RESEARCH ARTICLE

Effect of dietary fat modification on subcutaneous white adipose tissue insulin sensitivity in patients with metabolic syndrome

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Scope: To determine whether the insulin resistance that exists in metabolic syndrome (MetS) patients is modulated by dietary fat composition.

Methods and results: Seventy-five patients were randomly assigned to one of four diets for 12 wk: high-saturated fatty acids (HSFAs), high-MUFA (HMUFA), and two low-fat, high-complex carbohydrate (LFHCC) diets supplemented with long-chain *n*-3 (LFHCC *n*-3) PUFA or placebo. At the end of intervention, the LFHCC *n*-3 diet reduced plasma insulin, homeostasis model assessment of insulin resistance, and nonsterified fatty acid concentration ($p < 0.05$) as compared to baseline Spanish habitual (BSH) diet. Subcutaneous white adipose tissue (WAT) analysis revealed decreased EH-domain containing-2 mRNA levels and increased cbl-associated protein gene expression with the LFHCC *n*-3 compared to HSFA and HMUFA diets, respectively ($p < 0.05$). Moreover, the LFHCC *n*-3 decreased gene expression of glyceraldehyde-3-phosphate dehydrogenase with respect to HMUFA and BSH diets ($p < 0.05$). Finally, proteomic characterization of subcutaneous WAT identified three proteins of glucose metabolism downregulated by the LFHCC *n*-3 diet, including annexin A2. RT-PCR analysis confirmed the decrease of annexin A2 ($p = 0.027$) after this diet.

Conclusion: Our data suggest that the LFHCC *n*-3 diet reduces systemic insulin resistance and improves insulin signaling in subcutaneous WAT of MetS patients compared to HSFA and BSH diets consumption.

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Abbreviations: **Akt**, v-akt murine thymoma viral oncogene homolog; **Anxa2**, annexin A2; **BSH**, baseline Spanish habitual; **CAP**, cbl-associated protein; **cbl**, casitas B-linage lymphoma; **EHD2**, EH-domain containing-2; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **GLUT4**, glucose transporter type 4; **GPD1**, glycerol-3-phosphate dehydrogenase-1 (soluble); **HMUFA**, high MUFA; **HSFA**, high-saturated fatty acid; **HOMA-IR**, homeostasis model assessment of insulin resistance; **IRS-1**, insulin receptor substrate-1; **ISI**, insulin sensitivity index; **JNK**, jun N-terminal kinase; **LFHCC**, low-fat, high-complex carbohydrate; **LFHCC *n*-3**, LFHCC diet supplemented with long-chain *n*-3; **MetS**, metabolic syndrome; **NEFA**, nonsterified fatty acid; **PDK1**, 3-phosphoinositide-dependent protein kinase-1; **PEPCK1**, phosphoenolpyruvate carboxykinase-1; **PI3K**, phosphoinositide 3 kinase; **SFA**, saturated fatty acid; **TG**, triglycerides; **T2D**, type 2 diabetes; **WAT**, white adipose tissue

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1 Introduction

The prevalence of metabolic syndrome (MetS) is increasing worldwide and is associated with enhanced type 2 diabetes (T2D) and cardiovascular disease risk. MetS pathogenesis is not completely understood, although there is no doubt that genetic, metabolic, and dietary factors play an intrinsic role in its development and progression [1, 2]. The main causative component in the development of MetS is abdominal obesity [3], wherein insulin resistance is the central pathophysiologic state that leads to hyperglycemia, hepatic lipogenesis, and increased plasma nonesterified fatty acid (NEFA) [4, 5] when insulin-sensitive tissues (e.g. liver, skeletal muscle, and fat) no longer respond to normal circulating levels of insulin [6]. Insulin exerts its physiological action on these tissues upon binding to its receptor, and through a series of intracellular signaling events leads to translocation of the vesicles containing glucose transporter type 4 (GLUT4) from their intracellular pool to the plasma membrane, thus facilitating uptake of glucose into cells [7]. Defects in the components of the signal transmission pathway between the insulin receptor and its downstream effectors could lead to insulin resistance [8–12].

Diet and lifestyle modifications may be effective in reducing obesity-associated insulin resistance and MetS [13, 14]. In overweight subjects, isoenergetic substitution of saturated fatty acids (SFA) by MUFA or complex carbohydrates improves insulin sensitivity [15, 16], although this observation has not been confirmed in other studies [17]. Similarly, intervention studies have failed to show a consistent effect of long-chain *n*-3 PUFA on insulin resistance [18, 19]. Nevertheless, long-chain *n*-3 PUFA lowers triglycerides (TG) [20], which are an important component of MetS. Therefore, it is difficult to define the optimum diet to treat MetS. In this regard, the LIPGENE study has demonstrated that a low-fat, high-complex carbohydrate (LFHCC) diet supplemented with long-chain *n*-3 PUFA (LFHCC *n*-3) reduced the risk of MetS [21] and lowered atherogenic risk factors (LDL, TG, and NEFA) [22, 23], as compared to isoenergetic LFHCC and high-fat diets in MetS subjects. Importantly, the LIPGENE dietary intervention study showed that habitual dietary environment at baseline (preintervention) may modify responsiveness to dietary fat modification with respect to insulin sensitivity [23]. To further explore this issue, we performed a preliminary trial in a subcohort from LIPGENE study with a baseline Spanish habitual (BSH) diet consumption to investigate the metabolic advantage of adhering to a low-SFA diet enriched in MUFA or a low-fat, high-complex carbohydrate diet, as well as the effect of a low-fat diet supplemented with long-chain *n*-3 PUFA on systemic insulin sensitivity and expression of key genes in insulin signaling pathway on subcutaneous white adipose tissue (WAT).

2 Materials and methods

2.1 Study design

The current study was conducted within the framework of the LIPGENE study (Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis), a Framework 6 Integrated Project funded by the European Union. The intervention study design and dietary strategy protocol have been previously described in detail by Shaw et al. [24]. MetS was defined by published criteria [25], which conformed to the LIPGENE inclusion and exclusion criteria [26]. Full recruitment, randomization, and dietary strategy protocol details are described in the Supporting Information. The Spanish LIPGENE trial profile is illustrated in Supporting Information Fig. 1. This study was carried out in the Lipid and Atherosclerosis Research Unit at the Reina Sofia University Hospital from February 2005 to April 2006. The experimental protocol was approved by the local ethic committee of the intervention center, according to the Helsinki Declaration. The study was registered with the US National Library of Medicine Clinical Trials registry (NCT00429195).

The rationale for the LIPGENE dietary intervention study was to determine the relative efficacy of replacing or reducing dietary SFA on insulin sensitivity, by comparing the effect of four dietary treatments. Each volunteer was randomly stratified to one of four dietary interventions for 12 wk. The diets differed in fat quantity and quality while remaining isoenergetic. The composition of the four diets was as follows:

(i) High-SFA (HSFA) diet (38% energy:16% SFA, 12% MUFA, 6% PUFA; HSFA, $n = 17$).
(ii) High-MUFA (HMUFA) diet (38% energy:8% SFA, 20% MUFA, 6% PUFA; HMUFA, $n = 18$).
(iii) LFHCC (where low fat contains 28% energy) diet (8% SFA, 11% MUFA, 6% PUFA), including a control high-oleic acid sunflower-seed oil capsule (4×1 g capsule/day; LFHCC, $n = 20$).
(iv) LFHCC (where low fat contains 28% energy) diet (8% SFA, 11% MUFA, 6% PUFA), with 1.24 g/day long-chain *n*-3 PUFA, ratio of 1.4 eicosapentaenoic acid:1 docosahexaenoic acid (4×1 g capsule/day; LFHCC *n*-3, $n = 20$).

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Patients arrived at the clinical center at 08:00 h following a 12-h fast, were refrained from smoking during the fasting period, and abstained from alcohol intake during the preceding 7 days. In the laboratory and after cannulation, fasting blood and subcutaneous WAT samples were taken pre- and postintervention (at the end of intervention). Biochemical measurements and procedure to extract subcutaneous WAT are described in the Supporting Information.

2.2 Protein extraction, 2D-PAGE, and MALDI-TOF-MS analysis

For proteomic studies, adipose tissue biopsies obtained at pre- and postintervention periods from three MetS patients on the LFHCC *n*-3 diet were used. Protein isolation and 2-DE analysis of adipose tissue samples were carried out using the same protocol as previously employed by us for human adipose tissue [27]. Spots were analyzed in a 4800 MALDI-ToF/ToF Analyzer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada).

2.3 Immunoblotting

Protein isolation was performed with a commercial kit following the manufacturer's instructions (Applied Biosystems/Ambion, Austin, TX, USA) and processed for immunoblotting as described in the Supporting Information.

2.4 Total RNA isolation from adipose tissue and RT-qPCR analysis

Total RNA from samples of frozen subcutaneous adipose tissue at pre- and postintervention was extracted using the RiboPure kit (Applied Biosystems/Ambion). Next, RT-qPCR was conducted in two steps. RT was performed using MessageBOOSTER cDNA Synthesis Kit for qPCR (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's instructions. Real-time PCR techniques were carried out according to standard procedures. Full methodology details are described in the Supporting Information.

2.5 Data analysis

For conventional 2-DE gel analysis, we employed the PDQuest software version 8.0 (Bio-Rad Laboratories, Hercules, CA, USA). Spot volume values were normalized in each gel by dividing the raw quantity of each spot by the total volume of all the spots included in the same gel. Spots were verified visually to exclude artifacts and finally confirmed and quantified by density measurement with ImageJ 1.40 g software.

Statistical analysis was performed using SPSS statistical software, version 15.0 for WINDOWS (SPSS Inc., Chicago, IL, USA). All data in the text, figures, and tables are expressed as mean $\pm$ SEM. The normal distribution of variables to characterize differences in the dietary response was assessed using the Kolmogorov–Smirnov test and log-transformed if appropriate. Repeated measures ANOVA, univariate ANOVA, one-way ANOVA, and Pearson's linear correlation were used where appropriate. Repeated measures ANOVA and univariate ANOVA analyses were adjusted for age, gender, and BMI as covariates. A Bonferroni correction was applied for multiple testing. $p < 0.05$ was considered statistically significant. Full data analysis information is described in the Supporting Information.

3 Results

3.1 The LFHCC *n*-3 diet reduced insulin resistance at systemic level in MetS patients

The adherence to recommended dietary patterns and the main anthropometric and biochemical characteristics of the 75 MetS subjects at pre- and postintervention have been described elsewhere [28]. Briefly, dietary compliance was good, with close attainment of the dietary intervention diet. Nonsignificant differences in age, BMI, blood pressure, and lipid metabolism were observed among the patients assigned to the four isoenergetic diets at either pre- or postintervention [28]. To establish the effect of dietary intervention in insulin resistance, we analyzed whole-body insulin sensitivity and glucose metabolism. A significant diet $\times$ time interaction was evident for insulin ($p = 0.041$; Fig. 1A), homeostasis model assessment of insulin resistance (HOMA-IR; $p = 0.047$; Fig. 1C), and NEFA levels ($p = 0.047$; Fig. 1E) in MetS subjects, wherein lower levels for the LFHCC *n*-3 diet were observed compared to BSH diet ($p = 0.005$ (insulin), $p = 0.008$ (HOMA-IR), and $p = 0.009$ (NEFA)). Dietary fat modification had no significant effect on fasting plasma glucose (Fig. 1B), insulin sensitivity index (ISI; Fig. 1D), or plasma adiponectin (Fig. 1F) and resistin levels (Fig. 1G). Next, the analysis among diets of postintervention changes from BSH diet (Fig. 1) showed that long-term ingestion of the LFHCC *n*-3 diet decreased insulin levels ($p = 0.031$; Fig. 1A) without changes in plasma glucose concentration as compared to the HSFA group. Furthermore, the LFHCC *n*-3 diet tended to improve HOMA-IR ($p = 0.076$; Fig. 1C) with respect to that observed in patients who consumed the HSFA diet. In all, our data suggest a beneficial effect of the LFHCC *n*-3 diet in systemic insulin sensitivity of MetS patients as compared to BSH and HFSA diets.

3.2 The LFHCC *n*-3 diet improved insulin signaling in subcutaneous WAT from MetS patients

To establish if the improvement of insulin sensitivity at the systemic level exerted by the LFHCC *n*-3 diet

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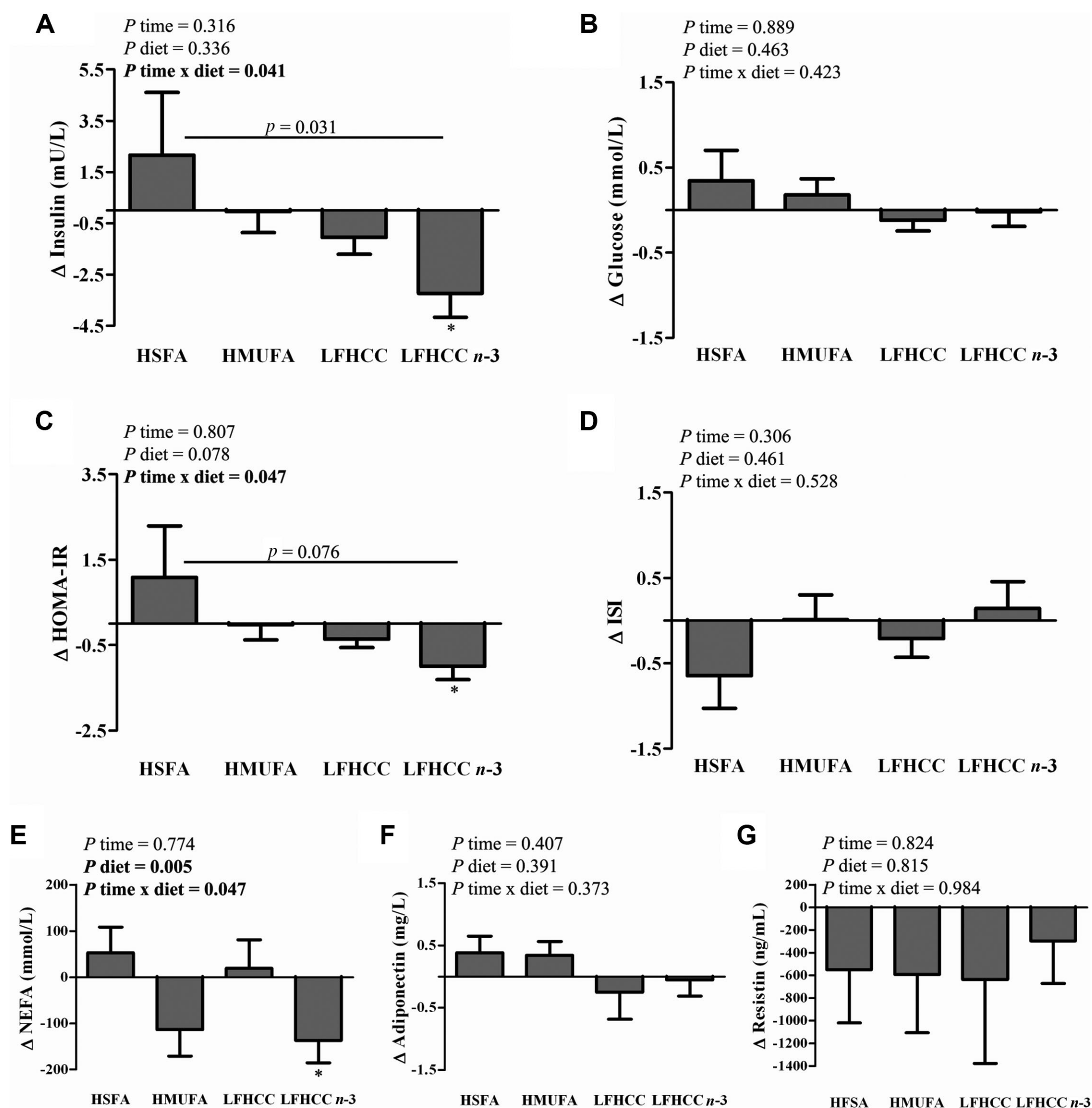

Figure 1. MetS patients fed the LFHCC *n*-3 diets showed improved systemic insulin sensitivity after 12 wk of dietary intervention. (A–G) Postintervention changes (Δ) from BSH diet (preintervention phase) of insulin levels (A), glucose concentrations (B), HOMA-IR (C), ISI (D), NEFA levels (E), adiponectin concentration (F), and resistin levels (G). Results are expressed as mean ± SEM; $n = 17$ HSFA, $n = 18$ HMUFA, $n = 20$ LFHCC, $n = 20$ LFHCC *n*-3. Data were analyzed using univariate ANOVA, and repeated measures ANOVA was used to calculate the time effect (*P* time), diet effect (*P* diet), and their interaction (*P* time × diet).
*Post- versus preintervention, $p < 0.05$. Glucose levels, HOMA-IR, and ISI were log-transformed before statistical analysis. HSFA, high-saturated fatty acids diet; HMUFA, high-MUFA diet; LFHCC, low-fat, high-complex carbohydrate diet; LFHCC *n*-3, low-fat, high-complex carbohydrate diet supplemented with *n*-3 PUFAs.

correlated with beneficial effects on glucose metabolism in subcutaneous WAT, we analyzed the expression levels of key proteins and enzymes involved in insulin signaling pathway, glycolysis, gluconeogenesis, and glyceroneogenesis in this fat depot at pre- and postintervention (Figs. 2 and 3). Compared to the BSH diet, increased adipose EH-domain containing-2 (EHD2) mRNA levels ($p = 0.005$; Fig. 2E) were observed after the ingestion of the HMUFA diet. The analysis among diets of postintervention changes from BSH diet (Fig. 2) showed that long-term intake of the HSFA diet induced a substantial increase in the gene expression levels of 3-phosphoinositide-dependent protein kinase 1 (PDK1; $p = 0.045$; Fig. 2D) and EHD2 ($p = 0.030$; Fig. 2E) as compared to the LFHCC and LFHCC *n*-3 diets, respectively. Furthermore, the LFHCC *n*-3 diet induced a significant increase in mRNA levels of the adaptor protein, cbl-associated protein (CAP; $p = 0.040$; Fig. 2F), compared to the HMUFA diet in subcutaneous WAT. No significant differences were found after the ingestion of the different diets in insulin receptor substrate (IRS)-1 protein expression (Fig. 2A), phosphorylated Jun

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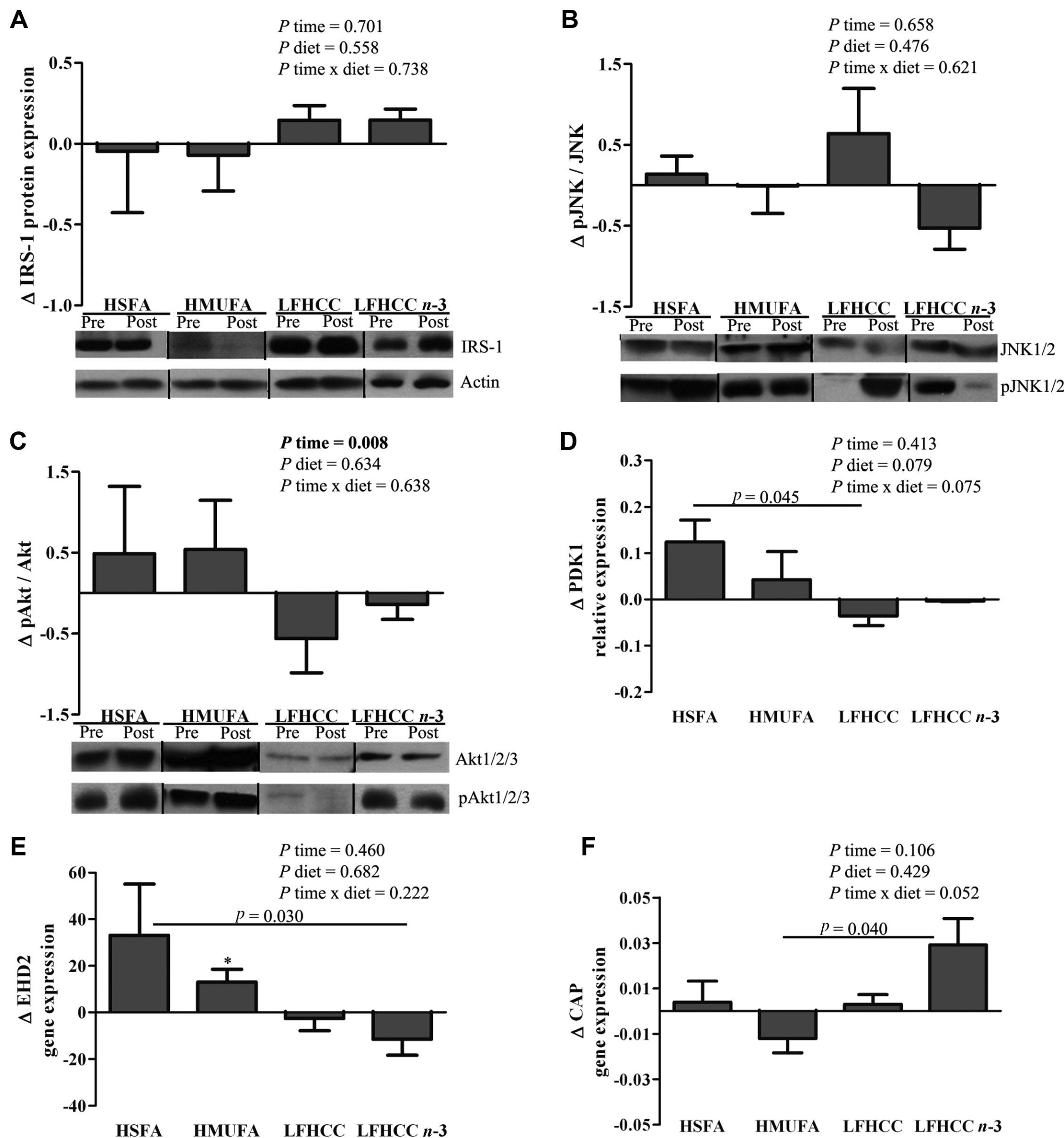

Figure 2. The long-term consumption of LFHCC *n*-3 diet reduces insulin resistance in subcutaneous WAT from MetS patients. (A–F) Postintervention changes (Δ) from BSH diet (preintervention phase) of IRS-1 protein expression (A), phosphorylated JNK/JNK ratio (B), phosphorylated Akt/Akt ratio (C), PDK1 gene expression (D), EHD2 gene expression (E), CAP mRNA levels (F). (A–C) Each subject was run on a different gel. Results are expressed as mean ± SEM; $n = 10$ HSFA, $n = 12$ HMUFA, $n = 18$ LFHCC, $n = 20$ LFHCC *n*-3. The data were analyzed using univariate ANOVA, and repeated measures ANOVA was used to calculate the time effect (*P* time), diet effect (*P* diet), and their interaction (*P* time × diet).
*Post- versus preintervention, $p < 0.05$. pJNK/JNK and gene expression of PDK1, CAP, and EHD2 were log-transformed before statistical analysis. HSFA, high-saturated fatty acids diet; HMUFA, high-MUFA diet; LFHCC, low-fat, high-complex carbohydrate diet; LFHCC *n*-3, low-fat, high-complex carbohydrate diet with *n*-3 PUFAs; pJNK, phosphorylated JNK; pAkt, phosphorylated Akt.

N-terminal kinase (pJNK)/JNK (Fig. 2B), and phosphorylated v-akt murine thymoma viral oncogene homolog (pAkt)/Akt (Fig. 2C).

Finally, we found a change in gene expression of key enzymes of glycolysis, gluconeogenesis, and glyceroneogenesis (i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoenolpyruvate carboxykinase-1 (PEPCK1)) in subcutaneous fat from MetS subjects who consumed the LFHCC *n*-3 diet (Fig. 3). Specifically, the LFHCC *n*-3 diet significantly decreased GAPDH mRNA ($p = 0.05$; Fig. 3A) and tended to reduce PEPCK1 mRNA expression levels ($p = 0.057$; Fig. 3B) in subcutaneous WAT from MetS patients as compared to the

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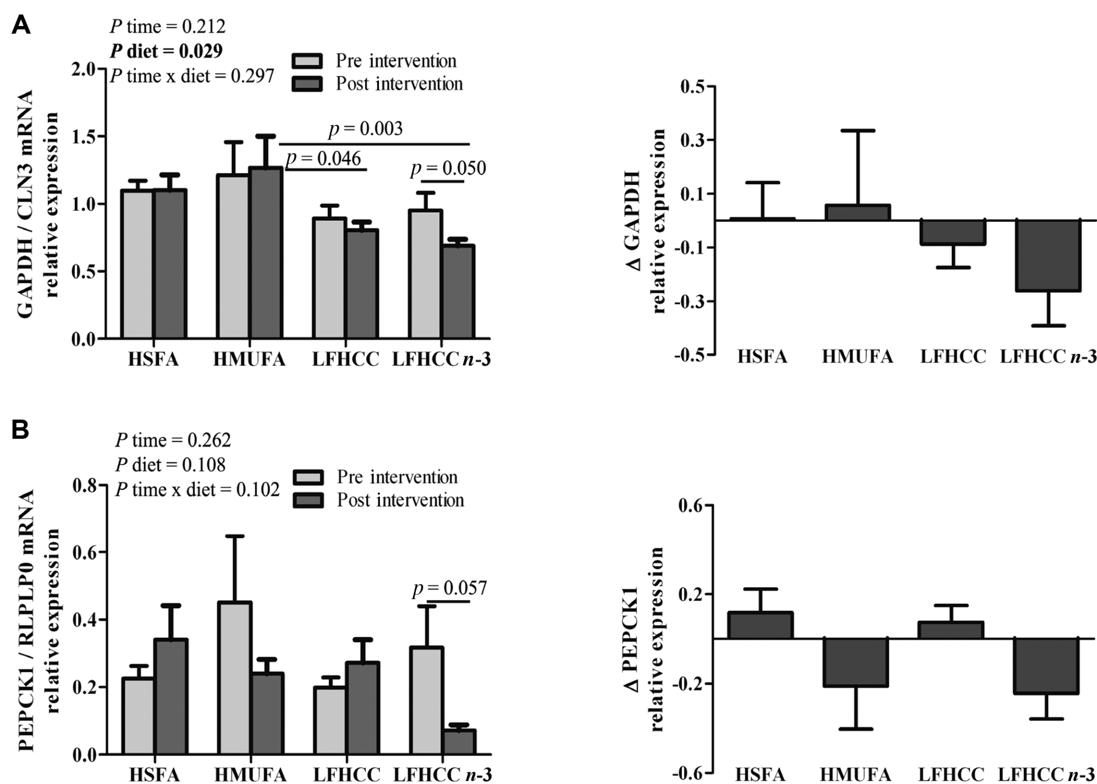

Figure 3. The expression of key enzymes of glycolysis, gluconeogenesis, and glyceroneogenesis is modulated by low-fat diets in subcutaneous WAT from MetS patients. (A) GAPDH mRNA levels (left panel) and postintervention changes (Δ) from BSH diet (preintervention phase) of GAPDH gene expression (right panel) in subcutaneous WAT. (B) PEPCK1 mRNA levels (left panel) and postintervention changes (Δ) from BSH diet (preintervention phase) of PEPCK1 gene expression (right panel) in subcutaneous WAT. Results are expressed as mean ± SEM; $n = 10$ HSFA, $n = 12$ HMUFA, $n = 18$ LFHCC, $n = 20$ LFHCC *n*-3. The data were analyzed using univariate ANOVA, and repeated measures ANOVA was used to calculate the time effect (*P* time), diet effect (*P* diet), and their interaction (*P* time × diet). HSFA, high-saturated fatty acids diet; HMUFA, high-MUFA diet; LFHCC, low-fat, high-complex carbohydrate diet; LFHCC *n*-3, low-fat, high-complex carbohydrate diet with *n*-3 PUFAs.

BSH diet. At the end of intervention, a decrease in GAPDH mRNA in this fat depot after consumption of LFHCC and LFHCC *n*-3 diets with respect to the HMUFA diet was observed ($p < 0.05$; Fig. 3A).

3.3 Annexin A2 (Anxa2), a protein involved in glucose metabolism, was modulated in subcutaneous WAT from MetS patients in response to the LFHCC *n*-3 diet

Figure 4A and Table 1 summarize the data on the characterization of the proteome of subcutaneous fat from MetS patients before and after the 12-wk intervention period with the LFHCC *n*-3 diet. Analysis of the 2D-PAGE data of the proteomes from subcutaneous WAT included in this study revealed five differentially expressed spots between pre- and postintervention in this fat depot (Fig. 4A). This pattern was reproducible in the three distinct sets of paired samples (i.e. pre- and postintervention) from patients on the LFHCC *n*-3 diet analyzed. To be more specific, all the identified proteins were downregulated at the end of dietary intervention period (Table 1). Three of the five identified proteins have been associated with glucose metabolism (gelsolin, glycerol-3-phosphate dehydrogenase-1 (soluble) (GPD1), and Anxa2). Presence of coagulation factor XIII A1 indicated contamination from blood.

In order to assess whether the observed differences in protein content were paralleled by changes at the transcript level, we compared the mRNA expression patterns of the three proteins identified by 2D-PAGE. A significant effect of the interaction between diet and time ($p = 0.045$) on Anxa2 mRNA levels (Fig. 4B, left panel) was observed in subcutaneous WAT from MetS subjects. In agreement with the proteomic studies, real-time PCR analysis revealed that the LFHCC *n*-3 diet decreased Anxa2 mRNA expression ($p = 0.027$) as compared to the BSH diet. Moreover, the MetS patients who consumed the HSFA diet showed enhanced, though not statistically significant, Anxa2 mRNA levels after the intervention period ($p = 0.060$), which were nevertheless significantly higher than those observed after ingestion of the HMUFA diet ($p = 0.040$). To test the long-term effect

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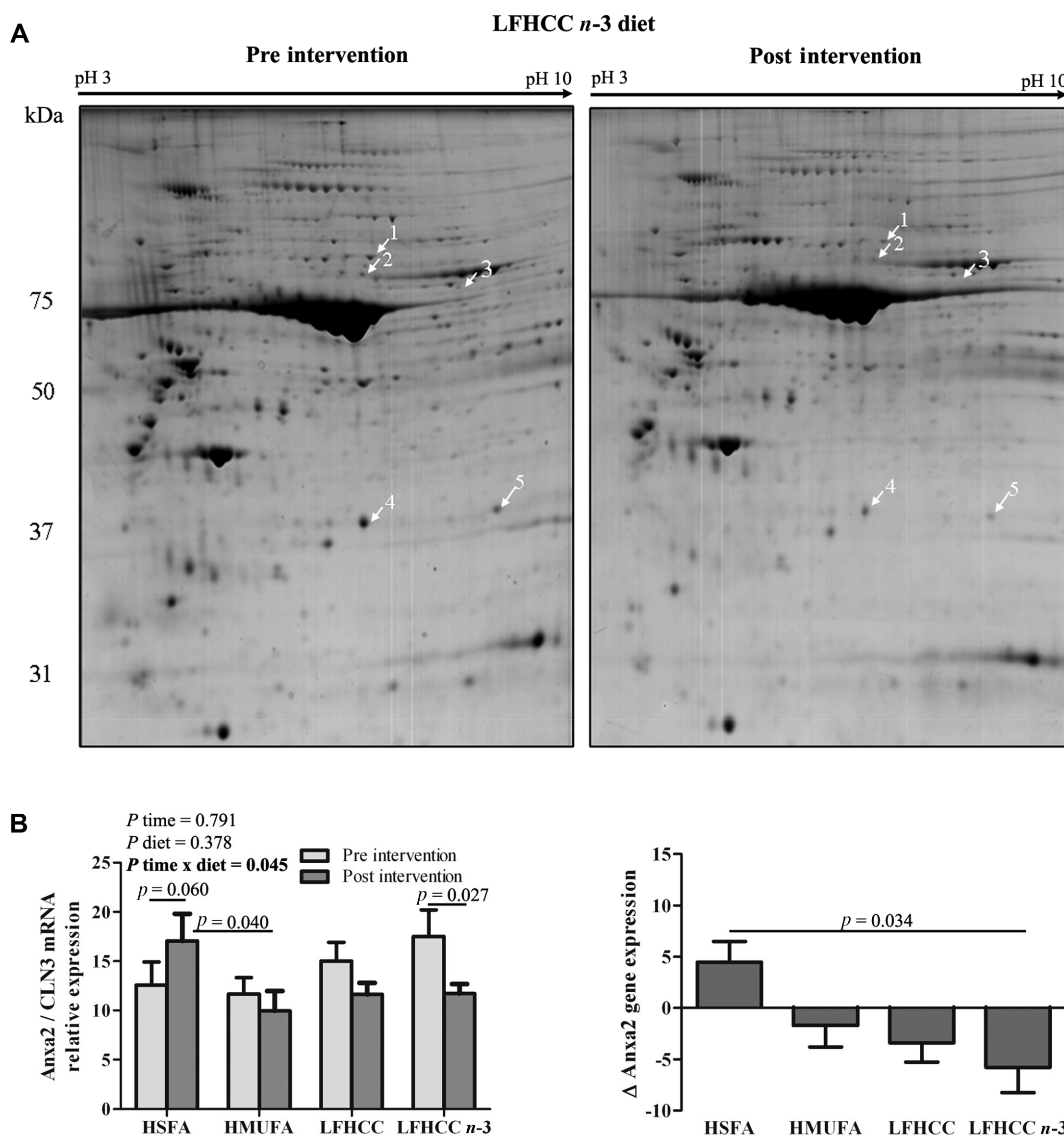

Figure 4. LHFCC *n*-3 diet induces changes in protein expression in subcutaneous WAT. (A) Representative 2-D PAGE of subcutaneous WAT samples from MetS patients fed the LFHCC *n*-3 diet at pre- (left panel) and postintervention (right panel). Proteins with significant downregulation after dietary intervention are indicated by arrows. The numbers correspond to the spot numbers that are listed in Table 1. (B) Anxa2 mRNA levels in subcutaneous WAT (left panel). Postintervention changes (Δ) from BSH diet (preintervention phase) of Anxa2 gene expression in subcutaneous WAT (right panel). Results are expressed as mean ± SEM; $n = 10$ HSFA, $n = 12$ HMUFA, $n = 18$ LFHCC, $n = 20$ LFHCC *n*-3. The data were analyzed using univariate ANOVA, and repeated measures ANOVA was used to calculate the time effect (*P* time), diet effect (*P* diet), and their interaction (*P* time × diet). HSFA, high-saturated fatty acids diet; HMUFA, high-MUFA diet; LFHCC, low-fat, high-complex carbohydrate diet; LFHCC *n*-3, low-fat, high-complex carbohydrate diet with *n*-3 PUFAs.

of dietary fat modification on Anxa2 gene expression, we performed the analysis among diets of postintervention changes from BSH diet (Fig. 4B, right panel). Long-term ingestion of the LFHCC *n*-3 diet decreased Anxa2 mRNA expression, compared to the HSFA diet ($p = 0.034$). Nonsignificant differences in GPD1 and gelsolin mRNA gene expression were found between pre- and postintervention (data not shown).

3.4 Whole-body insulin resistance and glucose metabolism gene expression correlated with Anxa2 mRNA content in subcutaneous WAT from MetS subjects

We further examined the relationship between Anxa2 expression in subcutaneous WAT and metabolic parameters in our

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Table 1. Proteins identified by MALDI-TOF/TOF significantly downregulated after LFHCC n-3 diet

Spot number[a)]	Name	NW	pI	Accession number	Peptide count[b)]	Total ion score[b)]	Fold change[c)]	p-Values[d)]
1	Gelsolin isoform 32	86	5.90	XP_00161890	15	191	2.78	0.031
2	Coagulation factor XIII A1 subunit	87	5.62	AAL12161	17	87	1.47	0.017
3	Enoyl-CoA hydratase alpha-subunit	83	9.16	BAA03941	14	56	1.53	0.030
4	GPD1 (soluble)	38	5.81	AAH32234	26	712	2.10	$<$0.001
5	Anxa2	39	7.57	AAH23990	17	63	1.68	0.027

a) Spot number corresponds to those in Fig. 4.
b) Peptide count and total ion score values correspond to MASCOT scores.
c) Fold change indicates the average volume ratio (post- versus preintervention) of three independent subjects.
d) p-Values of repeated measures ANOVA; $p < 0.05$.

MetS patients. There was a positive correlation of subcutaneous WAT Anxa2 mRNA levels with plasma resistin levels ($r = 0.292$, $p = 0.032$), plasma TG concentration ($r = 0.306$, $p = 0.024$), and EHD2 mRNA expression in this fat depot ($r = 0.378$, $p = 0.015$) at postintervention (Fig. 5A). Next, we studied the postintervention changes from BSH diet in these parameters and observed a negative correlation of Anxa2 gene expression in subcutaneous fat with ISI ($r = -0.320$, $p = 0.039$), and a positive correlation between GAPDH mRNA levels and Anxa2 transcript content ($r = 0.496$, $p = 0.001$) in subcutaneous WAT from MetS subjects (Fig. 5B). When MetS patients were classified by tertiles according to their Anxa2 expression levels (Fig. 5C), we observed that MetS patients in the lowest tertile of Anxa2 mRNA expression had lower plasma resistin levels ($p < 0.05$), compared to MetS patients in the first and second tertiles of Anxa2 mRNA levels postintervention. However, ISI exhibited a decreasing trend in the first tertile versus the third tertile when the comparative analysis of pre- and postintervention changes was carried out ($p = 0.069$).

4 Discussion

The MetS is associated with unhealthy lifestyles and its main features are dyslipidemia, hypertension, abdominal obesity, and insulin resistance. Approaches aimed to improve the MetS may be effective at reducing cardiovascular disease and T2D risk. In this regard, beneficial effects of long-chain n-3 PUFA on insulin sensitivity have been observed in overweight and obese subjects [29, 30]. Nevertheless, an analysis of randomized controlled trials in T2D patients (reviewed by Hartweg et al. [31]) concluded that n-3 PUFA supplementation had not significant effects on glycemic control. Inconsistencies among these studies could be due to the distinct n-3 PUFA dosages, duration, and nature or chain length of the n-3 PUFA sources employed, along with differences in the study populations. Herein, we demonstrate that consumption of the LFHCC (28% energy) diet supplemented with 1.24 g/day long-chain n-3 PUFA (1.4 eicosapentaenoic acid:1 docosahexaenoic acid ratio) for 12 wk induced an improvement in systemic insulin sensitivity compared to BSH diet, as evidenced by the decrease in plasma insulin, HOMA-IR, and NEFA concentration observed in our study.

Abdominal adipose tissue accumulation is strongly correlated with the development of the MetS [32]. Abnormal release of NEFA from fat depots may contribute to the development of insulin resistance, thus providing an important link between adiposity and MetS [33]. Our results demonstrated that LFHCC n-3 diet produced a decrease in plasma NEFA concentration as compared to BSH diet, which was coincident with a reduction of insulin resistance in these patients. Insulin exerts its physiological actions through two independent signaling pathways, the IRS/ phosphoinositide 3 kinase (PI3K)/PDK1/Akt pathway, which plays key roles in the regulation of cell survival, cell proliferation and growth, and metabolism, and the adaptor protein containing pleckstrin homology and SH2 domains (APS)/CAP/ casitas B-linage lymphoma (cbl) pathway, which regulates insulin-induced GLUT4 translocation from intracellular vesicles to the cell surface [7]. Defective insulin signaling, due to serine phosphorylation of IRS-1 [8, 34], chronic Akt activation [9], increased PDK1 mRNA levels [10], or decreased CAP expression [11, 35], results in insulin resistance. Furthermore, high expression levels of EHD2, a mediator of GLUT4 internalization, have been observed to disrupt this process in adipocytes [12]. Notably, our study suggests that the detrimental effect of high-fat diets on adipose insulin signaling might be prevented by the LFHCC diet, especially if supplemented with long-chain n-3 PUFA. Specifically, we observed that the long-term intake of the HSFA diet induced higher gene expression levels of PDK1 and EHD2 in subcutaneous WAT of MetS patients as compared to the LFHCC and LFHCC n-3 diet, respectively. Moreover, we observed that the LFHCC n-3 diet increased CAP mRNA levels compared to the HMUFA diet, while an increase in the mRNA levels of EHD2 was found in MetS patients fed with the HMUFA diet compared to the BSH diet. When viewed together, these data support the view that the consumption of LFHCC diets, especially those supplemented with long-chain n-3 PUFA, may exert a positive

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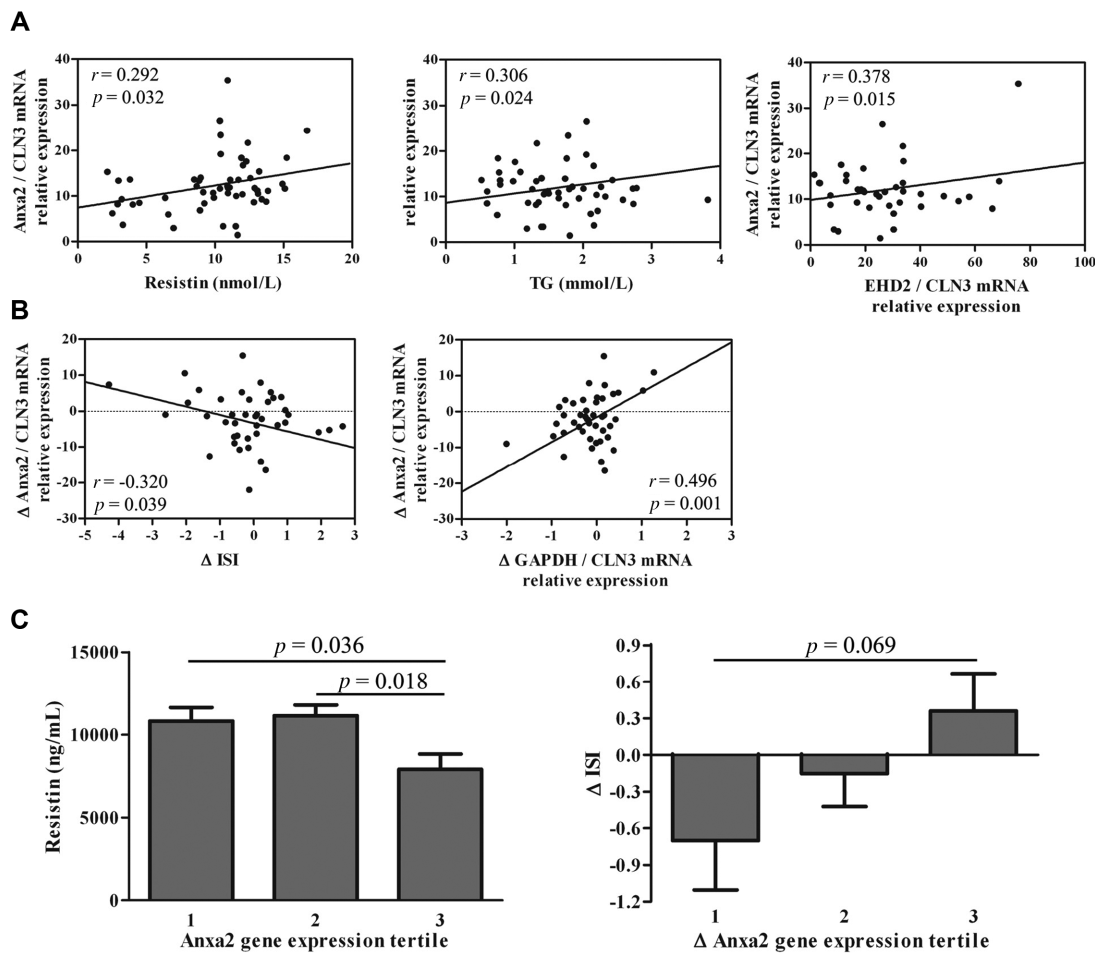

Figure 5. Anxa2 gene expression in subcutaneous WAT correlates with glucose metabolism parameters in MetS patients. (A) Correlation of postintervention Anxa2 gene expression with resistin, TG, and EHD2 mRNA levels. (B) Correlation of postintervention changes (Δ) from BSH diet (preintervention phase) of Anxa2 gene expression with ISI and GAPDH mRNA levels. (C) Resistin levels in relation to the Anxa2 gene expression tertiles (left panel) in postintervention period. Postintervention changes (Δ) from BSH diet of ISI in relation to the Δ Anxa2 gene expression tertiles (right panel). Results are expressed as mean ± SEM; $n = 60$. The data were analyzed using one-way ANOVA. First tertile, higher Anxa2 gene expression; second tertile, middle Anxa2 gene expression; third tertile, lower Anxa2 gene expression. HSFA, high-saturated fatty acids diet; HMUFA, high-MUFA diet; LFHCC, low-fat, high-complex carbohydrate diet; LFHCC *n*-3, low-fat, high-complex carbohydrate diet with *n*-3 PUFAs.

modulatory effect on insulin signaling in the MetS through their regulatory action on the adipose tissue gene expression profile.

Activation of insulin signaling pathways triggers multiple cellular responses including glycolysis, gluconeogenesis, and glyceroneogenesis. GAPDH is a glycolytic and gluconeogenesis enzyme whose expression is stimulated by insulin [36]. Among its functions, GAPDH negatively regulates insulin signaling by promoting the dephosphorylation of the downstream signaling component, phosphatidylinositol-3,4,5-triphosphate [37]. Interestingly, it has been shown that diet-induced obesity increases GAPDH expression levels in adipose tissue [38], while short-term administration of very low calorie diets decreases adipose tissue GAPDH mRNA in obese individuals [39]. Our findings show that patients fed the HMUFA diet exhibited increased GAPDH mRNA expression levels in comparison with those fed the low-fat diets. Moreover, a decrease in GAPDH mRNA levels with the LFHCC *n*-3 diet compared to BSH diet was demonstrated, coincident with the lower plasma insulin levels observed with this diet. Therefore, we speculate that long-term consumption of LFHCC diets, especially those supplemented with long-chain *n*-3 PUFA, could reduce insulin resistance in MetS patients by preventing the activation of GAPDH phosphoinositide phosphatase activity in adipose tissue.

PEPCK1, a cytosolic decarboxylase enzyme involved in gluconeogenesis, is regulated by hormones that are related to the maintenance of glucose homeostasis [40]. Moreover, PEPCK1 is key in glyceroneogenesis, or the production of glycerol, which is essential for the re-esterification

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of NEFA to synthesize TG [41]. A functional link between glyceroneogenesis and insulin sensitivity has been established in mice and humans. Thus, adipocyte-specific inactivation of PEPCK1 [42] in mice or the decrease of glyceroneogenesis efficiency in human subcutaneous WAT [43] increases systemic insulin resistance associated with enhanced circulating NEFA levels. By contrast, mice that overexpress PEPCK1 in adipocytes exhibit increased fat depots due to enhanced glyceroneogenesis and NEFA re-esterification, but have reduced circulating NEFA levels and normal insulin sensitivity [44]. However, when fed a high-fat diet, transgenic mice overexpressing PEPCK1 exhibit high body weight, glucose intolerance, and insulin resistance [45]. According to these findings, there seems to be a modulatory effect of the diet on this protein. In line with these observations, our data show that MetS subjects fed the LFHCC *n*-3 diet displayed a trend to lower PEPCK1 expression levels, as compared to BSH diet, which, in addition, exhibited decreased plasma NEFA levels. In all, these data suggest that the LFHCC *n*-3 diet might promote fatty acid clearance from plasma by normalizing adipocyte PEPCK1 expression, thus contributing to prevent insulin resistance in the MetS.

Along with the changes in signaling proteins and metabolic enzymes, proteomic analysis of subcutaneous WAT from MetS patients revealed that the LFHCC *n*-3 diet also decreased the expression levels of a member of the annexin family of membrane-binding proteins, Anxa2. Compared to BSH diet, our study showed that LFHCC *n*-3 diet reduces Anxa2 expression after 12 wk of dietary intervention. Anxa2 is a multifunctional protein that has been shown to play a major role in membrane-trafficking events, including exocytosis [46], endocitosis, and cell adhesion [47]. To be more specific, Anxa2 facilitates the transport of cholesterol ester from caveolae to internal membranes and appears to be involved in GLUT4 translocation in a way that has not been fully defined yet [48]. Previous observations indicated that mRNA and protein levels of Anxa2 are increased in cell exposed to oxidative stress [49]. In line with these observations, Anxa2 is overexpressed in WAT of obese mice [50] and reduced after exercise training in overweight and obese subjects [51]. Remarkably, we observed that dietary supplementation with long-chain *n*-3 PUFA, which has been consistently shown to exert anti-oxidative and anti-inflammatory effects in adipose tissue [52], reduced Anxa2 expression. Thus, downregulation of adipose Anxa2 by long-chain *n*-3 PUFA might be indicative of reduced oxidative stress in WAT of MetS patients, which would be in line with the improvement in insulin sensitivity observed after dietary supplementation with *n*-3 PUFA. Further data demonstrated that Anxa2 correlated positively with plasma resistin and TG levels and negatively with ISI. In fact, an analysis by tertiles showed that the higher expression of Anxa2 is associated with higher resistin levels and lower ISI. In all, our data suggest that Anxa2 may represent a potential biomarker of adipose tissue dysfunction that can be targeted by dietary interventions. Further studies are needed to unveil the specific contribution of Anxa2 to adipose tissue function.

Our study presents some limitations. Ensuring complete adherence to dietary instructions is difficult in a feeding trial. However, adherence to recommended dietary patterns was good, as judged as per dietary assessment. On the other hand, our design has the strength of reproducing real-life conditions with home-prepared foods, reflecting the subjects' usual practice. Another limitation, concerning the sample size, is that our trial was able to detect differences in insulin signaling to discern a relevant biological effect, but a smaller effect could not have been detected. Therefore, the evidence of specific effects of LFHCC *n*-3 diet on insulin signaling should be reevaluated in a future study with higher sample size per group. From the data in our trial, the statistical power calculations for a confirmatory study indicated that at least 18 participants per group would be needed to detect mean differences of 1 unit (SD 1.4) in the HOMA-IR among diets at a significant level of 0.05 and a power of 0.8.

In conclusion, our data suggest that long-term intake of an isocaloric, low-fat, high-complex carbohydrate diet supplemented with long-chain *n*-3 PUFA reduces insulin resistance at systemic level and improves insulin signaling in subcutaneous WAT of MetS patients, as compared to the consumption of HSFA and BSH diets. However, and due to the limitations imposed by our sample size, a confirmatory study would be necessary to support recommendations to consume this dietary pattern as a useful preventive measure against the insulin resistance that exists in MetS patients.

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The authors have declared no conflict of interest.

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