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Lipids and insulin regulate mitochondrial-derived peptide (MOTS-c) in PCOS and healthy subjects.

Running title: Lipids and insulin regulate MOTS-c.

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Abstract

OBJECTIVE:
Polycystic ovarian syndrome (PCOS) is a heterogeneous endocrine disorder associated with mitochondrial dysfunction and insulin resistance (IR). MOTS-c, a mitochondrial peptide, promotes insulin sensitivity (IS) through activating AKT and AMPK dependent pathways. The current study was designed to examine the response of MOTS-c to lipids (intralipid) followed by insulin in PCOS and healthy subjects.

METHODS:
All subjects underwent 5-hour intralipid/saline infusion with a hyperinsulinemic euglycaemic clamp in the final 2 hours. Plasma samples were collected to measure circulating MOTS-c using a commercial elisa kit. Subsequently this was repeated following an eight week exercise intervention.

RESULTS:
Intralipid significantly increased plasma MOTS-c both in controls and PCOS subjects, whilst the insulin infusion blunted the intralipid induced response seen for both lipids and MOT-c.
Intralipid elevated plasma MOTS-c to 232±124% of basal in control ($P<0.01$) and to 349±206% of basal in PCOS ($P<0.001$) subjects. Administration of insulin suppressed intralipid induced MOTS-c from 232±124% to 165±97% (NS) in control and from 349±206% to 183±177% ($P<0.05$) in PCOS subjects, respectively. Following exercise,

intralipid elevated plasma MOTS-c to 305±153% of basal in control (P<0.01) and to 215±103% of basal in PCOS (P<0.01) subjects; insulin suppressed intralipid induced MOTS-c only in controls.

CONCLUSIONS:
In conclusion, this is the first study to show increased lipid enhanced circulating MOTS-c whilst insulin attenuated the MOTS-c response in human. Further, eight weeks of moderate exercise training did not show any changes in circulating MOTS-c levels in healthy controls and in women with PCOS.

Key words:
Polycystic ovarian syndrome; mitochondrial open reading frame of the 12S rRNA type-c; metabolic syndrome, mitochondrial dysfunction, triglycerides and insulin resistance.

Introduction

Polycystic ovarian syndrome (PCOS) is a complex heterogeneous endocrine disorder affecting 5-20% of women in the reproductive age group and is one of the most common causes of infertility and metabolic disorder in these women [1] . In Qatar, the frequency of PCOS among women aged between 18–30 years is estimated to be 12% according to original NIH criteria for its diagnosis [2]. PCOS is a chronic pro-inflammatory state associated with obesity, diabetes and dyslipidemia [1]. Women with PCOS commonly have symptoms of oligomenorrhoea, polycystic ovaries, hirsutism, subfertility and insulin resistance (IR) [3]. PCOS women have elevated levels of cardiovascular disease risk (CVD) factors including type 2 diabetes (T2DM), carotid intima-media wall thickness and platelet dysfunction [4]. Weight loss improves many clinical features of PCOS, including menses regularity and fertility [5], and reduces CVD risk factors and IR [6]. Cellular mechanisms leading to IR in PCOS are not clearly understood. Adipose tissue dysfunction and dysregulated expression of adipokines have been implicated in the pathophysiology of PCOS. Adipose tissue may communicate with the brain, ovaries, and uterus through adipokines, to regulate reproductive functions and metabolic features of women with PCOS [7]. Over-production of pro-inflammatory adipokines such as TNFα, hyperstimulation of GLUT4 glucose transporter [8] and reduced expression of beneficial adipokines such as adiponectin have been observed in PCOS [9].

Mitochondrial transcriptome data analysis provided initial evidence for the presence of small RNAs and polypeptides originating from mtDNA [10] that possess significant biological activity [11, 12]. Humanin was the first mitochondria-derived peptide discovered from the 16s ribosomal region of mtDNA [11]. Recently, another mitochondrial peptide originating from the

12s ribosomal region of mtDNA was identified and named MOTS-c (mitochondrial open reading frame of the 12S rRNA type-c). As expected, due to its mitochondrial origin, MOTS-c is expressed in key metabolic organs such as heart, skeletal muscle, testes, liver and brain [12]. The expression of MOTS-c is down-regulated following calorie restriction in skeletal muscle in mice [12]. Studies in rodents demonstrated that small mitochondrial peptides promote mitochondrial metabolism, regulate critical processes such as aging, inflammation and reversed IR [12-15].

MOTS-c enhanced glucose utilization, promoted IS and restored metabolic homeostasis through activation of AMPK-dependent mechanisms in skeletal muscle and protected rodents against IR induced by obesity and ageing [12]. Some of the biological responses mediated by MOTS-c were due to its ability to upregulate cellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), a potent activator of the AMP-activated protein kinase **(**AMPK) pathway and mitochondrial metabolism (24). MOTS-c treatment suppressed bone loss following ovariectomy via activation of an AMPK-dependent pathway, suggesting the importance of MOTS-c in bone metabolism and osteoporosis [16].

The role of MOTS-c in human pathophysiological conditions such as PCOS that is associated with multiple metabolic abnormalities including hyperinsulinemia, IR, obesity, and hyperlipidemia, has not been studied previously; therefore the present study was designed to examine the functional effects of acute intravenous administration of lipids, insulin and a combination of both before and after 8 weeks of a moderate exercise intervention on the circulatory levels of MOTS-c in control and PCOS subjects.

Study design and methodology

Study Subjects

Twelve PCOS women were recruited from the endocrine clinics. PCOS was diagnosed based on the presence of two out of three criteria; oligomenorrhoea, clinical or biochemical hyperandrogenism and polycystic ovaries on ultrasound after exclusion of other endocrine causes of hyperandrogenism according to the Rotterdam criteria; however, all women with PCOS fulfilled all 3 criteria [17]. Ten healthy volunteers matched for weight and BMI were recruited through advertisements at the local University and the hospital intranet. The healthy women had regular menstrual cycles and no clinical signs of polycystic ovaries. All the participants were nonsmokers, took no regular medications, and had no concurrent illness. All the subjects were asked not to use drugs containing acetylsalicylic acid during the week

preceding the experiments. Subjects with impaired glucose tolerance identified by an oral glucose tolerance test at screening were excluded. All women had a pregnancy test prior to their inclusion in the study. Following overnight fasting (O/N) all the subjects underwent 5 hour saline infusion with IS assessed by a hyperinsulinemic euglycaemic clamp in the final 2 hours to determine IS (M value). A week later, the subjects underwent 5 hour intralipid/saline infusion (20% soybean oil, 1.2% egg yolk phospholipids, and 2.2% glycerol; Kabi Fresenius Pharmacia) with a hyperinsulinemic euglycaemic clamp in the last 2 hours to determine IS (M value) [18]. Following this procedure, all the participants underwent supervised moderate intensity (60% maximum oxygen consumption) exercise, 3 hours weekly for 8 weeks. During the second phase of this study, the phase 1 protocol was repeated in all the subjects. Normal control women had the initial clamp in the first week of their menstrual cycle, whilst PCOS women were clamped after 6 weeks amenorrhea.

Insulin clamps

After fasted blood samples were taken, either normal saline 1.5 mL/min or 20% intralipid 1.5 mL/min, along with unfractionated heparin sodium 0.3 unit/kg/min was infused for 5 h. At 180 minutes, a 2 h hyperinsulinemic-euglycemic clamp was started using intravenous soluble insulin (Humulin S, Eli Lilly and Co., Indianapolis, IN) at a rate of 80 mU/m2 surface area/min for the first 20 minutes, followed by a constant rate of 40 mU/m2 surface area/minute for the remaining 100 minutes. Plasma glucose was clamped at 5.0 mmol/L with a variable infusion rate of 20% dextrose, adjusted relative to arterialized blood glucose measurements undertaken every 5 minutes. Endogenous glucose production was more than 90% suppressed by an acute rise of insulin with the primed insulin infusion. The rate of insulin stimulated glucose disposal (mg/kg/min) (M value), a measure of IS, was calculated from the mean of the five 20 minute periods from 20-120 minutes during the clamp using the Defronzo method [19]. Overall study scheme is shown in Figure 1.

Ethical approval

The study was approved by the Yorkshire and the Humber Research Ethics Committee (reference number 10/H1313/44) and The Medical Research Center at Hamad Medical Corporation (reference number RP #17261/17) all study participants gave their written informed consent before participation. Intralipid and euglycemic clamp studies were conducted in the clinical research facility at Hull and East Yorkshire Hospital.

Biochemical measurements

Plasma MOTS-c concentrations were measured using a commercially available ELISA kit (Peninsula Laboratories International, Inc**,** CA 94070 USA) according to manufacturer's recommended protocol, with an intra-assay variation of less than 10%. Serum insulin was assayed using a competitive chemiluminescent immunoassay (Euro/DPC, Llanberis, UK). Plasma glucose was measured using a Synchon LX 20 analyzer (Bechman-Coulter, High Wycombe, UK). Triglycerides (TG) and total cholesterol were measured by **a** Synchon LX 20 analyzer (Beckman-Coulter). The free androgen index (FAI) was calculated by dividing the total testosterone by SHBG, and then multiplying by 100. Serum testosterone (nmol/L) was assessed by high performance liquid chromatography linked to tandem mass spectrometry (Waters Corporation, Manchester, UK), SHBG (nmol/L) levels were measured by immunometric assay with fluorescence detection on the DPC Immulite 2000 analyzer (Euro/DPC, Llanberis UK). FSH (iu/L) was measured by an Architect analyser (Abbott laboratories, Maidenhead, UK); TCH (mmol/L), TG (mmol/L) and HDL (mmol/L) were measured using a Synchon LX 20 analyzer (Beckman-Coulter); LDL (mmol/L) was calculated using the Friedewald equation. Plasma glucose was measured using a Synchon LX 20 analyzer (Beckman-Coulter). NEFA was measured using an enzymatic colorimetric method (Wako NEFA-H2) on a Konelab20 auto analyzer with a coefficient of variation of 1.4%. All the above measurements were done according to manufacturer's recommended protocol.

Statistical analyses

Power; for 11 subjects with an alpha of 0.05 and power of 80% a difference in means greater than 1.26 standard deviations for MOT-c would be required to show significance. Descriptive statistics and Means ± Standard Deviations (SD) were calculated for all continuous variables in the study. Student t-tests were applied to determine significant differences between the groups. Trend analysis in the form of repeated measures ANOVA with post hoc (Bonferroni) analysis was performed to evaluate significant differences within the variable. A P value <0.05 (two-tailed) was considered for statistical significance. In post hoc analysis comparing multiple groups are as follows: a= Baseline to saline comparison; b= Saline to saline+ insulin comparison; c = Base line to saline+ insulin comparison. * = P<0.05, ** = P<0.01 and *** = P<0.001. Spearman Rank correlation was used to evaluate significant associations between variables and MOTS-c. The SPSS 22.0 statistical package used for the study analysis and Graphpad prism 5software was used for the data analysis.

Results

Baseline characteristics of control and PCOS subjects

Anthropometric measurements for both PCOS and healthy control subjects are shown in Table 1. There was no significant difference in height, weight or BMI between the controls and PCOS subjects at the time of recruitment into the study. The mean age [23.4±5.9 in controls and 28.8±6.6 in PCOS, ($P<0.05$)], waist [78.4±11.9 in controls and 91.4±14.9 in PCOS, ($P<0.05$)] and hip size [97.5±13 in controls and 109.8±12.6 in PCOS, ($P<0.05$)] were significantly higher in the PCOS group compared to the healthy volunteers. However, the waist to hip ratio was not significantly different between the two groups. 8 weeks of exercise did not make any significant alterations to BMI, waist, hip or waist to hip ratio in either the healthy or the PCOS subjects (Table 1).

Basic hormonal measurements such as LH, FSH, estradiol, prolactin, TSH, DHEAS, androstenedione, lipid profiles (including TCH, TG, HDL, and LDL), creatinine, creatine kinase, platelets, FPG, 2hr PG and HbA1c levels were similar between PCOS and healthy subjects. However, blood parameters that are a characteristic feature of PCOS subjects, such as the androgen profile, were elevated in PCOS patients as assessed by the FAI [2.05±1.9 in controls and 6.12±3.1 in PCOS, ($P<0.001$)]. The elevated FAI was due to a significant decrease in SHBG in the PCOS subjects [70.42±28.9 in controls and 31±20.3 in PCOS, ($P<0.001$)] although total testosterone was not significantly different between the 2 groups (Table 1). ALT [16.75±5.1 in controls and 26.25±13.5 in PCOS, ($P<0.05$)] was elevated in PCOS compared to healthy subjects and may reflect fatty liver in the PCOS subjects.

Acute insulin administration does not affect plasma MOTS-c before exercise intervention

In the first phase of the study, we tested the effects of saline and acute insulin administration on blood metabolic and biochemical parameters in the study subjects (Table 2). Plasma MOTS-c levels were not affected by the saline or insulin infusion in either the PCOS or control subjects. Saline infusion did not alter TCH, TG, NEFA and MOTS-c levels either in control or PCOS subjects. Administration of insulin infusion along with saline during the last two hours of the euglycemic clamp procedure suppressed both TG (b^{***}) and NEFA (b^{***}) in healthy volunteers, but only NEFA (b^{***}) was reduced in PCOS subjects. One way ANOVA analysis comparing mean differences among baseline, saline and the combination of saline +

insulin showed significant changes in TG (P<0.001 in controls vs NS changes in PCOS) and NEFA (P<0.001 in controls vs P<0.001 in PCOS subjects). Mean IS index M value was significantly higher in controls (4.96 ± 2.0) compared to PCOS subjects (3.26±0.8, P<0.016, Table 2).

Acute intralipid infusion increases MOTS-c levels in both control and PCOS subjects

Intralipid infusion altered many of the blood biochemical parameters except TCH in both groups (Table 3). Descriptive statistics assessing the mean differences between each group and the overall group comparison showed that the lipid infusion significantly increased plasma MOTS-c (a^{**}, P<0.05 in controls vs a^{**}, P<0.01 in PCOS subjects), and also elevated TG (a^{***}, P<0.001 in controls vs a^{***}, P<0.001 in PCOS subjects) and NEFA (a^{***}, P<0.001 in controls vs a^{***}, P<0.001 in PCOS subjects). To assess the effects of supra-physiological insulin whilst maintaining normal plasma glucose at 5mmol/L, insulin was infused along with intralipid for 2 hours and the resulting metabolic changes were measured. Insulin suppressed lipid-induced MOTS-c more profoundly in PCOS subjects compared to controls (a^{*}, P<0.05 in controls vs a^{**}, P<0.01 in PCOS subjects). In addition to the reduction in plasma MOTS-c, insulin also suppressed NEFA (b^{***}, P<0.001 in controls vs b^{***}, P<0.001 in PCOS subjects), TG (b^{***}, P<0.001 in controls vs NS changes in PCOS subjects**),** insulin (b^{**}, P<0.05 in controls vs b^{***}, P<0.001 in PCOS subjects) and the IS index M value was significantly reduced both in control (2.4 ± 1.7, P=0.007) and PCOS subjects (1.16 ± 0.8, P<0.0001, Table 3) in the lipid infusion compared to the saline treatment group as shown in Table 2.

The percentage changes in plasma MOTS-c following intralipid and insulin before exercise intervention is depicted in Figure 1B. Baseline levels were adjusted to 100%. Intralipid elevated plasma MOTS-c from 100% to 232±124% in control subjects and in PCOS subjects to 349±206% respectively. Administration of insulin suppressed intralipid-induced MOTS-c significantly in both groups, to 165±97% and 183±177% in control and PCOS subjects, respectively. Thus, insulin suppressed the lipid-induced increase in MOTS-c levels.

Exercise intervention followed by acute insulin infusion does not affect plasma MOTS-c

Regular exercise has many beneficial and positive effects in PCOS women. Blood biochemical measurements and plasma MOTS-c were measured in response to saline and hyperinsulinemic euglycaemic clamp in our study subjects following 8 weeks of supervised

exercise intervention. Exercise intervention did not show any improvement in the IS index M value in either group (Table 4) compared to the sensitivity index M value prior to exercise intervention (Table 2).

Infusion of insulin following saline did not have any significant effects on circulating TG and MOTS-c; however, overall group analysis showed significant changes in TCH ($P< 0.001$) and NEFA ($P<0.001$) in healthy subjects. In PCOS subjects, the effects were more profound after exercise intervention compared to controls. In PCOS subjects, insulin infusion suppressed circulating NEFA (b^{***}, $P<0.001$) as assessed by post-hoc analysis comparison between each group and overall group comparison by ANOVA analysis (Table 4).

Acute intralipid infusion enhances, and insulin suppresses, intralipid-induced plasma MOTS-c following exercise intervention

In the second phase of the study, we compared the effects of lipid and the lipid + insulin combination on plasma MOTS-c and other blood parameters following 8 weeks of supervised exercise reaching VO_2 max of 60% in each of the participating subjects. We observed a small but insignificant increase in baseline plasma MOTS-c in both healthy and PCOS subjects following exercise.

The overall group ANOVA analysis revealed the effect of intralipid and intralipid + insulin insults following exercise on blood biochemical parameters, such as TCH, TG and NEFA, were similar to that found prior to exercise training in healthy subjects. The net change in plasma MOTS-c in the lipid and the intralipid + insulin treatment group was significantly elevated following exercise in healthy subjects ($P<0.01$) compared to ($P<0.05$) before exercise measurements in the same subjects (Table 3 compared to Table 5). However, this differed to what was found in the PCOS subjects where the combination of intralipid + insulin administration lowered MOTS-c ($P< 0.05$, Table 5) after exercise compared to observed changes ($P<0.01$, Table 3) in MOTS-c with the lipid and the lipid + insulin insult prior to the exercise intervention. The percentage changes in plasma MOTS-c following intralipid and insulin after exercise intervention in PCOS and control subjects is depicted in Figure 1 D. Baseline levels were adjusted to 100%. Intralipid elevated plasma MOTS-c from 100% to 305 ± 153% in control subjects and to 215 ± 103% in PCOS subjects. Administration of insulin suppressed intralipid-induced MOTS-c significantly in both groups to 183 ± 102 and 212 ± 231% in control and PCOS subjects, respectively.

Correlation of MOTS-c with covariates

In all participants (*n* =10 controls and n=12 PCOS subjects), Spearman Rank analysis showed that, prior to exercise, plasma MOTS-c negatively correlated with plasma glucose (PG) at baseline (0 min) only in the PCOS group (r = - 0.773; P = 0.01). Following intralipid + insulin administration following the lipid only infusion, MOTS-c showed a significant positive correlation with NEFA (r = 0.773; P = 0.005) in controls and negatively correlated to TCH in PCOS subjects (r = -.580; P = 0.048). In the same subjects following 8 weeks of endurance exercise, plasma MOTS-c showed no significant correlation in controls whilst a negative correlation was observed in the PCOS group (r = -0 .733; P = 0.013) at 0 min. Following intralipid administration, plasma MOTS-c positively correlated with TG (r = .745; P = 0.013) and NEFA (r = .758; P = 0.011) in controls but no significant correlation was observed in PCOS subjects. However, in the combination intralipid + insulin administration samples, MOTS-c showed a positive correlation with TG (r = .648; P = 0.043) and NEFA (r = .855; P = 0.002) in controls though no significant correlation was observed in PCOS subjects (Supplementary table S1).

Discussion

In this study the role of FFA in the regulation of MOTS-c was evaluated and showed that MOTS-c was increased in both PCOS and controls by the intralipid infusion, but that the MOTS-c levels did not fall following the infusion of insulin that reduced NEFA and TG levels. The hyperinsulinemic-euglycemic clamp technique is a well-accepted model for studying *in vivo* IR, skeletal muscle IS and glucose metabolism in human subjects [19]. Whilst there are studies in vitro and in animal models, human studies are scant and this is the first to report the response of MOTS-c in such an interventional study.

Intralipid administration elevates circulating FFA levels that have deleterious effects in humans [20], promotes proinflammatory cytokine TNFα secretion, interferes with glucose metabolism, obstructs glucose oxidation and reduces glucose disposal rate [21, 22]. As a consequence, this promotes hepatic and peripheral IR [23, 24]. More than 50% of women with PCOS have lower IS and higher IR [6] compared to healthy women. The metabolic pathways responsible for this contrasting IR profile are not completely understood. PCOS women who lead a sedentary lifestyle and consume high caloric diets develop higher insulin resistance compared to metabolically active PCOS women.

We hypothesized that intralipid and insulin may differentially regulate IR, resulting in altered metabolic processes and altered glucose metabolism that may influence plasma MOTS-c expression. Further, increasing plasma FFA concentrations through an intralipid infusion increases IR [25] in healthy subjects and aggravates preexisting IR in PCOS subjects. This study was designed to compare the effects of FFA elevation through intralipid infusion and the influence of insulin infusion on fasting and insulin-stimulated glucose metabolism and to understand the importance of this metabolic shift on circulating MOTS-c in PCOS and healthy women. In accord with previous reports [26, 27], acute insulin administration significantly suppressed NEFA both in control and PCOS subjects, but with no alteration in plasma MOTS-c in either group, suggesting that acute insulin administration does not affect circulating MOTS-c. However, one cannot rule out the possibility of alterations in MOTS-c following chronic insulin administration since this was not tested as part of our study protocol.

Acute intralipid infusion diminished whole-body IS and elevated FFAs, changes accompanied by a parallel rise in plasma TG and an increase in circulating MOTS-c in both groups. The elevated FFAs and MOTS-c levels were suppressed upon insulin administration during the final 2 hours of the clamp procedure. The pioneer study on MOTS-c suggested that MOTS-c may be released into the circulation by key metabolic organs, such as the brain, skeletal muscle, heart and testes, comprising the major sources of the circulating pool of MOTS-c [12]. This study did not address the source of the circulating MOTS-c levels; however, given MOTS-c is a mitochondrial ribosomal protein, one would assume that the majority of the recognized cell types could produce MOTS-c and contribute to the circulating pool.

Regular moderate exercise reduces diabetes risk by more than 50% in subjects at risk of developing diabetes [28]. For individuals with diabetes, exercise makes it easier to control blood glucose via a reduction in skeletal muscle IR and an improvement in IS, leading to a reduction in long-term diabetes related comorbidities [29]. Obese PCOS women have an increased risk of metabolic syndrome with elevated cardiovascular risk factors [30]. Lifestyle modification and weight reduction enhances sex hormone binding globulin (SHBG) levels, reduces testosterone, restores ovulation and improves pregnancy rates [31]. Increased physical activity and weight loss improves the biochemical and phenotypic features of PCOS, thus reducing metabolic complications and thereby improving quality of life [32-34].

Endurance exercise intervention did not reduce weight or alter plasma MOTS-c significantly in either group; however, enhanced glucose utilization, through an improvement in IS [35], and a reduction in IR with lower plasma glucose levels were seen following the hyperinsulimic euglycemic clamp in PCOS subjects (Table 2 and Table 4). Increasing physical activity is a therapeutic option for reducing abnormalities associated with metabolic syndrome [36]. Some of the beneficial effects of exercise could be attributed to alterations in the adipokine profile, as exercise lowers proinflammatory cytokines that are responsible for the chronic inflammatory state and increased IR, whilst levels of anti-inflammatory cytokines are increased in the circulation and have been implicated in reducing IR [37]. In our study, a small but insignificant elevation in plasma MOTS-c levels was observed following exercise in both the PCOS and the healthy groups of women. This would also suggest that the changes in IR seen following exercise do not have a direct effect on MOTS-c, but rather MOTS-c may reflect FFA changes.

Lee et al. showed that MOTS-c treated mice subjected to hyperinsulinemic-euglycemic clamps required more infused glucose and had a better insulin-stimulated glucose disposal rate compared to controls, suggesting an improvement in IS. MOTS-c levels in the circulation and in skeletal muscle decline following induction of IR in mice, and IR was reversed by MOTS-c administration showing improved insulin signaling in skeletal muscle and IS [12]. In this context, the small rise in circulating MOTS-c observed in our study following exercise may be physiologically beneficial in humans. It is difficult to explain the importance of our finding, given that our sample size is small and that there are no similar human studies available with which to compare our results. However, in rodents, MOTS-c treatment promotes energy expenditure through activation of AMPK pathways and enhances insulin signaling through activation of the AKT signaling pathway, thus promoting IS in skeletal muscle [12]. Intralipid infusion prior and following endurance exercise significantly increased circulating TG and NEFA levels and this was accompanied by a parallel rise in plasma MOTS-c, suggesting that circulating MOTS-c is regulated by lipids *in vivo.* Administration of the insulin infusion during the last 2 hours of intralipid administration suppressed intralipid, induced an elevation in NEFA and also reduced plasma MOTS-c. In a recent study in ovariectomy induced obese mice MOTS-c treatment decreased circulating free fatty acids, lowered hepatic triglyceride content, down regulated the genes involved in lipogenesis and prevented ovariectomy induced weight gain [38]. From the above study in mice it could be speculated that the rise in plasma MOTS-c observed following intralipid administration could be a defense mechanism kicking in as a compensatory response to counteract the deleterious

effects caused by the rise in NEFA and triglycerides. Furthermore, the changes in IR associated with MOTS-c may be indirectly due to alterations in FFA levels.

The main strengths of this study are this is the first *in vivo* study to show the relationship between circulating lipids and MOTS-c in humans and that gold standard methodology for this intensive interventional study and well-supervised exercise intervention were employed. However, the limitations were that this was a small group of women with PCOS, though all fulfilled all three of the diagnostic criteria for its diagnosis thereby reducing heterogeneity; however, the groups were not well matched for BMI and age. For the reduction of MOT-c following insulin during the lipid infusion that did not differ in the control subjects the study was underpowered to determine the significance of this finding that could represent a type 2 statistical error, given that the reduction of MOT-c in the PCOS group was significant. The diagnostic criteria used to define the PCOS group would likely not have altered the results here, as IS was reported to be no different between the original NIH criteria and Rotterdam criteria [39]. In addition, the findings may not be generalized to other populations; however, due to the intense nature of the study protocol requiring multiple visits, and the frequent blood sampling needed to stabilize plasma glucose at steady state levels during clamp conditions, it is not feasible to perform such studies in a large cohort of subjects [19]. For this reason, the results must be interpreted cautiously and thus further studies in large cohorts of subjects are warranted to confirm our findings.

In conclusion, this is the first study to show lipid increases circulating MOTS-c and insulin attenuates lipid induced MOTS-c in humans. Further eight weeks of moderate exercise training did not show any changes in circulating MOTS-c levels in healthy controls and in women with PCOS.

Clinical trial registration Number: ISRCTN42448814

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Disclosure summary
The authors have no conflict of interest to declare.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions
MR: designed the experiments, performed the experiments, analyzed, interpreted data and prepared the manuscript;
JJ: performed ELISA studies prepared graphical presentation;
IB: performed ELISA studies with subsequent analysis;
MB: performed ELISA measurements and analyzed the data;
MA: Assisted with study design, performed the study protocol and revised the manuscript;
TS: Assisted with study design, performed the study protocol and revised the manuscript;
SS: performed ELISA studies prepared graphical presentation and revised manuscript;
MS: verified all obtained results and revised the manuscript;
SA**:** Designed the experiments, provided the samples, analyzed data and revised the manuscript;
AB: Designed the experiments analyzed data and revised the manuscript;
All authors took part in preparation and modification of figures and the manuscript.

Abbreviations

PCOS = Type 2 diabetes
IR = insulin resistance
IS = Insulin sensitivity
MOTS-c = mitochondrial open reading frame of the 12S rRNA type-c
BMI= body mass index
SBP = systolic blood pressure
DBP = diastolic blood pressure
TCH = total cholesterol
HDL = high density lipoprotein
LDL = low density lipoprotein
TG = triglycerides
HbA1c = glycated hemoglobin
ALT = alanine aminotransferase
TCH = total cholesterol
AMPK = 5' AMP-activated protein kinase

Figure and table legends

Figure 1: Effect of acute administration of intra lipid and insulin on plasma MOTS-c before and after exercise in control and PCOS subjects. A) Saline and insulin before exercise; B) Intralipid and Insulin before exercise; C) Saline and insulin after exercise; D) Intralipid and insulin after exercise. ***-$p<0.0001$; **-$p<0.001$; *- $p<0.05$.

Table 1: Anthropometric, biochemical and hormonal measurement of study subjects. . Wt - weight; bf- before; af- after; BMI- Body mass index; ex- exercise; WHR- Waist to hip ratio; NS-not significant. FAI-Free androgen index; SHBG-Sex hormone-binding globulin; LH-Luteinizing hormone; FSH-Follicle stimulating hormone; TCH-Total cholesterol; TG; Triglycerides; HDL-High density lipoprotein; LDL-Low density lipoprotein; CK-Creatine kinase; ALT-Alanine transaminase; FPG-Fasting plasma glucose; PG-Plasma glucose; HbA1c-hemoglobin A1c; TSH-Thyroid stimulating hormone; DHEAS-Dehydroepiandrosterone; NA-not significant.

Table 2: Effects of acute insulin administration on blood TCH, TG, NEFA, M-value and MOTS-c levels in study subjects before exercise intervention. a= Baseline to saline comparision; b= Saline to saline+ insulin comparision; c = Base line to saline+ insulin comparision. TCH-Total cholesterol; M-value – Insulin sensitivity index; TG; Triglycerides; NEFA- non-esterified fatty acid; MOTS-c- mitochondrial open reading frame of the 12S rRNA-c; NA-not significant.

Table 3: Effects of acute intralipid and insulin administration on blood TCH, triglycerides, NEFA, M-value **and MOTS-c levels in study subjects before exercise intervention.** a= Baseline to lipid comparision; b= Lipid to Lipid+ insulin comparision; c = Base line to lipid+ insulin comparision. TCH-Total cholesterol; M-value – Insulin sensitivity index; TG; Triglycerides; NEFA- non-esterified fatty acid; MOTS-c- mitochondrial open reading frame of the 12S rRNA-c; NA-not significant.

Table 4: Effects of acute insulin administration on blood TCH, NEFA, M-value and MOTS-c levels in study subjects after exercise intervention. a= Baseline to saline comparision; b= Saline to saline+ insulin comparision; c = Base line to saline+ insulin comparision. TCH-Total cholesterol; M-value – Insulin sensitivity index; TG; Triglycerides; NEFA- non-esterified fatty acid; MOTS-c- mitochondrial open reading frame of the 12S rRNA-c; NA-not significant.

Table 5: Effects of acute intralipid and insulin administration on blood TCH, TG, NEFA, M-value and MOTS-c levels in study subjects after exercise intervention. a= Baseline to lipid comparison; b= lipid to lipid + insulin comparison; c = Baseline to lipid + insulin comparision. TCH-Total cholesterol; M-value – Insulin sensitivity index; TG; Triglycerides; NEFA- non-esterified fatty acid; MOTS-c- mitochondrial open reading frame of the 12S rRNA-c; NA-not significant.

Supplementary table S1: Spearman Rank correlation of MOTS-c with variables. TCH-Total cholesterol; TG; Triglycerides; PG-Plasma glucose; NEFA- non-esterified fatty acid; M-value – Insulin sensitivity index.

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Table 1: Anthropometric, biochemical and hormonal measurement of study subjects

Anthropometric measurements	**Control (n=10)**	**PCOS (n=12)**	
	Mean ± SD	**Mean ± SD**	**Significance**
Age (y)	23.4±5.9	28.8±6.6	p<0.05
Height (m)	1.7±0.03	1.7±0.1	NS
Wt-bf-ex (kg)	69.7±15.5	82.9±17.1	NS
Wt-af-ex (kg)	70.6±16.2	85.0±19.6	NS
BMI-bf-ex (kg/ m^2)	25.1±5.2	29.3±6	NS
BMI-af-ex (kg/ m^2)	25.4±5.4	30.6±6.3	NS
Waist-bf-ex (cm)	78.4±11.9	91.4±14.9	p<0.05
Waist-af-ex (cm)	76.8±13.2	91.6±15.7	p<0.05
Hip-bf-ex (cm)	97.5±13	109.8±12.6	p<0.05
Hip-af-ex (cm)	99.9±13.3	111.6±12.8	NS
WHR-bf-ex (cm)	0.8±0.1	0.8±0.1	NS
WHR-af-ex (cm)	0.8±0.1	0.8±0.1	NS
Baseline blood biochemistry and hormonal measurements			
FAI	2.05±1.9	6.12±3.1	p<0.001
Testosterone (nmol/L)	1.08±0.3	1.48±0.8	NS
SHBG (nmol/L)	70.42±28.9	31±20.3	p<0.001
LH (iu/L)	6.97±11.5	5.123±3.8	NS
FSH (iu/L)	4.9±1.8	6.72±10.2	NS
Oestradiol (pmol/L)	168.50±195.1	178.23±129	NS
TCH (mmol/L)	4.6±0.9	4.15±0.6	NS
TG (mmol/L)	0.91±0.4	1.08±0.5	NS
HDL (mmol/L)	1.5±0.4	1.25±0.4	NS

LDL (mmol/L)	2.63±0.6	2.37±0.5	NS
TCH/HDL ratio	3.19±0.8	3.44±0.9	NS
Creatine (nmol/L)	63.83±7.5	64.77±10.9	NS
CK (U/L)	72.73±27	90.25±33.05	NS
ALT (U/L)	16.75±5.1	26.25±13.5	p<0.05
Platelets x10^9/L	267±72.08	285.67±49.2	NS
FPG (nmol/L)	4.72±0.5	4.74±0.4	NS
2 hrPG (nmol/L)	4.69±1.9	5.56±1.7	NS
HbA1c (mmol/mol)	32.8±5.8	33.36±2.7	NS
Prolactin (mU/L)	436±230.3	324.23±112	NS
TSH (mU/L)	2.15±1.6	1.6±0.6	NS
DHEAS (umol/l)	8±2.6	5.46±3.53	NS
Androstenedione (nmol/L)	9.94±3.7	10.66±5.3	NS

Table 2: Effects of acute insulin administration on blood TCH, TG, NEFA, MOTS-c and M-value in study subjects before exercise intervention.

Before exercise		**Control (N=10)**	**ANOVA**	**Post-Hoc analysis**	**PCOS (N=12)**	**ANOVA**	**Post-Hoc analysis**
Biochemistry	**Infusion**	**Mean ± SD**	**Significance**	**Significance**	**Mean ± SD**	**Significance**	**Significance**
TCH (mmol/L)	Base line	4.10±0.7	NS	NS	3.64±0.9	NS	NS
	Saline	3.87±0.6			3.62±0.9		
	Saline + Ins	3.84±0.7			3.56±0.9		
TG (mmol/L)	Base line	0.83±0.2	P<0.001	b^{***},c^{***}	1.09±0.7	NS	NS
	Saline	0.75±0.3			1.02±0.6		
	Saline + Ins	0.50±0.2			0.85±0.6		
NEFA (µmol/L)	Base line	469.28±174.6	P<0.001	a^{*},b^{***},c^{***}	462.35±150.4	P<0.001	b^{***},c^{***}
	Saline	640.77±183.1			629.86±281.4		
	Saline + Ins	50.50±32.4			55.46±40.6		
MOTS-c (pg/ml)	Base line	476.11±261.4	NS	NS	389.74±230.3	NS	NS
	Saline	488.21±382.4			449.3		
	Saline + Ins	518.68±296.6			528.6		
M-value		4.96± 2.0			3.26± 0.8		

a= Baseline to saline comparison; b= Saline to saline+ insulin comparison; c = Base line to saline+ insulin comparison

Table 3: Effects of acute intralipid and insulin administration on blood TC, TG, NEFA, MOTS-c levels and M-Value in study subjects before exercise intervention.

		Control (N=10)	ANOVA	Post-Hoc analysis	PCOS (N=12)	ANOVA	Post-Hoc analysis
Biochemistry	**Infusion**	**Mean ± SD**	**Significance**	**Significance**	**Mean ± SD**	**Significance**	**Significance**
TCH (mmol/L)	Base line	4.15±0.9			3.62±0.8		
	Lipid	3.95±0.8			3.61±0.6		
	Lipid + Ins	4.19±0.7	NS	NS	3.65±0.6	NS	NS
NEFA (µmol/L)	Base line	527.30±207.1			516.39±227.1		
	Lipid	3122.25±1023.5			3569.91±1147		
	Lipid + Ins	1427.63±727.7	P<0.001	a^{***},b^{***},c^{**}	1784.77±869.2	P<0.001	a^{***},b^{***},c^{***}
TG (mmol/L)	Base line	0.88±0.3			0.96±0.5		
	Lipid	4.94±2.4			5.55±3.05		
	Lipid + Ins	5.2±2.2	P<0.001	a^{***}, c^{***}	6.17±3.6	P<0.001	a^{***}, c^{***}
MOTS-c (pg/ml)	Base line	469.91±217.6			482.34±258.4		
	Lipid	927.89±638.9			1139.38±618.9		
	Lipid + Ins	793.10±811.3	P<0.05	a^{*}	626.37±260.7	P<0.01	a^{**},b^{*},c^{*}
M-value		2.44± 1.7			1.16± 0.8		

a= Baseline to lipid comparison; b= Lipid to Lipid+ insulin comparison; c = Base line to lipid+ insulin comparison.

Table 4: Effects of acute insulin administration on blood TCH, TG, NEFA, MOTS-c and M-Value in study subjects after exercise intervention.

		Controls (N=10)	ANOVA	Post-Hoc analysis	PCOS (N=12)	ANOVA	Post-Hoc analysis
Biochemistry	**Infusion**	**Mean ± SD**	**Significance**	**Significance**	**Mean ± SD**	**Significance**	**Significance**
TCH (mmol/L)	Base line	4.13±0.6	p<0.001	NS	3.92±0.9	NS	NS
	Saline	3.9±0.5			3.63±0.7		
	Saline + Ins	3.86±0.6			3.64±0.7		
TG (mmol/L)	Base line	0.84±0.4	NS	NS	1.085±0.7	NS	NS
	Saline	0.73±0.4			0.9±0.5		
	Saline + Ins	0.65±0.6			0.82±0.4		
NEFA (µmol/L)	Base line	483±150.3	p<0.001	b^{***},c^{***}	445.2±54.7	p<0.001	a^{***},b^{***},c^{***}
	Saline	524±98.3			604.7±112		
	Saline + Ins	40.2±35.2			79.1±54.2		
MOTS-c (pg/ml)	Base line	501± 220	NS	NS	490±301	NS	NS
	Saline	840±1001			450±305		
	Saline + Ins	620±400			520±313		
M-value		5.5± 1.9			3.92± 1.5		

a= Baseline to saline comparison; b= Saline to saline+ insulin comparison; c = Base line to saline+ insulin comparison

Table 5: Effects of acute intralipid and insulin administration on blood TCH, TG, NEFA, MOTS-c and M-Value in study subjects after exercise intervention.

		Control (N=10)	ANOVA	Post-Hoc analysis	PCOS (N=12)	ANOVA	Post-Hoc analysis
Biochemistry	**Infusion**	**Mean ± SD**	**Significance**	**Significance**	**Mean ± SD**	**Significance**	**Significance**
TCH (mmol/L)	Base line	4.33±0.7	NS	NS	3.63±0.8	NS	NS
	Lipid	4.23±0.6			3.51±0.6		
	Lipid + Ins	4.2±1			3.67±0.7		
TG (mmol/L)	Base line	0.78±0.3	p<0.001	a^{***},c^{***}	0.97±0.4	p<0.001	a^{***},c^{***}
	Lipid	4.07±1.4			4.9±2.4		
	Lipid + Ins	5.52±2.6			5.16±3.1		
NEFA (µmol/L)	Base line	387.5±149.3	p<0.001	a^{***},b^{**},c^{**}	469±152	p<0.001	a^{***},b^{***},c^{**}
	Lipid	3106±976			2989±905		
	Lipid + Ins	1727±1034			1326±636		
MOTS-c (pg/ml)	Base line	481± 402	p<0.01	a^{**},b^{*},c^{*}	510±322	p<0.05	a^{**},c^{**}
	Lipid	1006± 610			960±504		
	Lipid + Ins	698±503			800±301		
M-value		3.01± 1.4			1.53± 0.8		

a= Baseline to lipid comparison; b= lipid to lipid + insulin comparison; c= Baseline to lipid + insulin comparison.

Figure 1