**Title**

Palmitoyl-carnitine production by blood cells associates with the concentration of circulating acyl-carnitines in healthy overweight women

**Authors**

Chondronikola, Maria
Asghar, Rabia
Zhang, Xiaojun
et al.

# PALMITOYL-CARNITINE PRODUCTION BY BLOOD CELLS ASSOCIATES WITH THE CONCENTRATION OF CIRCULATING ACYL-CARNITINES IN HEALTHY OVERWEIGHT WOMEN

**Maria Chondronikola**[1,3], **Rabia Asghar**[2], **Xiaojun Zhang**[1], **Edgar L. Dillon**[2], **William J. Durham**[2], **Zhanpin Wu**[4], **Craig Porter**[1,3], **Maria Camacho-Hughes**[2], **Yingxin Zhao**[2], **Allan R. Brasier**[2], **Elena Volpi**[2], **Melinda Sheffield-Moore**[2], **Nicola Abate**[2], **Labros Sidossis**[1,3], and **Demidmaa Tuvdendorj**[2]

[1]Department of Surgery, University of Texas Medical Branch, Galveston, TX 77555

[2]Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555

[3]Metabolism Unit, Shriners Hospitals for Children, Galveston, TX 77555

Zoex Corporation, Houston, TX 7703

## Abstract

**Background**—Circulating acyl-carnitines (acyl-CNTs) are associated with insulin resistance (IR) and type 2 diabetes (T2D) in both rodents and humans. However, the mechanisms whereby circulating acyl-CNTs are increased in these conditions and their role in whole-body metabolism remains unknown. The purpose of this study was to determine if, in humans, blood cells contribute in production of circulating acyl-CNTs and associate with whole-body fat metabolism.

**Methods and Results**—Eight non-diabetic healthy women (age: $47\pm19$ y; BMI: $26\pm1$ kg•m$^{-2}$) underwent stable isotope tracer infusion and hyperinsulinemic-euglycemic clamp study to determine *in vivo* whole-body fatty acid flux and insulin sensitivity. Blood samples collected at baseline (0 min) and after 3h of clamp were used to determine the synthesis rate of palmitoyl-carnitine (palmitoyl-CNT) *in vitro*. The fractional synthesis rate of palmitoyl-CNT was significantly higher during hyperinsulinemia ($0.788\pm0.084$ *vs.* $0.318\pm0.012$ %•hr$^{-1}$, $p=0.001$); however, the absolute synthesis rate (ASR) did not differ between the periods ($p=0.809$) due to ~30% decrease in blood palmitoyl-CNT concentration ($p=0.189$) during hyperinsulinemia. The ASR of palmitoyl-CNT significantly correlated with the concentration of acyl-CNTs in basal ($r=0.992,p<0.001$) and insulin ($r=0.919,p=0.001$) periods; and the basal ASR significantly correlated with plasma palmitate oxidation ($r=0.764,p=0.027$).

**Conclusion**—In women, blood cells contribute to plasma acyl-CNT levels and the acyl-CNT production is linked to plasma palmitate oxidation, a marker of whole-body fat metabolism. Future

studies are needed to confirm the role of blood cells in acyl-CNT and lipid metabolism under different physiological (i.e., in response to meal) and pathological (i.e., hyperlipidemia, IR and T2D) conditions.

**Keywords**

acyl-carnitines; blood cells; stable isotope tracer; plasma palmitate oxidation; insulin resistance

## INTRODUCTION

Type 2 diabetes and atherosclerotic CVDs (aCVDs) are significant contributors to morbidity and mortality in the US (5,6,11). Obesity and insulin resistance (IR) predispose one to the development of T2D and aCVDs. IR is accompanied by excessive accumulation of lipid metabolites, e.g., acyl-carnitine (acyl-CNT) in skeletal muscle (2,10,13,19,27). Several studies have shown associations between excessive accumulation of intramuscular lipids and the development of IR in muscle (2,7,10,12,13,15,18,27). Recently, elevated plasma concentrations of acyl-CNTs have been reported in insulin-resistant individuals and patients with T2D when compared to healthy adults (1,17). However, the source of these acyl-CNTs remains unknown. To address this question, Schooneman et al. (21) measured plasma and tissue acyl-CNT profiles in mice and showed that, in general, the plasma acyl-CNT profile did not reflect the acyl-CNT profile of any specific tissues, including skeletal muscle or the liver. However, these authors did not determine the contribution (if any) of blood cells (i.e., peripheral blood mononuclear cells; PBMC, and platelets) to plasma acyl-CNTs (21). Acyl-CNTs are produced by the outer mitochondrial membrane enzyme carnitine palmitoyl transferase 1 (CPT-1), meaning that any cells that contain mitochondria, such as PBMCs and platelets, may contribute to plasma acyl-CNT levels (3,8,23,27). Since the plasma acyl-CNT profile is used as a marker of inborn metabolic disorders, most studies to date have focused on the pediatric population, with few data available on adults (3,8,23). The purpose of this study was to determine the potential contribution of blood cells (i.e., PBMC and platelets) to fatty acid oxidation (FAO) and insulin sensitivity in healthy women. We combined indirect calorimetry, stable isotope tracer and hyperinsulinemic-euglycemic clamp approaches to measure palmitate oxidation and systemic insulin sensitivity *in vivo* with *in vitro* assessment of the synthesis rate of palmitoyl-carnitine (palmitoyl-CNT) by blood cells. We hypothesized that the production of palmitoyl-CNT by blood cells contributes to circulating acyl-CNT concentration. Further, we theorize that the production of palmitoyl-CNT by blood cells correlates with whole body fatty acid metabolism.

## MATERIALS AND METHODS

Healthy women were eligible for the study. Exclusion criteria included any evidence of acute illnesses, diabetes mellitus [defined as fasting plasma glucose > 126 mg•dl$^{-1}$ or taking any hypoglycemic agents], taking medications that affect lipid metabolism, pregnancy or lactation, a history of substance abuse, and the inability to provide informed consent. Upon enrollment the subjects underwent a history and physical. All study procedures were approved by the Institutional Review Board at the University of Texas Medical Branch

(UTMB), Galveston, TX, and all participants provided signed informed consent and the studies were conducted at Clinical Research Center (CRC), UTMB.

### In vivo infusion study

Volunteers participated in a 6 h infusion study, as described in Figure 1. The infusion study consisted of two periods, each 3 h long. The first period of the study was designed to determine the basal fatty acid turnover. Indirect Calorimetry was performed at 120 min to determine whole-body fat oxidation, using Vmax Encore (CareFusion Corporation, San Diego, CA), as described previously (28). Plasma palmitate oxidation was measured using an infusion of stable isotope-labeled palmitate tracer, as described below. After the collection of baseline blood and breath samples to determine background enrichments, a bolus of $NaH^{13}CO_3$ (55 mmol/kg, dissolved in 0.9% NaCl) was given to prime the bicarbonate pool. Thereafter, a constant infusion of $U^{13}C_{16}$-palmitate (Cambridge Isotopes, Andover, MA) in 5% human albumin (Albuminar-5, CSL Behring LLC., Kankakee, IL) was administered (infusion rate: 0.06 $\mu mol \bullet kg^{-1} \bullet min^{-1}$) to assess plasma palmitate oxidation (28). Blood samples were obtained every 30 min for the first 150 min, and then every 10 min until 180 min. Breath samples were obtained at 150, 160, 165 and 170 mins after the start of stable isotope tracer infusion. At 180 min, a hyperinsulinemic-euglycemic clamp was conducted for the last 3 h of the infusion study, as previously described (14). Briefly, insulin (Lilly, Indianapolis, IN), dissolved in sterile NaCl 0.9%, was administered at a rate of 1 $mU \bullet kg^{-1}$ of fat free mass (FFM)$\bullet min^{-1}$. The glucose infusion rate (GIR) during the steady-state period of the clamp was used as a marker for whole-body insulin sensitivity.

### Clinical Chemistry Measurements

Fasting levels of plasma very low density lipoprotein cholesterol (VLDL-C), triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol(HDL-C) and low density lipoprotein cholesterol (LDL-C) were measured using a Vitros 5600 analyzer (Ortho Clinical Diagnostic, Rochester, NY) in the Clinical Pathology Laboratory at UTMB. Plasma glucose concentrations were determined using an automated glucose analyzer (Stat 2300; Yellow Spring Instruments). Serum insulin concentrations were determined using an Immulite 2000 Insulin (Siemens Medical Solutions USA, Inc., Norwood MA).

### Analyses of plasma lipid and breath samples

Plasma lipids were extracted using a heptane-propanol extraction buffer and free fatty acids (FFAs) were separated using thin-layer chromatography plates (Partisil LK5D, Silica Gel 150 Å, Schleicher & Schuell, Maidstone, England). After the samples were methyl-esterified, the tracer-to-tracee ratio of $U^{13}C_{16}$-palmitate in plasma FFAs was measured using GC-MS (MSD system, Agilent, Santa Clara, CA) monitoring the mass-to-charge ratios of 270, 285 and 286 for methyl palmitate. Eight fatty acids (FAs) in plasma FFAs were measured using a GC system with flame ionization detection (GC-FID 6890, Agilent, Santa Clara, CA); the relative contribution of palmitate to the total 8 FAs, which were measured, was expressed as percent contribution (25). The 8 FAs were myristate (C14:0), palmitate (C16:0), palmitoleate [C16:1(n-1)], stearate (C18:0), oleate [C18:1(n-9)], linoleate [C18:2(n-6)], linolenate [(C18:3(n-3)], and arachidonic acid [(C20:4(n-6)]. Total FFA concentrations were measured by means of internal standards using a GC-FID system (28).

$CO_2$ enrichment in breath samples was measured using GC combustion isotope ratio mass spectrometry (GC-C-IRMS, Delta V Advantage/Finnigan Gas Bench II, Thermo Fisher, Waltham, MA).

### In vitro studies

*In vitro* studies were designed to determine the rate of incorporation of $U^{13}C_{16}$-palmitate into palmitoyl-CNT under basal and insulin conditions (8). Blood samples (~ 2 ml) were collected in EDTA-containing tubes at two time points - at baseline (0 min) and at the end of the hyperinsulinemic-euglycemic clamp (360 min). After obtaining samples to measure the background parameters (i.e., the enrichment of $U^{13}C_{16}$-palmitate, the total concentrations of FFAs and acyl-CNTs), the remaining samples were mixed with 1 μL•mL$^{-1}$ of 2mM $U^{13}C_{16}$-palmitate dissolved in 5% human albumin. The samples were then incubated in a 37°C water bath with periodic mixing and aliquots were collected at 5, 10, 20, 40 and 60 min after the start of incubation. All aliquots were immediately frozen in LN2 to arrest all biochemical reactions.

### Analyses of blood lipids and acyl-CNTs

Blood FFAs were isolated and analyzed as described above. Blood acyl-CNTs were isolated using a 1 ml of an acetonitrile-methanol mixture (3:1) with 50 μL of 1M $KH_2PO_4$ (24, 29). To measure the concentrations of specific and total acyl-CNT species, $D_3$-C16:0-carnitine was added as an internal standard (45 μM). Samples were then analyzed to determine the total concentration of acyl-CNTs using a Liquid Chromatograph Mass Spectrometer (LC-MS 6130 Quadruple, Agilent, Santa Clara, CA), as previously described (24, 29). The measured acyl-CNT species were myristoyl-(C14:0), palmitoyl- (C16:0), palmitoleoyl-[C16:1(n-1)], stearoyl- (C18:0), oleoyl-[C18:1(n-9)], linoleoyl- [C18:2(n-6)] and linolenoyl-carnitines [(C18:3(n-3)]. The enrichment of $U^{13}C_{16}$-palmitoyl-CNT was measured to determine the tracer-to-tracee ratio of the label incorporated into palmitoyl-CNT. The white blood cell count was done using a Sysmex XE 5000/XT 2000i analyzer (Sysmex America, Inc., Lincolnshire, IL) in the Hematology Laboratory at UTMB.

### Calculations

Whole-body FAO was calculated using the whole-body oxygen consumption and carbon dioxide production rates, and the percent contribution of palmitate in total plasma FFAs, as described previously (28). For *in vivo* studies, rates of oxidation of plasma palmitate and fatty acids were calculated using the tracer enrichment data in breath and plasma FFA samples, as previously described (28). An acetate correction factor of 0.37 was used.

For *in vitro* studies, to calculate the kinetic parameters the tracer-to-tracee ratios of labeled palmitic acid and palmitoyl-CNT were converted to molar percent excess (MPE). The fractional synthesis rate (FSR) of palmitoyl-CNT was calculated by dividing the slope of incorporation of the label into palmitoyl-CNT by the area under the curve (AUC) of labeled palmitic acid (MPE) over 60 min *in vitro* experimental period, and is expressed in %•min$^{-1}$. The absolute synthesis rate (ASR) of palmitoyl-CNT was calculated by multiplying the FSR by the concentration of palmitoyl-CNT and expressed as nMol•L$^{-1}$•min$^{-1}$ (28).

## Statistical analyses and data presentations

Data are presented as means ± SE. The differences in parameters between basal and insulin periods from *in vivo* studies were evaluated using a two-tailed, paired, equal variance *Student's t*-test. The differences in parameters from *in vitro* studies were evaluated using two-way ANOVA repeated measures with the factors period (i.e., basal or insulin) and time (0, 5, 10, 20, 40 and 60 min). Correlations between the outcomes of interest were performed using a linear regression model. The GIR parameters were log-transformed for non-normal distribution prior to use in linear regression analyses. $p < 0.05$ was considered statistically significant.

# RESULTS

Nine healthy non-diabetic women were studied; however, due to technical problems results from one subject were unavailable, thus the data presented herein are from total of eight subjects. None of the subjects reported taking any medications known to affect glucose or lipid metabolism. The demographic and clinical laboratory data for these individuals are presented in Table 1. All the subjects participated in the same isotope infusion study, as described in Figure 1, to determine the *in vivo* parameters of whole-body FAO and systemic insulin sensitivity.

## *In vivo* study

Whole-body FAO parameters in the basal state were measured during the first 180 min of the infusion study (Fig. 1) are presented in Table 2. Thirty min after the start of the clamp, the plasma concentration of insulin significantly increased ($p < 0.001$), and remained elevated until the end of the infusion study (Fig. 2). The plasma glucose concentration was clamped at pre-insulin levels ($77 \pm 8$ mg•dL$^{-1}$). The average glucose infusion rate, a marker of whole-body systemic insulin sensitivity, was $20.95 \pm 2.17$ mMol•kg$^{-1}$•min$^{-1}$.

## *In vitro* study

The blood samples for *in vitro* acyl-CNT studies were collected at 0 min (i.e., basal period) and at 360 min or after 3 h of hyperinsulinemic-euglycemic clamp (i.e., insulin period; Fig. 1). The blood concentration of FFAs was significantly lower at all time points during the insulin period when compared to the basal levels (Fig. 3A). However, because blood concentration of FFAs did not change significantly within 1 h of *in vitro* experiments in either periods (Fig. 3A), the FFA concentration is presented as an average of all time points within each period. As expected, the average concentration of blood FFAs was significantly lower in the insulin period when compared to the basal period ($p = 0.003$, Fig. 3B).

Total blood acyl-CNTs were reduced by ~ 50% during the insulin period compared with basal; however, at any time point during the *in vitro* experiment, the differences did not reach statistical significance ($p$ = NS; Fig. 4A). The concentrations of individual acyl-CNTs species decreased to a similar extent (~ 50%), but the differences between the periods varied by species. For example, no significant differences were observed in the concentrations of C14:0 (Fig. 4B), C16:0 (Fig. 4C), C18:1 (Fig. 4F) and C18:2 (Fig. 4G) species, while the concentration of C16:1 at 40 min was significantly higher ($p = 0.017$) than baseline (0 min)

time point within the basal period (Fig. 4D). At the 20 min time point, the concentration of C18:0 was significantly lower ($p = 0.043$) during the insulin period compared to the basal period (Fig. 4E). Within the basal period, the concentration of C18:3 at baseline was significantly higher than at 20 ($p = 0.02$), 40 ($p = 0.003$) and 60 ($p < 0.001$) min. Also, during the insulin period the concentration of C18:3 was significantly lower in the insulin period at 0 ($p = 0.005$) and 5 min ($p = 0.038$) time points when compared to the basal period (Fig. 4H).

The steady state enrichments of $U^{13}C_{16}$-palmitate in the basal and insulin periods were 13.04±1.01 and 37.85±4.02%, respectively. The baseline enrichment of $U^{13}C_{16}$-palmitoyl-CNT in the basal period was zero percent, while in the insulin period it was 0.55±0.19%, which was accounted in the calculation of the actual enrichments in further time points. The increase in incorporation of $U^{13}C_{16}$-palmitate into palmitoyl-CNT is presented in Figure 5. There was a significant difference in the slope of production of $U^{13}C_{16}$-palmitoyl-CNT between the basal and insulin periods (Basal vs. Insulin: 0.041±0.003 vs. 0.292±0.032, $p < 0.001$). The FSR of palmitoyl-CNT was significantly higher in the insulin period ($p = 0.001$; Fig. 6A). However, the ASR of palmitoyl-CNT was not different between the groups ($p = 0.809$; Fig. 6B). This was due to ~30% decrease in the blood concentration of palmitoyl-CNT, although the difference did not reach statistical significance (Basal vs. Insulin: 0.538±0.175 vs. 0.298±0.102 μMol•L$^{-1}$, $p = 0.189$). The blood white cell count ($10^{-3}$•μL$^{-1}$) was not different between the basal and insulin periods (Basal vs. Insulin: 5538±446 vs. 6014±2273 cells, $p = 0.591$).

### Correlation analyses

Linear regression analyses demonstrated that the ASR of palmitoyl-CNT was significantly correlated with the blood concentration of FFAs during the basal ($p = 0.004$) and insulin ($p = 0.014$) periods (Fig. 7A). The ASR of palmitoyl-CNT also significantly correlated with blood concentration of acyl-CNTs in both the basal ($p < 0.001$) and insulin ($p = 0.001$) periods (Fig. 7B). The regression analyses also demonstrated that ASR of palmitoyl-CNT significantly correlated with blood concentration of all other measured acyl-CNT species, but not with C18:2(n-6)-CNT under hyperinsulinemic conditions, the data are presented in Table 3. Moreover, the ASR of palmitoyl-CNT was significantly correlated with the plasma palmitate oxidation rate in the basal period ($p = 0.007$; Fig. 7C). However, no significant correlation was observed between ASR of palmitoyl-CNT and whole-body palmitate oxidation, estimated using indirect calorimetry measurement in the basal period ($r = 0.091$, $p = 0.830$). The whole-body FAO, measured using indirect calorimetry, did not correlate the plasma FAO, measured using isotopic approach ($r = 0.661$, $p = 0.074$). Finally, correlation analyses demonstrated that GIR tended to be inversely correlated with the ASR of palmitoyl-CNT during the insulin period ($r = 0.703$, $p = 0.051$) but not during the basal period ($r = 0.305$, $p = 0.463$; Fig. 7D).

## DISCUSSION

The main finding of the current study is that in healthy women, the ASR of palmitoyl-CNT by blood cells is significantly correlated with blood concentration of total acyl-CNTs and the

plasma palmitate oxidation. Additionally, it was also significantly dependent upon the availability of blood FFAs during both fasting and insulin conditions.

The association of increased amounts of acyl-CNT species and the development or the progression of IR and thus T2D and aCVDs has been well documented (1,2,17,20). The production of acyl-CNTs occurs inside the cell, in the level of mitochondrion. Once FAs enter the cell they are activated by esterification to acyl-Coenzyme As (acyl-CoAs). Long-chain acyl-CoAs are then converted into acyl-CNTs via the activity of CPT1, which is located on the outer mitochondrial membrane. Long-chain acyl-CNTs are then transported through the inner mitochondrial membrane, where they are transesterified by CPT2 back to long chain acyl-CoAs and free carnitine. Long-chain acyl-CoA can then participate in β-oxidation. Recent reports have demonstrated increased levels of acyl-CNT species in the plasma of patients with IR and T2D (1,17). These data also suggest that acyl-CNTs can be exported across the cell membrane into the blood. Since ~90 % of body carnitine is found in skeletal muscle, most of the body's acyl-CNT esters are thought to be produced within myocytes (26). However, recent reports demonstrated that in mice there is no correlation between the profile of acyl-CNTs in plasma and other tissues, including skeletal muscle (21). Similarly, in pigs the contribution of skeletal muscle acyl-CNTs to plasma acyl-CNTs was shown to be insignificant (22). Other reports have demonstrated that blood cells (i.e., leukocytes and platelets) can also produce acyl-CNTs (3,8,23). Our current data demonstrating that the production of palmitoyl-CNT by blood cells is associated with the total concentration of blood acyl-CNTs indicates that blood cells contribute to the circulating acyl-CNT pool. Indeed, since the rate of palmitoyl-CNT production by blood cells correlates with total acyl-CNT concentration, it is tempting to speculate that blood cells may be somewhat responsible for the plasma acyl-CNT pool. However, it should be noted that palmitoyl-CNT is a fleeting metabolite, typically trans-esterified back to acyl-CoAs or exporting from the intracellular space almost immediately after its formation. Additionally, Shooneman et al., (22) conducted an *in vivo* study to evaluate the association of transorgan acyl-CNT fluxes and systemic acyl-CNT metabolism. The results of this robust study demonstrated that under fasting conditions liver plays an important role in whole body acyl-CNT metabolism. Thus, it is difficult to accurately determine the source of circulating acyl-CNTs from the measure of CPT1 flux alone. Nonetheless, our data suggest that blood cells play a prominent role in systemic lipid metabolism. If this is possible, the research in this field may be advanced tremendously due to the easier access to blood cells compared to muscle samples.

The activity of CPT1 is considered to be the rate-limiting step in long-chain FAO, thereby it has been suggested to be contributing in modulating FAO (4,9). Our data demonstrating that the *in vitro* absolute production of palmitoyl-CNT is correlated with both the blood FFA concentration and plasma palmitate oxidation rate suggests that blood cells could be a significant contributor in whole-body lipid metabolism. Moreover, our data may suggest that under physiological conditions the blood cell lipid metabolism may still be well maintained in relatively healthy women. However, whether this is true under pathological conditions (i.e., IR, T2D and aCVDs) is yet to be determined.

The capacity for FAO of a cell/tissue typically reflects the health and metabolic flexibility of that given cell/tissue. Currently, to estimate *in vivo* FAO one can utilize an indirect calorimetry approach and/or infusion of carbon-labeled FAs (28). Indirect calorimetry provides measurements of whole-body fat oxidation, which includes both the plasma and intracellular FAO; although the procedure that is required to perform indirect calorimetry is simple and widely used, the results do not necessarily reflect the rate of oxidation in individual tissue (28). Mensink et al., (16) demonstrated that the whole-body FAO was not different between lean and glucose intolerant or diabetic individuals. However, there was a redistribution of the source of oxidized FAs. Thus, indirect calorimetry may not provide us with detailed and physiologically, in some cases, significant data. Our data demonstrating that there is no significant correlation between the whole-body and plasma FAO, measured using indirect calorimetry and isotopic approaches, respectively, suggests that novel methods of estimating *in vivo* FAO would be beneficial to advance research in this area. Additionally, the fact that the *in vitro* ASR of palmitoyl-CNT did not correlate with whole-body palmitate oxidation measured using indirect calorimetry, but was significantly correlated with plasma palmitate oxidation measured using the tracer approach, may suggest that this application more precisely reflects lipid metabolism in blood cells. If this is the case, it may also suggest that blood cells significantly contribute in utilization of circulating FFAs and thus systemic lipid metabolism. However, we have attempted to estimate the contribution of blood cells' palmitate oxidation in total plasma palmitate oxidation. Thus, we multiplied the plasma palmitate oxidation ($\mu Mol \cdot kg^{-1} \cdot min^{-1}$) by body weights of the subjects and determined that the average total plasma palmitate oxidation was $31 \pm 3$ µmol·$min^{-1}$, and ranged from 23 to 45 $\mu Mol \cdot min^{-1}$. We also multiplied the ASR of palmitoyl-CNT ($nMol \cdot L^{-1} \cdot min^{-1}$) by estimated whole-body blood volume and determined that the absolute production of palmitoyl-CNT was $8 \pm 3$ nMol·$min^{-1}$, and ranged from 0.8 to 26 nMol·$min^{-1}$. Interestingly, the two variables still significantly correlated ($r = 0.865$, $p = 0.006$). If these estimations are correct, these data may suggest that blood cells' contribution in FAO may significantly vary even within the tightly chosen healthy people, like our study population. However, these results need to be further validated and, thus further studies are warranted. Therefore, blood cells may be of significant importance to the study of whole-body lipid metabolism, and the assaying of blood cell oxidative capacity may represent a novel biomarker of IR and metabolic disease.

The slope of incorporation of tracer into palmitoyl-CNT was different between the basal and insulin periods, which was not due to the number of blood cells, because there was no difference in the cell count between the two periods. This finding can be explained in part by a higher enrichment of $U^{13}C_{16}$-palmitic acid in the insulin period due to the insulin-related suppression of lipolysis and the decline in blood FFA concentration. However, FSR of palmitoyl-CNT, which accounts for precursor enrichment (i.e., $U^{13}C_{16}$-palmitic acid) was still higher during the insulin period. Given that at the same time the concentration of total acyl-CNTs was almost 50% lower, we can only hypothesize that during hyperinsulinemia, the next step of acyl-CNT metabolism (i.e., beta-oxidation) was increased. Thus, to understand the complete mechanism of blood cell fatty acid turnover, it is necessary to estimate the degradation of palmitoyl-CNT, which can perhaps be done by measuring the rate of production of uniformly labeled myristoyl-CNT (C14:0). Unfortunately, our current

methods did not allow us to measure these metabolites, so we were unable to estimate the turnover of palmitoyl-CNT or the actual *in vitro* FAO. This will be one of our goals for future studies.

There are a number of limitations in our study. Firstly, although our rationale for studying women only was to eliminate any possible sex effects, since it is well established that lipid metabolism differs between men and women, the fact that gender differences exist strongly argues that similar studies in men are warranted. This is a proof of concept study and further larger-scale studies, including both men and women with various conditions of impaired lipid metabolism, such as obesity, IR, T2DM and others, are warranted. Due to the small sample size we also did not observe a significant association between GIR, a marker for systemic insulin sensitivity, and the absolute synthesis of palmitoyl-CNT. The sample size analyses demonstrated that we will need fourteen subjects to achieve significance with power of 0.8 and alpha = 0.05. Lastly, we observed variability in hyperinsulinemia-related changes in the concentration of acyl-CNT species, which is in accordance with reports from other investigators (17,28), and may reflect individualized responses to hyperinsulinemia.

In conclusion, our data confirmed our hypothesis that the *in vitro* production of palmitoyl-CNT by blood cells contributes to circulating acyl-CNT concentration and may represent or contribute *in vivo* FAO (i.e., plasma palmitate oxidation). Additionally, we demonstrated that the production of palmitoyl-CNT depends on the availability of circulating FFAs and that whole blood samples can be used for these analyses, which may be useful in developing novel approaches to identify individuals with impaired FAO and thus at higher risk of developing IR, T2D and aCVDs. Future larger studies in a more diverse group of individuals are warranted to confirm the validity of our methods and results.

## Acknowledgments

## REFERENCES

1. Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, Hwang DH, Newman JW, Garvey WT. Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. J Nutr. 2009; 139(6):1073–10781. [PubMed: 19369366]

2. Aguer C, McCoin CS, Knotts TA, Thrush AB, Ono-Moore K, McPherson R, Dent R, Hwang DH, Adams SH, Harper ME. Acylcarnitines: potential implications for skeletal muscle insulin resistance. FASEB J. 2015; 29(1):336–345. [PubMed: 25342132]

3. Brivet M, Slama A, Saudubray JM, Legrand A, Lemonnier A. Rapid diagnosis of long chain and medium chain fatty acid oxidation disorders using lymphocytes. Ann Clin Biochem. 1995; 32(Pt 2): 154–159. [PubMed: 7785942]

4. Bruce CR1, Hoy AJ, Turner N, Watt MJ, Allen TL, Carpenter K, Cooney GJ, Febbraio MA, Kraegen EW. Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance. Diabetes. 2009; 58(3):550–558. [PubMed: 19073774]

5. CDC National Diabetes Fact Sheets. 2011. http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2011.pdf

6. CDC National Heart Disease Fact. http://www.cdc.gov/heartdisease/facts.htm

7. Consitt LA, Bell JA, Houmard JA. Intramuscular lipid metabolism, insulin action, and obesity. IUBMB Life. 2009; 61(1):47–55. [PubMed: 18839419]

8. Dessein AF, Fontaine M, Dobbelaere D, Mention-Mulliez K, Martin-Ponthieu A, Briand G, Vamecq J. Deuterated palmitate-driven acylcarnitine formation by whole-blood samples for a rapid diagnostic exploration of mitochondrial fatty acid oxidation disorders. Clin Chim Acta. 2009; 406(1–2):23–26. [PubMed: 19422814]

9. Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD. Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. Diabetes. 2001; 50(1):123–130. [PubMed: 11147777]

10. Ellis BA, Poynten A, Lowy AJ, Furler SM, Chisholm DJ, Kraegern EW, Cooney GJ. Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle. Am J Physiol Endocrinol Metab. 2000; 279:E554–E560. [PubMed: 10950822]

11. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 21. 2014; 129(3):399–410.

12. Houmard JA. Intramuscular lipid oxidation and obesity. Am J Physiol Regul Integr Comp Physiol. 2008; 294(4):R1111–R1116. [PubMed: 18256136]

13. Hulver MW, Berggren JR, Cortright RN, Dudek RW, Thompson RP, Pories WJ, MacDonald KG, Cline GW, Shulman GI, Dohm GL, Houmard JA. Skeletal muscle lipid metabolism with obesity. Am J Physiol Endocrinol Metab. 2003; 284:E741–E747. [PubMed: 12626325]

14. Kanaley JA, Shadid S, Sheehan MT, Guo Z, Jensen MD. Hyperinsulinemia and skeletal muscle fatty acid trafficking. Am J Physiol Endocrinol Metab. 2013; 305(4):E540–E548. 15. [PubMed: 23820622]

15. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 2008; 7:45–56. [PubMed: 18177724]

16. Mensink M, Blaak EE, van Baak MA, Wagenmakers AJM, Saris WHM. Plasma free fatty acid uptake and oxidation are alredy diminish in subjects at high risk for developing type 2 diabetes. Diabetes. 2001; 50:2548–2554. [PubMed: 11679433]

17. Mihalik SJ, Goodpaster BH, Kelley DE, Chace DH, Vockley J, Toledo FG, DeLany JP. Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity. Obesity (Silver Spring). 2010; 18(9):1695–1700. [PubMed: 20111019]

18. Ramos-Roman MA, Sweetman L, Valdez MJ, Parks EJ. Postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight and obesity. Metabolism. 2012; 61(2):202–212. [PubMed: 21820684]

19. Reaven GM. Pathophysiology of insulin resistance in human disease. Physiol Rev. 1995; 75(3): 473–486. [PubMed: 7624391]

20. Schooneman MG, Vaz FM, Houten SM, Soeters MR. Acylcarnitines: Reflecting or Inflicting Insulin Resistance? Diabetes. 2013; 62(1):1–8. [PubMed: 23258903]

21. Schooneman MG, Achterkamp N, Argmann CA, Soeters MR, Houten SM. Plasma acylcarnitines inadequately reflect tissue acylcarnitine metabolism. Biochim Biophys Acta. 2014; 1841(7):987–994. [PubMed: 24747043]

22. Schooneman MG, Ten Have GA, van Vlies N, Houten SM, Deutz NE, Soeters MR. Transorgan fluxes in a porcine model reveal a central role for liver in acylcarnitine metabolism. Am J Physiol Endocrinol Metab. 2015; 309(3):E256–E264. [PubMed: 26037250]

23. Seargeant LE, Balachandra K, Mallory C, Dilling LA, Greenberg CR. A simple screening test for fatty acid oxidation defects using whole-blood palmitate oxidation. J Inherit Metab Dis. 1999; 22(6):740–746. [PubMed: 10472534]

24. Sun D, Cree MG, Zhang XJ, Bøersheim E, Wolfe RR. Measurement of stable isotopic enrichment and concentration of long-chain fatty acyl-carnitines in tissue by HPLC-MS. J Lipid Res. 2006; 47(2):431–439. [PubMed: 16301738]

25. Tuvdendorj D, Zhang XJ, Chinkes DL, Wang L, Wu Z, Rodriguez NA, Herndon DN, Wolfe RR. Triglycerides produced in the livers of fasting rabbits are predominantly stored as opposed to secreted into the plasma. Metabolism. 2015; 64(5):580–587. [PubMed: 25682063]

26. Wall, BT., Porter, C. Carnitine Metabolism and Human Nutrition. CRC press, Taylor and Francis Group; 2014.

27. Wolf M, Chen S, Zhao X, Scheler M, Irmler M, Staiger H, Beckers J, de Angelis MH, Fritsche A, Häring HU, Schleicher ED, Xu G, Lehmann R, Weigert C. Production and release of acylcarnitines by primary myotubes reflect the differences in fasting fat oxidation of the donors. J Clin Endocrinol Metab. 2013; 98(6):E1137–E1142. [PubMed: 23633211]

28. Wolfe, RR., Chinkes, DL. Isotopic Tracers in Metabolic Research: Principle and practice of kinetic analysis. 2nd. New Jersey: Wiley-Liss; 2004.

29. Zhang XJ, Rodriguez NA, Wang L, Tuvdendorj D, Wu Z, Tan A, Herndon DN, Wolfe RR. Measurement of precursor enrichment for calculating intramuscular triglyceride fractional synthetic rate. J Lipid Res. 2012; 53(1):119–125. [PubMed: 21934122]

**Figure 1.**
A schematic presentation of the *in vivo* infusion study.
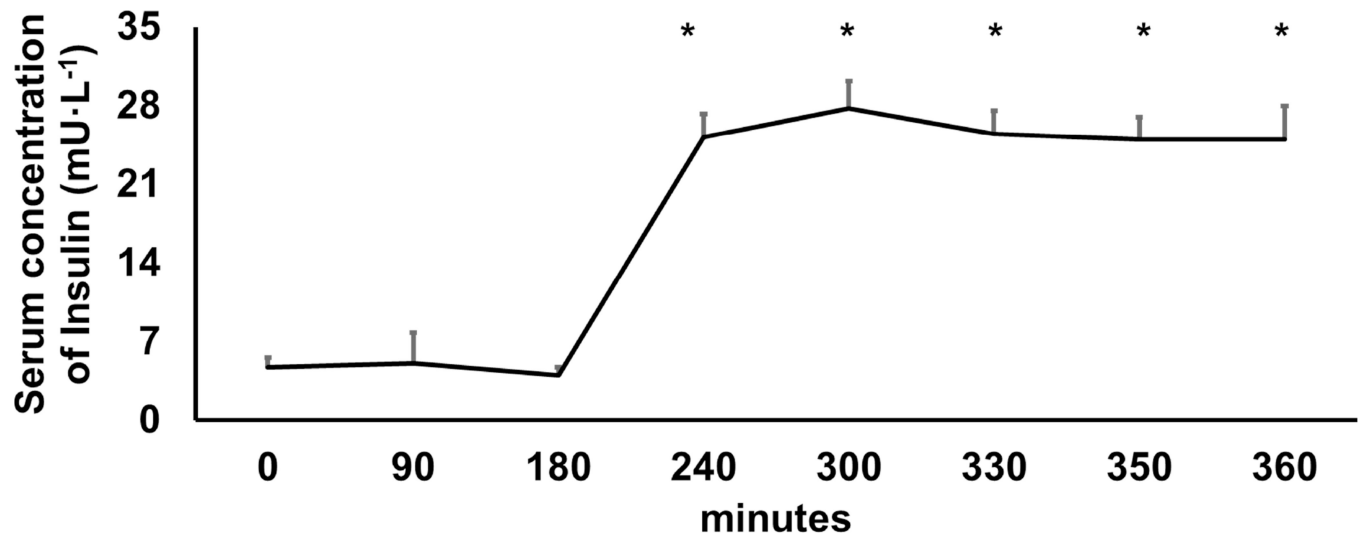
**Figure 2.**
Serum concentration of insulin significantly increased at 30 min after the start of hyperinsulinemic-euglycemic clamp and remained elevated until the end of the clamp. *, significantly higher than in baseline or at 0, 90 and 180 min ($p < 0.05$). Analyses were performed using Two-way ANOVA repeated measures with factors of period and time points, n = 8.
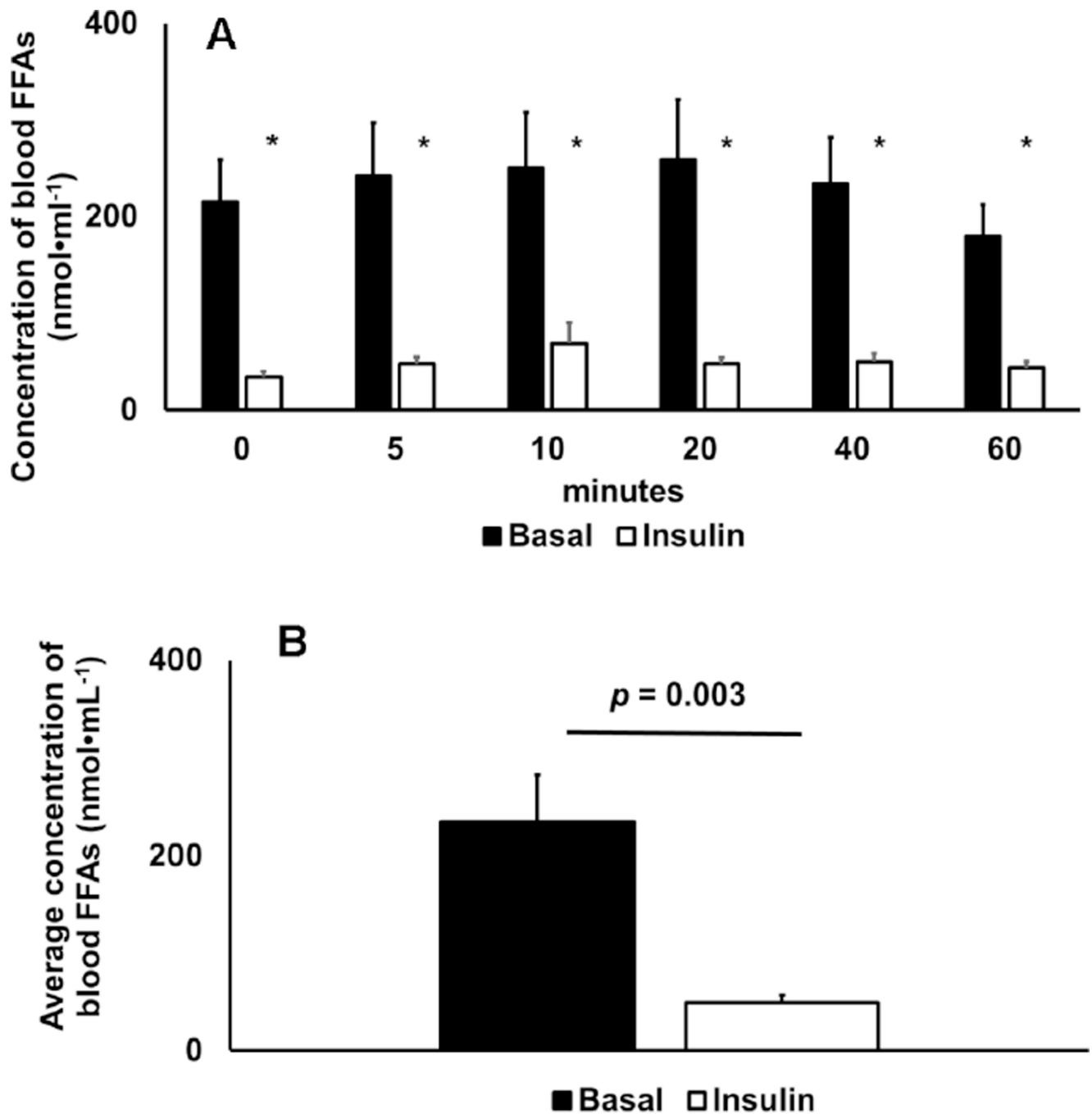
**Figure 3.**
Blood concentration of Free Fatty Acids (FFAs) was significantly lower at each time point of the insulin period (**A**) when compared to the same time point of the basal period (*, $p < 0.05$). However, the concentrations did not differ between the time points within the each period (i.e., basal or insulin periods), thus an average of FFAs at different time points within the same period was used for data analyses, which was significantly lower during the insulin period when compared to the basal (**B**). The difference between the periods and time points was determined using Two-way ANOVA repeated measures with factors of period and time
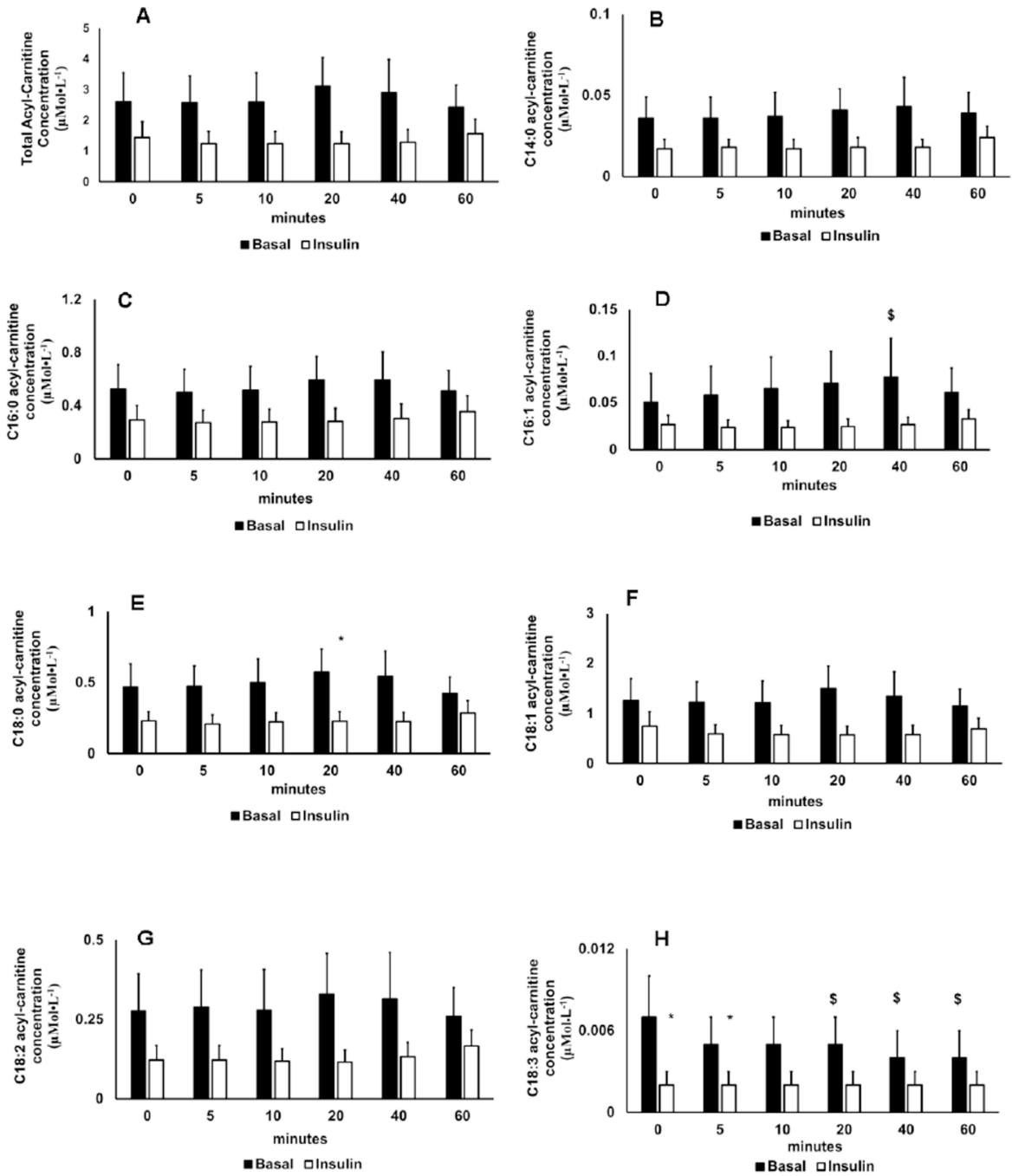
points, n = 8. The difference between the averaged values in basal and insulin periods was evaluated using two-tail, paired variance *Student t*-test.
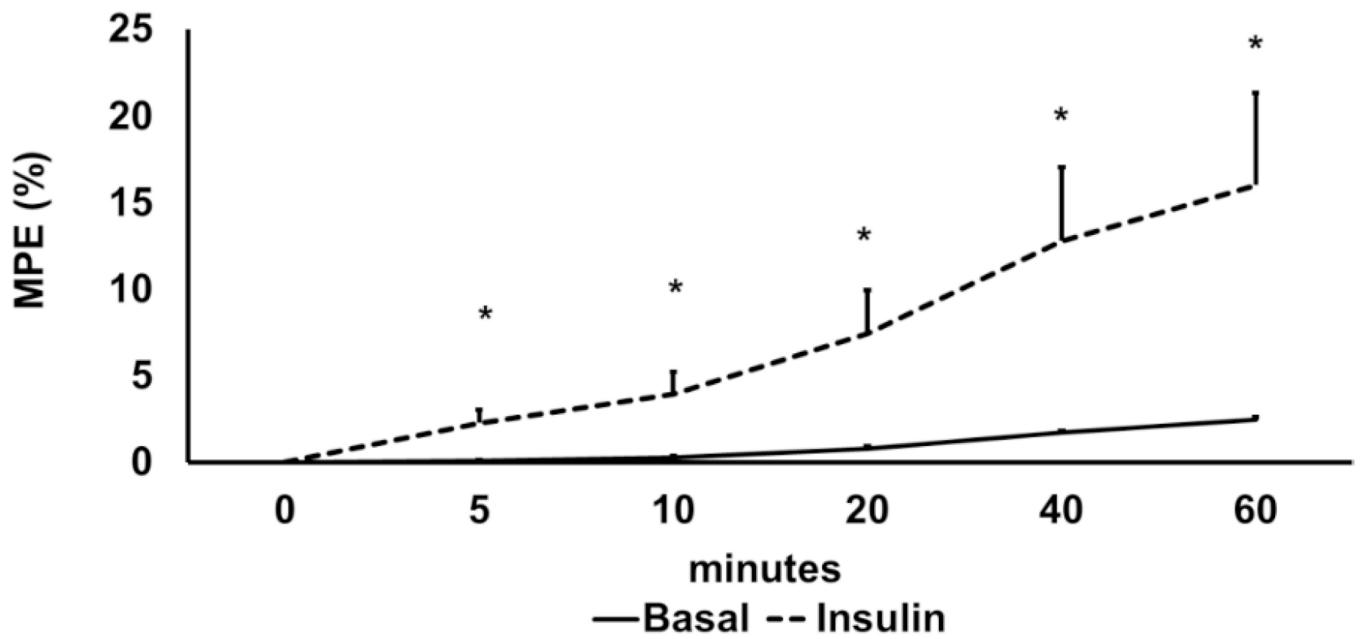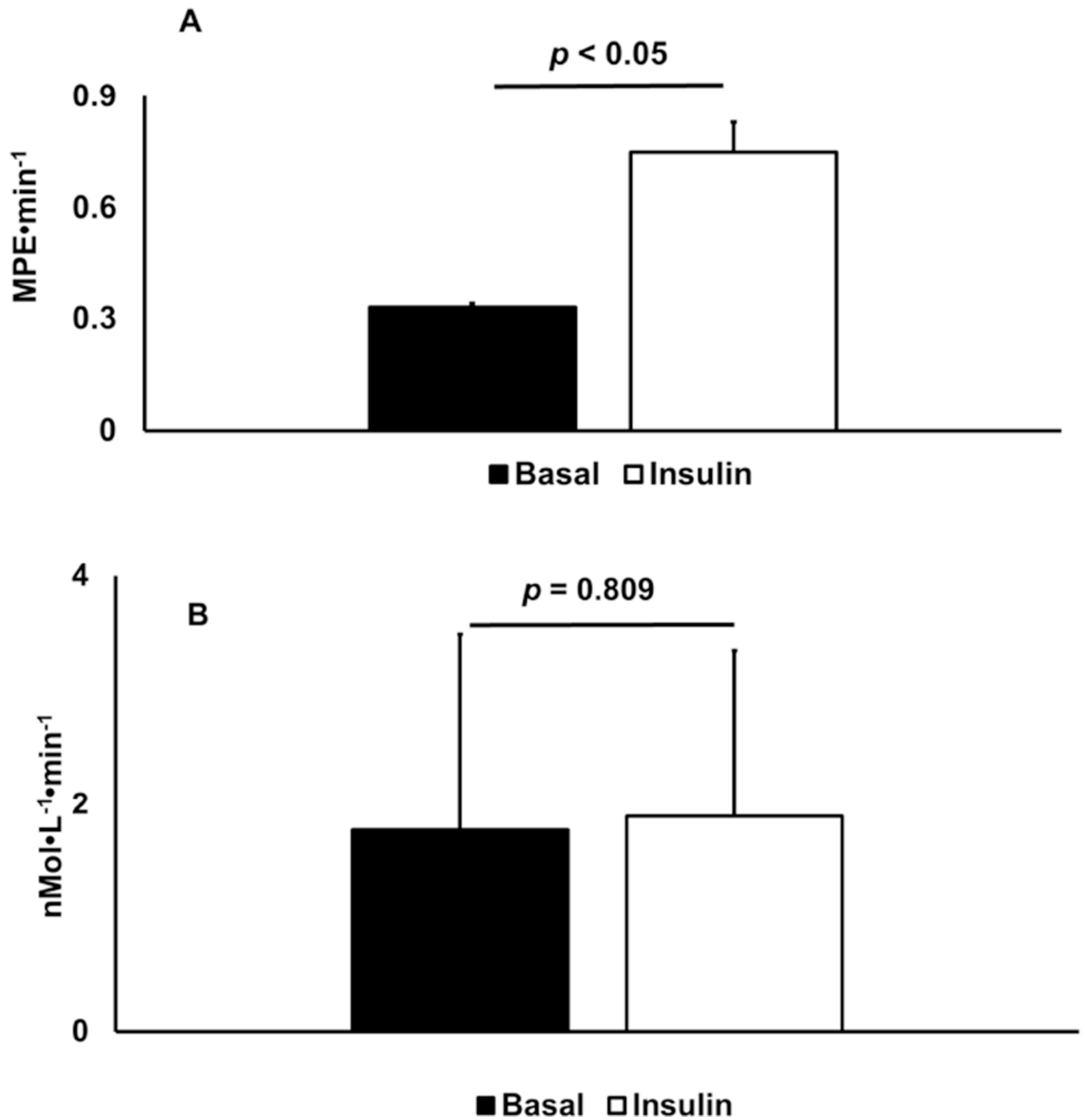
**Figure 4.**

The total concentration of blood acyl-CNTs was not different between the insulin and basal periods (**A**). No significant differences were observed in concentrations of C14:0 (**B**), C16:0 (**C**), C18:1 (**F**), C18:2 (**G**) species; while the concentration of C16:1 at 40 min was significantly higher ($p = 0.017$) than at 0 min time point within the basal period (**D**). At 20 min time point, the concentration of C18:0 was significantly lower ($p = 0.043$) during the insulin period when compared to the basal period (**E**). Within the basal period, the concentration of C18:3 at 0 min was significantly higher than at 20 ($p = 0.02$), 40 ($p =$

0.003) and 60 ($p < 0.001$) min, and during the insulin period the concentration of C18:3 was significant lower in the insulin period at 0 ($p = 0.005$) and 5 ($p = 0.038$) min time points when compared to the basal period (**H**). The difference between the periods and time points was determined using Two-way ANOVA repeated measures with factors of period and time points, n = 8.
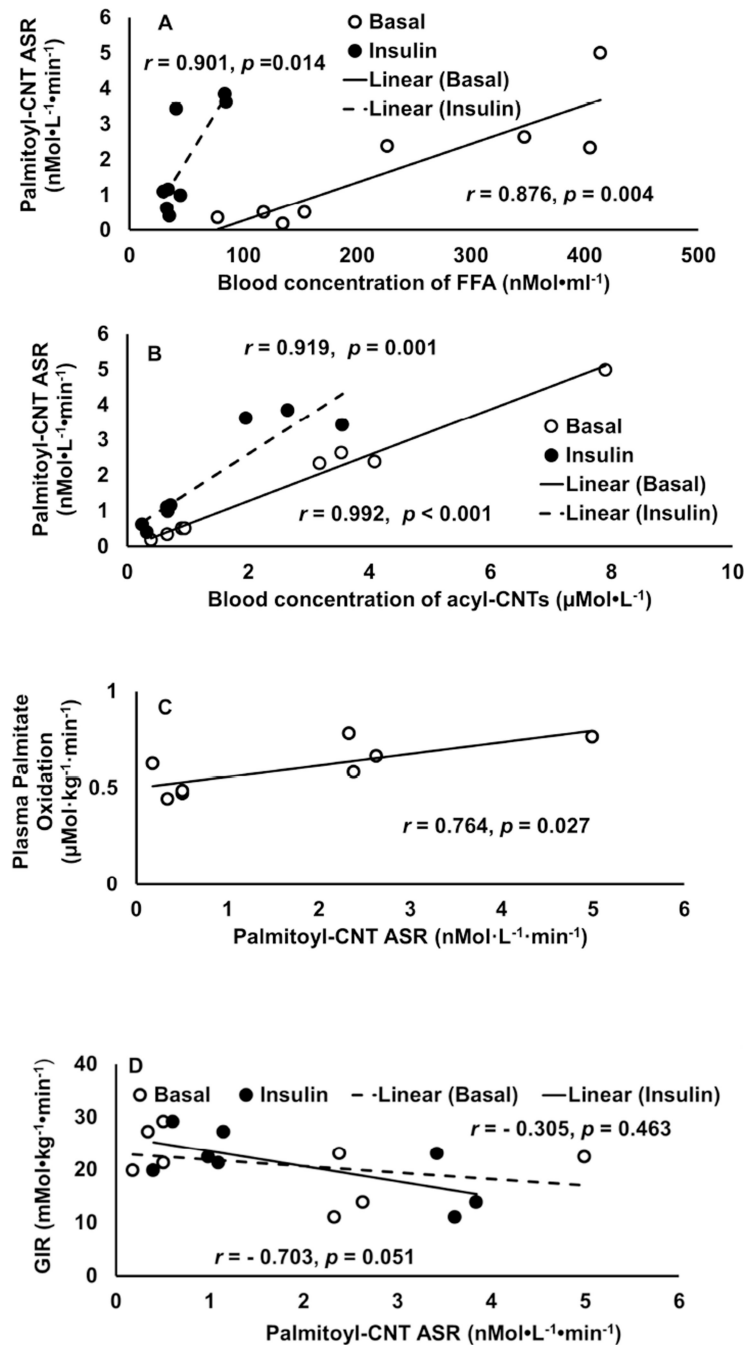
**Figure 5.**
The slope of incorporation of $U^{13}C_{16}$-palmitate into palmitoyl-carnitine (palmitoyl-CNT) was significantly higher during the insulin period. The enrichments are expressed as Mole Percent Excess (MPE). The difference between the periods and time points was determined using Two-way ANOVA repeated measures with factors of period and time points, n = 8.

**Figure 6.**
Fractional synthesis rate of palmitoyl-CNT was significantly higher ($p = 0.001$) during the insulin period (**A**), while the absolute synthesis rate was not different ($p = 0.809$) between the periods (**B**).

**Figure 7.**
Linear regression analyses demonstrated that the absolute synthesis rate (ASR) of palmitoyl-carnitine (palmitoyl-CNT) significantly depended on blood concentration of Free Fatty Acids (FFAs) in either periods (Basal: $r = 0.876$, $p = 0.004$; Insulin: $r = 0.901$, $p = 0.014$; **A**). The ASR of palmitoyl-CNT significantly associated with blood concentration of total acyl-CNT under either periods (Basal: $r = 0.992$, $p < 0.001$; Insulin: $r = 0.919$, $p = 0.001$; **B**). The basal ASR of palmitoyl-CNT significantly correlated with plasma palmitate oxidation ($r = 0.764$, $p = 0.027$; **C**). The correlation between the absolute synthesis rate of palmitoyl-CNT

and the glucose infusion rate (GIR), a marker of systemic insulin sensitivity, tended to be significant during the insulin period ($r = -0.703$, $p = 0.051$) but not during the basal period ($r = -0.305$, $p = 0.463$; **D**).

**Table 1**

Demographic and clinical chemistry parameters of study subjects.

| Parameters | Values |
| --- | --- |
| Age, y | 47±19 |
| Body weight, kg | 71±6 |
| Body mass index, kg•m$^2$ | 26±1 |
| Plasma fasting glucose, mg•L$^{-1}$ | 85±16 |
| Plasma fasting insulin, mU•dL$^{-1}$ | 7±5 |
| Plasma Cholesterol, mg•dL$^{-1}$ | 165±41 |
| Plasma Triglyceride, mg•dL$^{-1}$ | 103±97 |
| Plasma High Density Lipoprotein Cholesterol, mg•dL$^{-1}$ | 50±8 |
| Plasma Low Density Lipoprotein Cholesterol, mg•dL$^{-1}$ | 95±31 |
| Plasma Very Low Density Lipoprotein Cholesterol, mg•dL$^{-1}$ | 20±19 |

Data are presented as Means±SE.

**Table 2**

Body fat oxidation parameters measured using indirect calorimetry and infusion of $U^{13}C_{16}$-palmitate.

|  | Values |
|---|---|
| Indirect calorimetry |  |
| Respiratory exchange ratio | 0.75±0.02 |
| Total fatty acid oxidation | 3.27±0.24 |
| Isotopic determination: |  |
| Plasma palmitate oxidation | 0.43±0.04 |
| Plasma fatty acid oxidation | 0.99±0.20 |

Data (n = 8) are presented as Means±SE.

The oxidation rates are presented in $\mu M \bullet kg^{-1} \bullet min^{-1}$.

**Table 3**

Palmitoyl-CNT Absolute synthesis rate significantly correlated with blood concentration of all measured acyl-carnitine species under both, fasting and hyperinsulinemic conditions, except for C18:2-carnitine under hyperinsulinemic conditions.

|  | Basal | | Insuliin | |
| --- | --- | --- | --- | --- |
|  | *r* | *p* | *r* | *p* |
| C14:0- carnitine | 0.966 | < 0.001 | 0.979 | < 0.001 |
| C18:3- carnitine | 0.721 | 0.0436 | 0.951 | < 0.001 |
| C16:1- carnitine | 0.908 | 0.0018 | 0.871 | 0.005 |
| C18:2- carnitine | 0.895 | 0.0028 | 0.676 | 0.066 |
| C18:1- carnitine | 0.993 | < 0.001 | 0.901 | 0.002 |
| C18:0- carnitine | 0.988 | < 0.001 | 0.960 | < 0.001 |