitivity was accompanied by a significant increase in acylcarnitine levels. The changes in individual chain lengths of acylcarnitines are important because they reveal some insight on physiological mechanisms in acylcarnitine metabolism during weight loss. Carnitine availability depends on dietary intake an endogenous synthesis. Both carnitine uptake via OCTN2 and carnitine synthesis are PPAR-alpha mediated and stimulated under hypocaloric conditions (16). The initial absence of increased free carnitine after 28 days may reflect increased esterification to

fatty acids. This effect probably became less after 84 days when weight loss declined and free carnitine levels increased. Indeed the increase in lipid derived acylcarnitines, including C2-carnitine, was most pronounced after the first 4 weeks. These changes over time were not seen for the amino acid derived acylcarnitines, which shows that under hypocaloric conditions lean body mass is more protected than fat mass (30).

Overall, acylcarnitines increased and insulin sensitivity improved in our study and so our results dissociate insulin sensitivity from plasma acylcarnitines. We speculate that the elevated acylcarnitine levels on day 28 and day 84 again might again reflect lipolysis, due to the caloric deficit of the weight loss intervention as detailed above. Additionally it could reflect high rates of fatty acid oxidation, although we could not confirm this with measurements of acylcarnitines in tissues or our whole body lipid oxidation data.

The effects on both acylcarnitine levels and the clinical parameters, such as weight loss, were greatest after 28 days and became weaker after 84 days. It is often seen in weight loss studies that the initial effects outweigh the later effects, which is not only due to changes in compliance over time (31-33). Under hypocaloric conditions, whole body energy expenditure decreases hampering further weight loss once a lower body weight is attained (34-36). This corresponds with the finding that the increase in acylcarnitines over time also showed a relation with weight loss. Here, a stronger decrease in total and lean body weight correlated with higher C4-OH-, C16- and C18:1- acylcarnitine levels.

We analysed and reported on whole group changes over time because of the total number of subjects. However, the weight loss intervention consisted of three arms (diet, sibutramine and exercise) (10). At baseline, the three groups differed in energy expenditure, the oxidation rates of the different substrates and in fasting plasma glucose levels. Therefore we performed within and not between group analyses. Nevertheless, the effects of the different interventions are intriguing and reveal subtle, but clear effects on clinical parameters and acylcarnitine metabolism in particular. Over time the sibutramine group lost the most weight and had the highest acylcarnitine levels. The other two groups, diet and exercise, showed modest weight loss

and a more moderate increase in acylcarnitine levels. This could confirm the idea that lipolysis, which is apparently greater when weight loss is greater, is reflected as higher levels of plasma acylcarnitines. Weight loss in the other two groups was comparable, but the rise in acylcarnitines was nearly absent upon weight loss in the exercise group. This could reflect more efficient mitochondrial fatty acid oxidation due to exercise (27, 37, 38), which can relieve the plasma compartment from accumulating acylcarnitines. For subgroups, the Bayesian hierarchical model showed that the change in plasma C4OH- and C18:1-carnitine remained significantly correlated with weight change in the sibutramine group but not in the other groups. Adding the subgroups together (see above) increased significance. This implies that both the diet alone and exercise group contributed significantly. Although the different interventions were extremely instructive for our understanding of acylcarnitine metabolism, the subgroups do have limitations, as groups were relatively small, the exercise intervention was only modest and sibutramine was pulled from the market due to side effects of the drug (39).

In conclusion, we have found an increase in several acylcarnitine species upon weight loss in obese human subjects, despite improvements in insulin sensitivity. It is likely that the level of acylcarnitines in plasma is driven by the rate of lipolysis and not by deranged mitochondrial FAO as insulin signalling is not impaired. Diet derived acylcarnitines as C16- and C18:1-carnitine seem the most relevant acylcarnitines at baseline and during weight loss. However the importance of plasma acylcarnitines as clinical markers for insulin resistance seems negligible and the interpretation of these lipid intermediates in relation to clinical parameters remains challenging.

TABLE 1 Baseline characteristics and clinical parameters for the total group and subgroups.

	Total	Diet	Exercise	Sibutramine
Subjects (*N)*	60	20	21	19
Age (years)	40 (± 8.6)	41 (± 7)	41 (± 8)	40 (± 11)
Sex (*m/f*)	23/37	10/10	7/14	6/13
Body weight (kg)	100.9 (± 12.6)	105.0 (± 12.7)	98.7 (± 11.7)	99.2 (± 13.0)
BMI (kg/m2)	34.8 (± 2.7)	35.2 (± 2.2)	34.5 (± 3.0)	34.8 (± 2.8)
Fat mass (kg)	43.8 (± 7.6)	43.0 (± 9.1)	41.4 (± 11.5)	43.2 (± 8.4)
Lean mass (kg)	52.2 (±10.9)	56.4 (± 11.7)	52.2 (± 10.9)	51.0 (± 9.7)
BMR (kJ/min)	5.2 (± 0.8)	5.4 (± 0.7)	5.2 (± 0.9)	5.1 (± 0.8)
RER	0.81 (± 0.05)	0.81 (± 0.04)	0.79 (± 0.04)	0.84 (± 0.06)*a*
FPG (mmol/L)	5.6 (± 0.5)	5.8 (± 0.5)	5.4 (± 0.3)	5.7 (± 0.6)*a*
Insulin (pmol/L)	14.7 (± 10.1)	13.2 (± 11.3)	13.2 (± 6.2)	17.9 (± 11.9)
HOMA-IR	3.7 (± 2.9)	3.5 (± 3.4)	3.2 (± 1.6)	4.6 (± 3.3)
NEFA (mmol/L)	0.5163 (±0.18)	0.49 (± 0.16)*b*	0.50 (± 0.18)	0.56 (± 0.19)*a*

BMI, body mass index; BMR, basal metabolic rate; RER, Respiratory Exchange Ratio; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment of insulin resistance; NEFA, non-esterified fatty acids. Data are represented as mean ± standard deviation; *a* = statistical significant between exercise and sibutramine, *b* = significant between exercise and placebo.

TABLE 2 Baseline profiles of the main plasma acylcarnitines of interest.

	Total	Diet	Exercise	Sibutramine
Subjects (*N)*	60	20	21	19
C0 (μmol/L)	32.9 (± 7.8)	33.5 (± 7.6)	34.3 (± 6.8)	31 (± 9.1)
C2 (μmol/L)	4.86 (± 1.68)	4.55 (± 0.96)	5.06 (± 1.13)	4.96 (± 2.56)
C4OH (μmol/L)	0.026 (± 0.021)	0.02 (± 0.012)	0.027 (± 0.012)	0.03 (± 0.031)
C10 (μmol/L)	0.18 (±0.11)	0.16 (± 0.10)	0.020 (± 0.13)	0.18 (± 0.08)
C14:1 (μmol/L)	0.079 (±0.035)	0.07 (± 0.03)	0.085 (± 0.036)	0.08 (± 0.036)
C16 (μmol/L)	0.024 (± 0.012)	0.021 (± 0.008)	0.026 (± 0.014)	0.024 (± 0.011)
C18:1 (μmol/L)	0.028 (±0.009)	0.029 (± 0.008)	0.028 (± 0.009)	0.028 (± 0.012)

Acylcarnitines are depicted as C followed by chain length and degree of saturation. No statistical significant differences were found between subgroups. The full profile is given in the online supplemental data.

TABLE 3 Correlations of HOMA-IR and plasma FFA with acylcarnitines

	HOMA-IR		Plasma FFA	
	Pearsons **ρ**	p	Pearsons **ρ**	p
C0 (µmol/L)	-0.21	0.11	0.31	0.12
C2 (µmol/L)	-0.18	0.18	0.49	0.00 *a*
C4OH (µmol/L)	0.00	0.98	0.26	0.05
C10 (µmol/L)	-0.21	0.12	0.27	0.043
C14:1 (µmol/L)	-0.24	0.07	0.52	0.00 *a*
C16 (µmol/L)	-0.37	0.00 *a*	0.59	0.00 *a*
C18:1 (µmol/L)	-0.031	0.017	0.49	0.00 *a*

Acylcarnitines are depicted as C followed by chain length and degree of saturation. P-values are shown before Bonferoni correction where as *a* refers to p-values that remained significant after Bonferoni correction. The full correlation matrix is given in the online supplemental data (table x).

TABLE 4 Multiple regression analysis of plasma FFA levels and selected plasma acylcarnitines

Analysis including C4OH-carnitine			Analysis excluding C4OH-carnitine		
	Unstandardized Coefficient B	*p*		Unstandardized Coefficient B	*p*
C0	-0.005	0.19	C0	-0.002	0.54
C2	0.045	0.12	C2	0.007	0.72
C4OH	-3.404	0.08			
C10	-0.389	0.16	C10	-0.248	0.36
C14:1	2.548	0.035	C14:1	1.840	0.11
C16	2.506	0.037	C16	3.071	0.01
C18:1	-0.132	0.91	C18:1	-0.159	0.89

TABLE 5a

Acyl-carnitine	*P*-value
C0	0.585886
C2	0.010468
C4	0.162672
C4-3-OH	0.000156
C5	0.162672
C12	0.618356
C14	0.231255
C16	0.005568
C16:1	0.002865
C18	0.193855
C18:1	0.000376

TABLE 5b

Acyl-carnitine	*P*-value
C0	0.900378
C2	0.085511
C4	0.558735
C4-3-OH	0.004058
C5	0.558735
C12	0.161710
C14	0.082855
C16	0.012940
C16.1	0.001734
C18	0.080707
C18.1	0.001972

FIGURE LEGENDS

FIGURE 1 Clinical parameters for the total group on day 0, 28 and 84. BMI: body mass index. Bars en whiskers represent mean and standard deviation. * = P < 0.05 after Bonferoni correction.

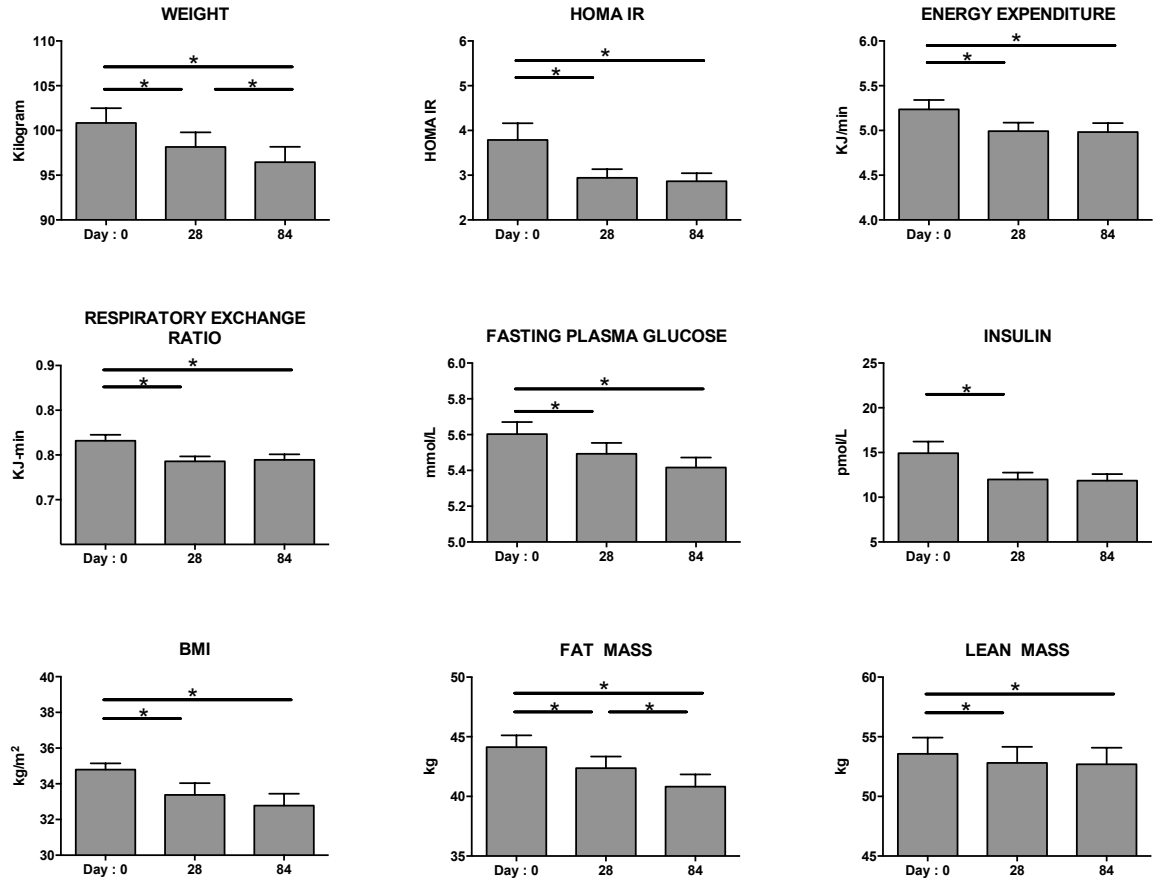


FIGURE 2 Plasma acylcarnitine levels for the total group on day 0, 28 and 84. BMI: body mass index. Bars en whiskers represent mean and standard deviation. Acylcarnitines are depicted as C where the following number depicts the chain length. * = P < 0.05 after Bonferoni correction.

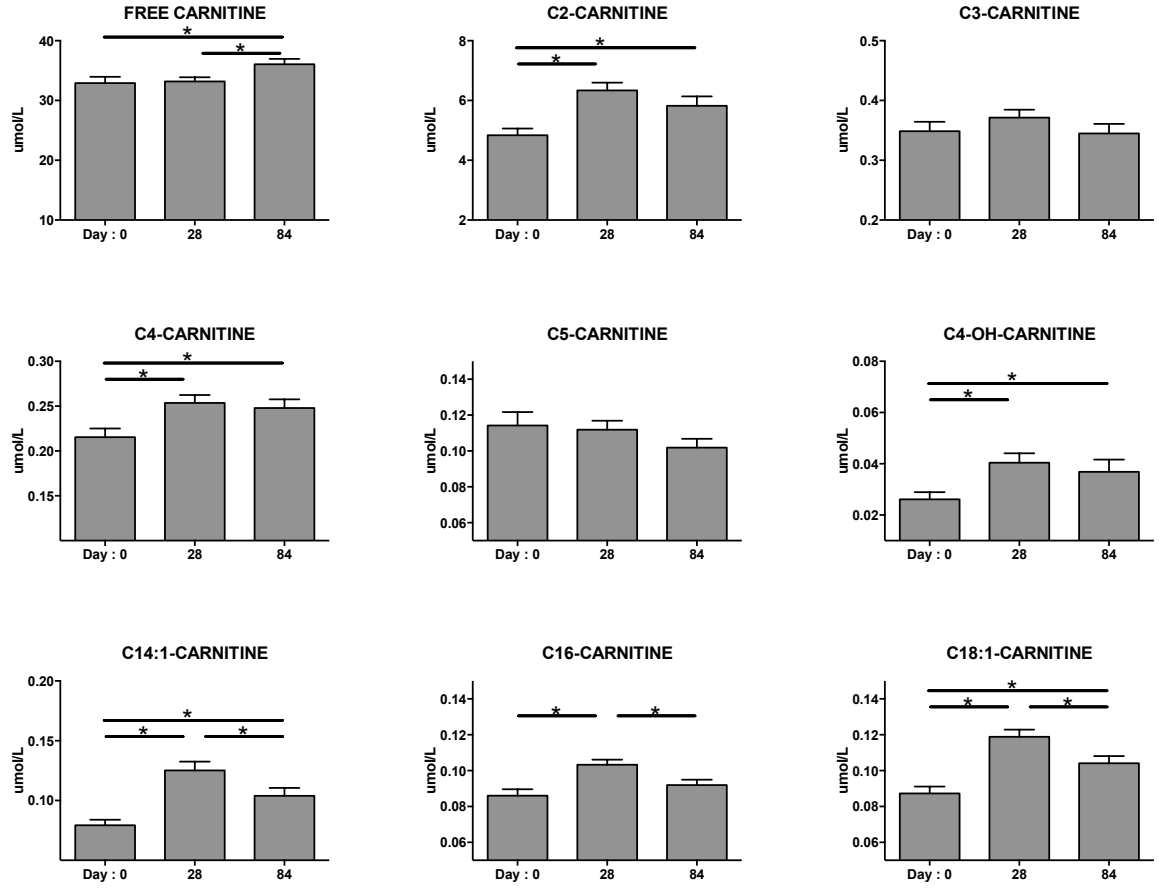


FIGURE 3 Pearsons Correlations between specific plasma acylcarnitine levels and weight for the total group on day 0. Acylcarnitines are depicted as C where the following number depicts the chain length.

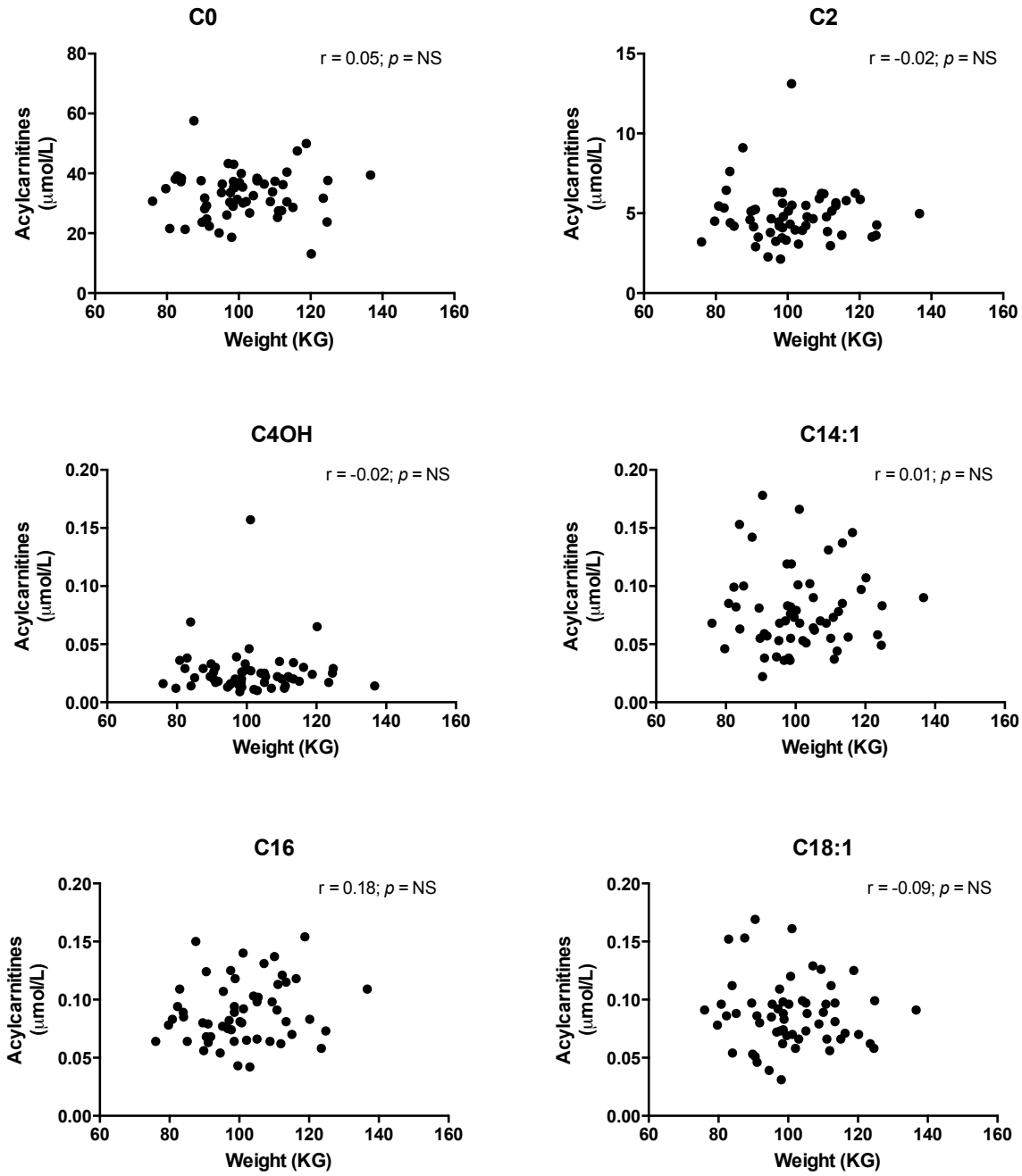


FIGURE 4 Pearsons Correlations between specific plasma acylcarnitine levels and homeostatic model assessment of insulin resistance (HOMA IR) for the total group on day 0. Acylcarnitines are depicted as C where the following number depicts the chain length. Depicted significance has been corrected by Bonferoni.

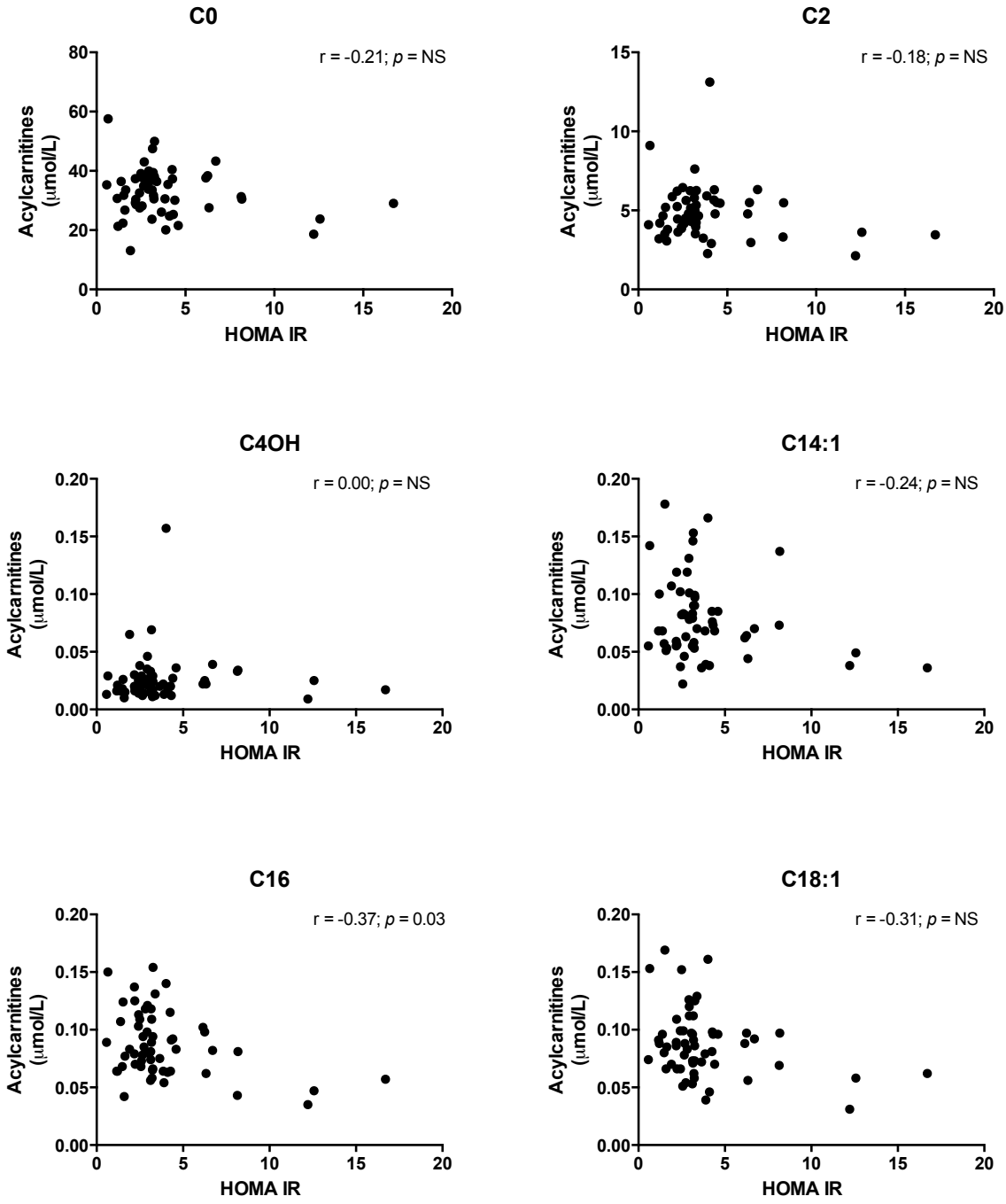


FIGURE 5 Pearsons Correlations between specific plasma acylcarnitine levels and plasma free fatty acids (FFA) for the total group on day 0. Acylcarnitines are depicted as C where the following number depicts the chain length. Depicted significance has been corrected by Bonferoni.

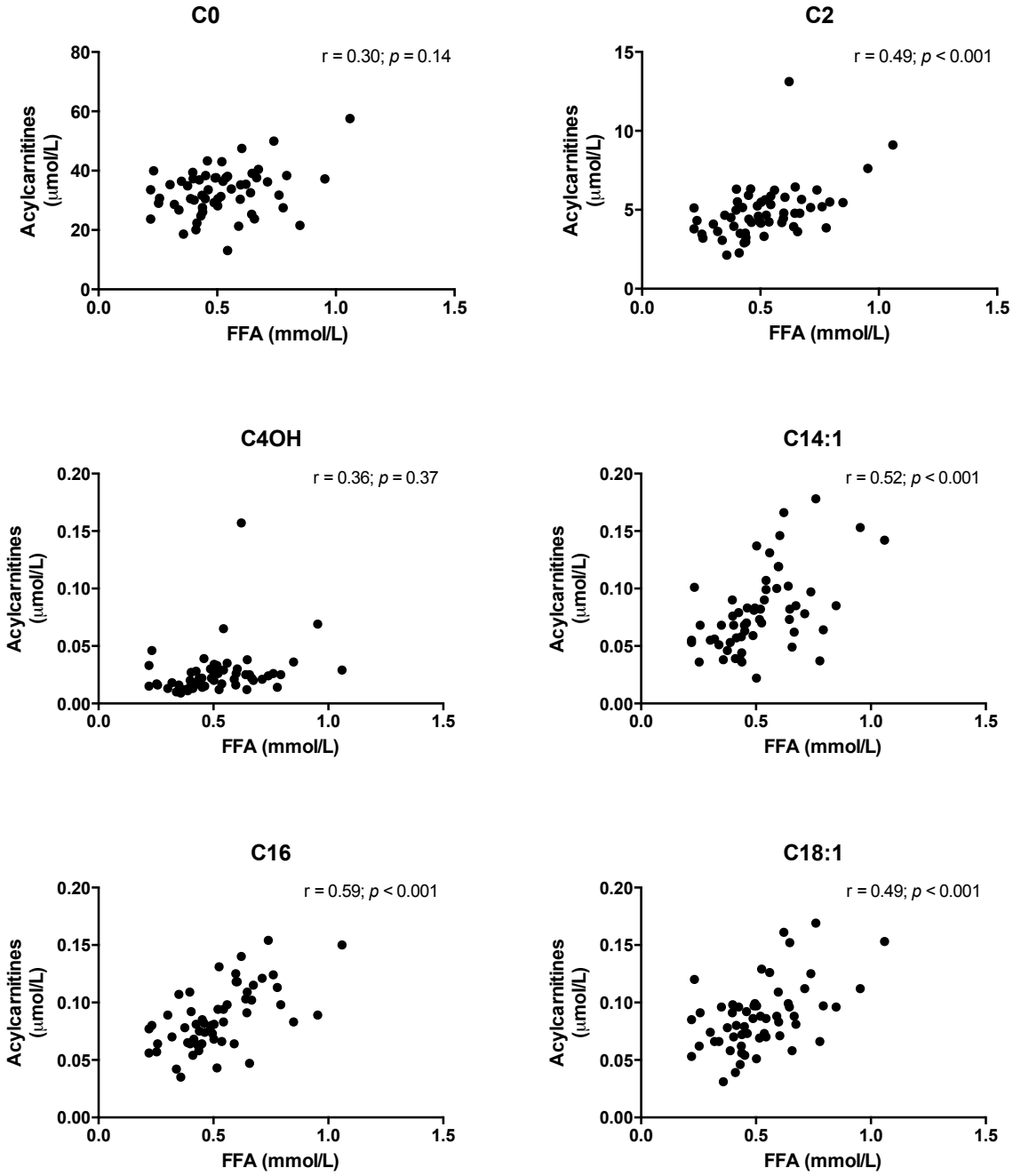
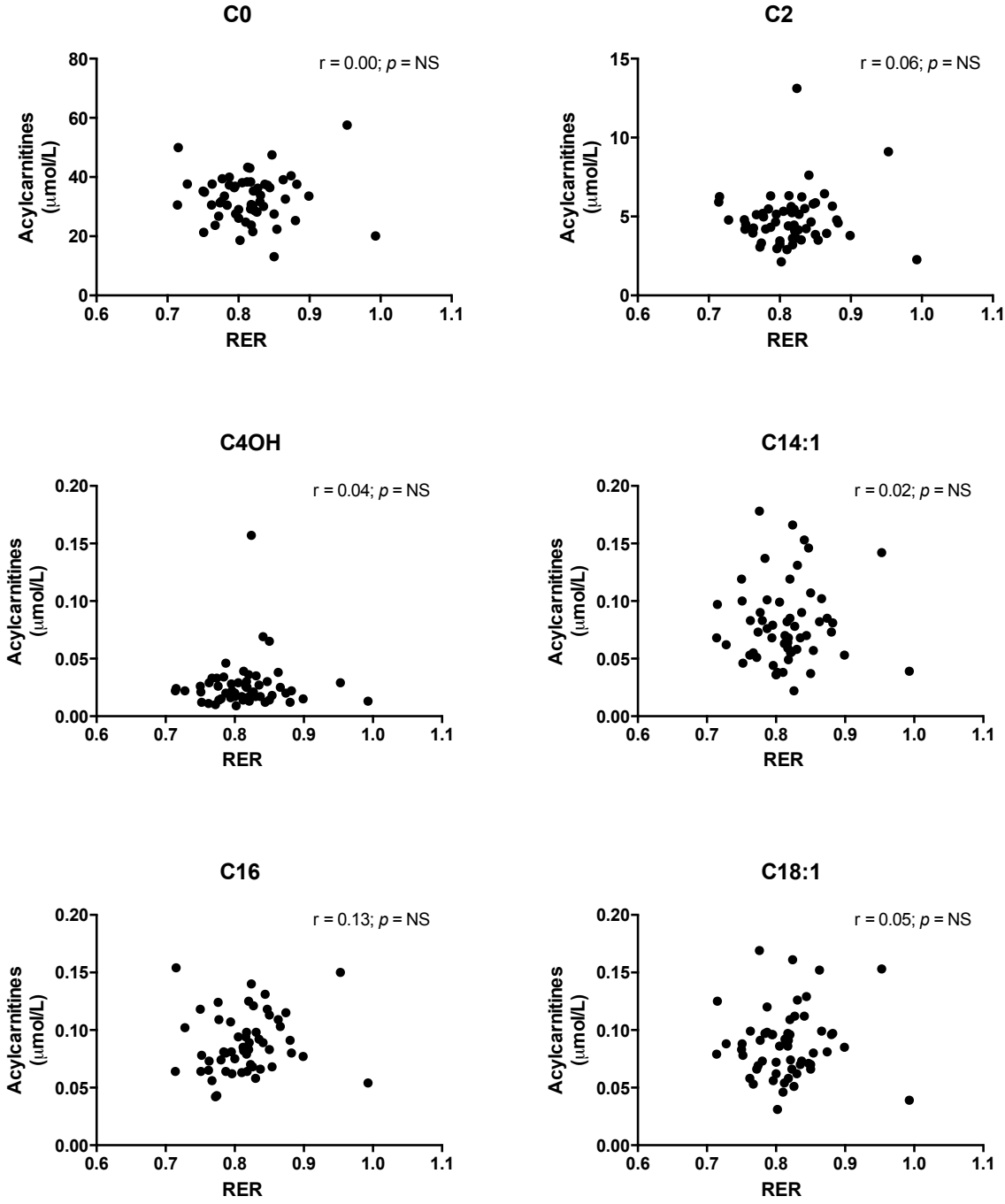


FIGURE 6 Pearsons Correlations between specific plasma acylcarnitine levels and respiratory exchange ratio (RER) for the total group on day 0. Acylcarnitines are depicted as C where the following number depicts the chain length.



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