# Effect of supplementation with an 80:20 cis9,trans11 conjugated linoleic acid blend on the human platelet proteome.

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RESEARCH ARTICLE

# Effect of supplementation with an 80:20 *cis*9,*trans*11 conjugated linoleic acid blend on the human platelet proteome

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**Scope:** The dietary fatty acid *cis*9,*trans*11 conjugated linoleic acid (*cis*9,*trans*11 CLA) has been shown to modify the function of endothelial cells, monocytes, and platelets, all of which are involved in the development of atherosclerosis. Potential mechanisms for the platelet effects have not been assessed previously. In this study, we assessed how supplementation of the diet with an 80:20 *cis*9,*trans*11 CLA blend affects the platelet proteome.

**Methods and results:** In a double-blind, randomized, placebo-controlled, parallel-group trial, 40 overweight but apparently healthy adults received either 4 g per day of *cis*9,*trans*11 CLA-enriched oil or placebo oil, consisting of palm oil and soybean oil, for 3 months. Total platelet proteins were extracted from washed platelets, separated using two-dimensional gel electrophoresis and differentially regulated protein spots were identified by LC-ESI-MS/MS. Supplementation with the CLA blend, compared with placebo, resulted in significant alterations in levels of 46 spots ($p < 0.05$), of which 40 were identified. Network analysis revealed that the majority of these proteins participate in regulation of the cytoskeleton and platelet structure, as well as receptor action, signaling, and focal adhesion.

**Conclusion:** The platelet proteomics approach revealed novel insights into regulation of cellular biomarkers of atherogenic and thrombotic pathways by an 80:20 *cis*9,*trans*11 CLA blend.

**Keywords:**
Conjugated linoleic acid / Cytoscape / Dietary supplementation trial / Platelet proteomics

## 1 Introduction

Consumption of dietary conjugated linoleic acids (CLAs), which belong to the group of trans fatty acids and are present in ruminant products including milk, cheese, and beef [1], may beneficially influence inflammation [2], eicosanoid metabolism in both platelets and endothelium [3], and lipid metabolism [4]. In addition, these fatty acids inhibit the development of atherosclerosis, at least in animal models [4–9]. The effect in animal models is thought to be isomer-specific, with *cis*9,*trans*11 CLA being the active isomer inhibiting atherogenesis [10–14].

Atherosclerosis is a multifactorial inflammatory arterial disease involving various cell types including endothelial cells, monocytes, and platelets [15]. Indeed, activated blood platelets contribute to the early stages of plaque formation within blood vessels, as well as to thrombus formation [16]. Therefore, platelets can be considered as a useful model to

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**Abbreviations: CDC42hs**, cell division control protein 42 homolog; **cFXIII**, cellular coagulation factor XIII; **CLA**, conjugated linoleic acid; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **PRP**, platelet rich plasma

assess the effects of *cis*9,*trans*11 CLA on mechanisms involved in atherogenesis. A few studies have indicated that the *cis*9,*trans*11 CLA isomer in particular may prevent platelet activation and aggregation in vitro, and may display anticoagulant properties [3, 17–20]. The effects of *cis*9,*trans*11 CLA on platelet function could be mediated through multiple mechanisms. Blood platelets appear an ideal cell type to apply proteomics and study the atheropreventive potential of *cis*9,*trans*11 CLA. First, because they play a pivotal role in cardiovascular disease (CVD) progress and second, since they synthesize proteins and modify the proteins post-translationally [21]. Therefore, we investigated the effects of supplementing the diet with *cis*9,*trans*11 CLA on regulation of the platelet proteome in humans, by visualizing the biological functions of the regulated proteins as a network [22]. This platelet proteomics approach could extend the availability of relevant biomarkers to properly assess the physiological and biochemical effects of *cis*9,*trans*11 CLA in humans.

## 2 Materials and methods

### 2.1 Subjects and intervention

The study was carried out in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the University Medical Center Utrecht in Utrecht, The Netherlands. All participants provided written informed consent. This trial was registered at www.clinicaltrials.gov as NCT00706745. The first 40 overweight but apparently healthy subjects that were recruited for participating in a larger trial investigating the effects of *cis*9,*trans*11 CLA supplementation for 6 months on aortic stiffness [23], who were eligible based on the additional selection criteria for this study, and who were willing to donate an additional blood sample for platelet proteomic analysis, were invited to participate. In these volunteers, blood samples were obtained at baseline and after 3 months of intervention. Details on recruiting and randomization in the core study are described elsewhere [23]. Inclusion criteria at screening for this study were apparently healthy men and women, aged 40–70 years, with a body mass index (BMI; in $kg/m^2$) of $\geq$25. The main exclusion criteria at screening were: a systolic blood pressure of $\geq$160 mm Hg or a diastolic of $\geq$90 mm Hg, or current use of blood pressure lowering drugs; a total cholesterol concentration of $\geq$8 mmol/L or current use of lipid lowering drugs; inability to perform pulse wave velocity measurements; clinical signs of renal, hepatic, or hematological diseases; currently taking any medication or dietary supplements known to alter platelet function or the hemostatic system; undertaking $\geq$6 h of vigorous exercise per week; having donated blood within a month of blood sampling; taking contraceptives or hormone replacement therapy or having an abnormal menstrual cycle.

Eligible participants were randomly assigned in the core study to receive either the 80:20 *cis*9,*trans*11 CLA blend or placebo supplements. The intervention and placebo oil supplements were given as four soft gel capsules of 1 g oil each daily. This supplied about 1.5 energy% based on a daily energy intake of 10 MJ. The CLA capsules provided 3.1 g CLA isomers or 1.1 energy%, of which 80% was in the form of *cis*9,*trans*11 CLA and 20% in the form of *trans*10,*cis*12 CLA. The placebo capsules consisted of a blend of palm oil and soybean oil, which resembles the average fatty acid composition of the fat consumed by a Western population. Both supplements included 0.05% (v/v) Tocoblend$^{TM}$ L50 IP (IOI Loders Croklaan, Wormerweer, NL) containing a mixture of $\alpha$ (5–9%), $\beta$ (1–2%), $\gamma$ (25–33%), and $\delta$ (10–15%) tocopherols in sunflower oil. Capsules were produced and supplied by Lipid Nutrition, Wormerveer, The Netherlands. Fatty acid composition of both types of capsules has been described previously [23].

### 2.2 Blood sampling and platelet isolation

The volunteers were asked to provide a fasted blood sample (55 mL) at baseline and after 3 months of intervention. The time frame of 3 months has been chosen as the incorporation of dietary fatty acids is known to happen as fast as 6 days after the onset of intervention and the lifespan of a platelet is only 10 days [24]. Therefore, any change in platelet function as a result of either incorporation of dietary fatty acids into the membrane of platelets or the direct action of the fatty acids on inflammatory or thrombogenic pathways should be apparent after 20 days. As we were interested in long-term established effects of the 80:20 *cis*9,*trans*11 CLA blend, a period of 3 months would be sufficient to detect any effects on levels of platelet proteins.

Blood sampling for platelet isolation in monovettes with trisodium citrate as anticoagulant was performed as described previously [25] and the first 5 mL of blood was discarded. We obtained platelets for protein isolation from platelet rich plasma (PRP), which was prepared within 30 min by centrifuging samples at 100 $\times$ *g* for 17 min at room temperature. The platelets contained in the upper two-thirds of PRP were carefully collected and precipitated by a second centrifugation step at 900 $\times$ *g* for 12 min at room temperature. $PGI_2$ (50 ng/mL final concentration) was added in all washing steps to minimize platelet activation during preparation of platelet pellet. The platelet pellet was resuspended in cold Tyrodes buffer (140 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4$, 2 mM $MgCl_2$, 0.1% (w/v) glucose, adjusted to pH 7.33 with HEPES), centrifuged at 900 $\times$ *g* for 12 min at 4°C and stored at −80°C until further analysis. The platelet pellet typically contained less than 1% white blood cells and ~7% red blood cells, as assessed by a differential full blood count analyzer (model KX-21N, Sysmex, Milton Keynes, UK).

### 2.3 Protein extraction and proteome analysis

Total platelet proteins were extracted according to a protocol described by us previously [25] with modifications. The pellets were homogenized with a sonicator for two times 10

 

s on ice in 100 μL extraction buffer containing 7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.06% proteinase inhibitor cocktail (Roche, Burgess Hill, UK) and the homogenates were centrifuged at 1100 × *g* for 10 min at 4°C. Protein concentrations in the supernatant were measured with a RC DC protein assay (Bio-Rad, Hemel Hempstead, UK) applying a reducing agent compatible (RC) and detergent compatible (DC) modification of Lowry's method. Samples for two-dimensional (2D) gel electrophoresis were run in batches of 12 that were randomized for intervention group and subject to prevent running order bias for proteome analysis, as described by us previously [10, 26] with modifications. Briefly, immobilizing pH gradient (IPG) strips (17 cm, pI 4–7, Bio-Rad) were rehydrated passively in 340 μL extraction buffer containing 15 μL freshly prepared 30% (w/v) DTT and 250 μg protein per sample for 1 h at 20°C followed by active rehydration for 16 h under low voltage (50 V per strip). To separate proteins in the first dimension the recommendations of Bio-Rad were followed. SDS-PAGE was performed on 18 × 18 cm acrylamide gradient (8–16%) gels for the separation of proteins in the second dimension. Flamingo (Bio-Rad) fluorescent stained gels were scanned using a Bio-Rad FX scanner, set at medium intensity with a resolution of 100 μm, and analyzed using the automatic matching tool within PDQuest software (Bio-Rad). Spots were excised from the gel using a robotic spot cutter (Bio-Rad), trypsinized using a MassPrep Station (Micromass, Manchester, UK). Liquid chromatography-electrospray ionization/multistage mass spectrometry (LC-ESI-MS/MS) was performed using a Q-Trap triple quadrupole mass spectrometer fitted with a nanospray ion source (Applied Biosystems/MDS Sciex, Framingham, USA). The total ion current (TIC) data were submitted for database searching using the MASCOT search engine (Matrix Science Ltd., London, UK) using the MSDB database (version 20060831) with the following search criteria: allowance of 0 or 1 missed cleavages; peptide mass tolerance of ± 1 Da; fragment mass tolerance of ± 0.8 Da, trypsin as digestion enzyme; carbamidomethyl modification of cysteine; methionine oxidation as partial modification; and charged state as MH+. Proteins were considered identified when at least two matched peptides with individual ion scores > 41 were found, indicating identity or extensive homology with 95% certainty (Supporting Information Table S1).

### 2.4 Visualization of pathway annotations with Cytoscape

To visualize the regulated proteins in the context of their biological function, a network was generated using pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG), which is generally considered one of the most complete sets of biological pathways available [27]. The network is a bipartite graph containing a set of nodes representing regulated proteins and a set of nodes representing pathways. An edge is added between a protein and pathway node when the protein plays a role in that pathway. To map the measured proteins with the protein and gene identifiers in the pathways, both were translated to UniProt identifiers using the BridgeDb library [28]. The constructed network was visualized using Cytoscape (version 2.8.2) [22]. Protein nodes were colored according to their differential up- or down-regulation. In a few cases where more than one protein isoform was identified per UniProt identifier, the protein node was segmented and each segment was colored according to the regulation of the corresponding isoform. Cytoscape plots were used to identify potential regulatory protein hubs (defined as a protein that plays a role in high number of different pathways) regulated by supplementation with the 80:20 *cis*9,*trans*11 CLA blend.

## 2.5 Statistical analysis

Data are presented as mean ± standard deviation (SD). Normalized data, as calculated by the PDQuest software (Bio-Rad) were used. Data were log-transformed and the changes in intensities for each spot between baseline and 3 months for CLA supplementation and for placebo were compared using an unpaired *t*-test and assuming equal variances. Tests were calculated in R (R foundation for Statistical Computing, Vienna, Austria). Spots significant at $p < 0.05$ were selected for identification. False discovery rates were calculated using the method described by [29]. The *q*-values ranged from about 0.01 for the most significant proteins to 0.31 at a *p*-value of 0.05. This indicates that while some nonregulated proteins will inevitably have been included, more than two-thirds are likely to be true positive results from the statistical testing.

# 3 Results

The baseline characteristics of the 40 participants in this study were similar between both intervention groups (Table 1).

2D gel electrophoresis revealed in total 584 protein spots from the protein extract of washed platelets. Intervention with

**Table 1.** Characteristics of the study population by treatment assignment at baseline

| | 80:20 CLA blend | Placebo |
|---|---|---|
| Age (year) | 60.0 ± 4.1 | 58.9 ± 3.9 |
| Gender (male/female) | 12/8 | 10/10 |
| Current smoking (*n* (%)) | 2 (10%) | 2 (10%) |
| BMI (kg/m$^2$) | 27.9 ± 2.9 | 28.7 ± 3.0 |
| Mean systolic blood pressure (mm Hg) | 126.7 ± 12.3 | 124.5 ± 10.0 |
| Mean diastolic blood pressure (mm Hg) | 75.3 ± 9.2 | 75.5 ± 6.1 |

Values represent mean ± SD for age, BMI, and blood pressure outcomes.

 

an 80:20 *cis*9,*trans*11 CLA blend for 3 months compared with placebo oil was associated with significant changes ($p < 0.05$) in 74 protein spots, of which 46 single valid protein spots were cut for identification. Of these, 40 protein spots were identified using LC-ESI-MS/MS resulting in 31 different proteins, some with more than one changed spot. The proteins were classified in categories according to their currently recognized major function based on the protein knowledge base UniProtKB [30] (Table 2). Additional data on the LC-ESI-MS/MS identification of proteins are available in Supporting Information Table S1. CLA-regulated platelet proteins were mainly those involved in platelet structure, receptor action, and cell signaling.

In order to assess the impact of changes in protein levels on the regulation of major pathways in platelets, we explored the results on protein levels with Cytoscape. Cytoscape [22] is an open source software platform to visualize complex networks in general, which has already been successfully utilized for the network analysis of MicroArray datasets to elucidate mechanisms in CVD [31]. We used this tool in a novel approach to visualize a network of regulated proteins assessed by 2D gel electrophoresis and their pathway annotations. Twenty two out of the 31 regulated and identified proteins were part of 52 different pathways annotated in KEGG, and these pathways were connected by 93 edges (Fig. 1). This network revealed that the majority of regulated proteins was involved in pathways that are very relevant for platelet function, such as focal adhesion, regulation of the actin cytoskeleton, aggregation, and the coagulation cascade. The central regulatory protein hubs affected by the 80:20 *cis*9,*trans*11 CLA blend were GTP-binding protein CDC42hs, integrin alpha-IIb precursor, and two cytoskeletal proteins, i.e., alpha-actinin-1 and a hypothetical protein of the actin family. GTP-binding protein CDC42hs (UniProt accession number P60953, change in spot intensity −2.011, $p = 0.007$) with the corresponding gene was involved in 22 biochemical pathways. Integrin alpha-IIb precursor (UniProt accession number P08514, change in spot intensity −1.184, $p = 0.036$) with the corresponding gene was involved in nine biochemical pathways. Alpha-actinin-1 (UniProt accession number P12814, change in spot intensity 2.348, $p = 0.047$) with the corresponding gene was involved in eight biochemical pathways and the hypothetical protein of the actin family (UniProt accession number Q8WVW5, change in spot intensity 2.610, $p = 0.030$) with the corresponding gene, annotated as actin, was involved in 16 biochemical pathways.

## 4 Discussion

Platelets in their roles in hemostasis, atherogenesis, and thrombosis undergo shape change, adhesion, secretion of chemokines and coagulation components, fibrinogen binding to receptors, and thromboxane $A_2$ formation leading to aggregation. Although platelets are enucleate they are yet capable of translation of selected proteins and post-translational modification of a large number of proteins, as well as uptake and storage of plasma components. Therefore, blood platelets appear an ideal cell type for the application of proteomics [21] in order to study the beneficial potential of the 80:20 *cis*9,*trans*11 CLA blend on platelets function and CVD progression.

Nutritional supplementation of healthy overweight and obese subjects with the 80:20 *cis*9,*trans*11 CLA blend for 3 months significantly altered the regulation of platelet proteins in unstimulated platelets when compared with those taking placebo oil. Albeit that our investigational product consisted mainly of *cis*9,*trans*11 CLA, we cannot exclude the possibility that our results may be partly attributable to the small content of the *trans*10,*cis*12 CLA content of the product. CLA-regulated platelet proteins were mainly those involved in platelet structure, receptor action, and cell signaling. This may be important as the protein distribution in resting platelets can influence the response after platelet stimulation. Data from in vitro studies and human intervention trials assessing the effect of CLA on platelet aggregation are very limited. Incubation with the CLA isomers *cis*9,*trans*11 and *trans*10,*cis*12 CLA, or a mix of both CLA isomers, reduced arachidonic acid-induced platelet aggregation as well as thromboxane $B_2$ production ex vivo, and the isomeric mix was also effective in inhibiting collagen-induced platelet aggregation compared with linoleic acid [17]. In one study in healthy women, no effect on agonist-induced platelet aggregation or other blood clotting parameters was observed upon supplementation with 3.9 g/day CLA ($n = 10$), compared with sunflower oil ($n = 7$) [19], but this study may have lacked statistical power to reveal any relevant antiplatelet effects. However, in patients with type 2 diabetes, supplementation with 13.0 g/day of 50:50 CLA mix ($n = 16$), compared with placebo oil ($n = 16$), significantly decreased fibrinogen levels [32]. In postmenopausal women, fibrinogen and plasminogen activator inhibitor-1 levels were significantly reduced upon intervention with CLA milk (4.7 g/day *cis*9,*trans*11 CLA and 0.4 g/day *trans*10,*cis*12 CLA; $n = 25$), compared with 50:50 CLA mix (2.3 g/day *cis*9,*trans*11 CLA and 2.2 g/day *trans*10,*cis*12 CLA; $n = 25$), or olive oil [33]. Furthermore, a human trial with naturally *cis*9,*trans*11 CLA-rich pecorino cheese showed a reduction in platelet aggregation induced with arachidonic acid [20]. Both in vitro as well as in vivo studies have reported that upon exposure, CLA is actually incorporated into the membranes of platelets and other blood cells [17–19, 34, 35]. Unfortunately, the setting of the human intervention did not allow us to measure the effect of the 80:20 *cis*9,*trans*11 CLA blend on measures of in vivo platelet function. But by and large, existing evidence indicates that CLA, and especially the *cis*9,*trans*11 CLA isomer, may modify platelet activation and aggregation, and display anticoagulant properties. However, mechanisms for these potentially antithrombotic or antiatherosclerotic effects have not been studied in detail.

 

**Table 2.** Differentially regulated proteins after 3 months supplementation with an 80:20 *cis*9,*trans*11 conjugated linoleic acid blend identified by LC-ESI-MS/MS

| Accession no. | Protein name | Kegg pathway | SSP[a)] | Mr exp [kDa] | Mr theor [kDa] | Change | *p*-value |
|---|---|---|---|---|---|---|---|
| *(A) Cell structure* | | | | | | | |
| P09493 | Tropomyosin alpha-1 chain | Hypertrophic cardiomyopathy [hsa05410]; (3)<br>Dilated cardiomyopathy [hsa05414]; (3)<br>Cardiac muscle contraction [hsa04260]; (2) | 405<br>1304 | 33.3<br>29.8 | 32.8<br>32.8 | −2.880<br>−0.128 | 0.009<br>0.026 |
| P19105 | Myosin regulatory light chain 12A | Focal adhesion [hsa04510]; (7)<br>Regulation of actin cytoskeleton [hsa04810]; (7)<br>Tight junction [hsa04530]; (5)<br>Leukocyte transendothelial migration [hsa04670]; (4) | 108<br>105<br>1102 | 17.9<br>18.0<br>18.1 | 19.7<br>19.7<br>19.7 | −2.323<br>−2.004<br>−2.459 | 0.003<br>0.026<br>0.034 |
| P35579 | Myosin-9 heavy chain, nonmuscle | Regulation of actin cytoskeleton [hsa04810]; (7)<br>Tight junction [hsa04530]; (5)<br>Viral myocarditis [hsa05416]; (2) | 6102 | 16.3 | 227.5 | −1.788 | 0.001 |
| Q6ZNL4 | FLJ00279 protein fragment of myosin-9 | Regulation of actin cytoskeleton [hsa04810]; (7)<br>Tight junction [hsa04530]; (5)<br>Viral myocarditis [hsa05416]; (2) | 5508<br>6501 | 47.6<br>47.5 | 65.8<br>65.8 | −3.229<br>2.018 | 0.006<br>0.046 |
| P21333 | Filamin-A | Focal adhesion [hsa04510]; (7)<br>MAPK signaling pathway [hsa04010]; (2) | 6514 | 42.1 | 280.7 | −3.673 | 0.001 |
| Q8WVW5 | Hypothetical protein (belongs to actin family) | Focal adhesion [hsa04510]; (7)<br>Regulation of actin cytoskeleton [hsa04810]; (7)<br>Tight junction [hsa04530]; (5)<br>Leukocyte transendothelial migration [hsa04670]; (4)<br>Hypertrophic cardiomyopathy [hsa05410]; (3)<br>Dilated cardiomyopathy [hsa05414]; (3)<br>Adherens junction [hsa04520]; (3)<br>Arrhythmogenic right ventricular cardiomyopathy [hsa05412]; (3)<br>Viral myocarditis [hsa05416]; (2)<br>Pathogenic *Escherichia coli* infection [hsa05130]; (2)<br>Phagosome [hsa04145]; (2)<br>Shigellosis [hsa05131]; (2)<br>Bacterial invasion of epithelial cells [hsa05100]; (2)<br>Vibrio cholerae infection [hsa05110] | 3503 | 45.0 | 40.5 | 2.610 | 0.030 |
| P12814 | Alpha-actinin-1 | Focal adhesion [hsa04510]; (7)<br>Regulation of actin cytoskeleton [hsa04810]; (7)<br>Tight junction [hsa04530]; (5)<br>Leukocyte transendothelial migration [hsa04670]; (4)<br>Adherens junction [hsa04520]; (3)<br>Arrhythmogenic right ventricular cardiomyopathy [hsa05412]; (3)<br>Amebiasis [hsa05146]<br>Systemic lupus erythematosus [hsa05322] | 7305 | 29.1 | 103.1 | 2.348 | 0.047 |
| Q86UX7 | Fermitin family homolog 3 FERM domain (talin head) | | 7405 | 35.5 | 76.0 | −2.251 | 0.021 |
| P06396 | Gelsolin precursor | Regulation of actin cytoskeleton [hsa04810]; (7)<br>Fc gamma R-mediated phagocytosis [hsa04666]; (2) | 6804<br>7703 | 88.1<br>79.3 | 85.7<br>85.7 | −2.474<br>−3.673 | 0.003<br>0.000 |
| P37802 | Transgelin-2 | | 6101 | 16.5 | 22.4 | 1.508 | 0.030 |
| Q9Y490 | Talin-1 | Focal adhesion [hsa04510]; (7) | 5704<br>1407 | 58.2<br>35.1 | 269.8<br>269.8 | −2.085<br>−2.320 | 0.037<br>0.001 |

 

**Table 2.** Continued.

| Accession no. | Protein name | Kegg pathway | SSP | Mr exp [kDa] | Mr theor [kDa] | Change | *p*-value |
|---|---|---|---|---|---|---|---|
| *(B) Platelet receptor action* | | | | | | | |
| P08514 | Integrin alpha-IIb precursor | Focal adhesion [hsa04510]; (7)<br>Regulation of actin cytoskeleton [hsa04810]; (7)<br>Hypertrophic cardiomyopathy [hsa05410]; (3)<br>Dilated cardiomyopathy [hsa05414]; (3)<br>Arrhythmogenic right ventricular cardiomyopathy [hsa05412]; (3)<br>Pathways in cancer [hsa05200]; (2)<br>Hematopoietic cell lineage [hsa04640]<br>ECM-receptor interaction [hsa04512]<br>Small cell lung cancer [hsa05222] | 3511 | 41.9 | 113.4 | −1.184 | 0.036 |
| P02675 | Fibrinogen beta chain | Complement and coagulation cascades [hsa04610]; (3) | 7608 | 50.4 | 55.9 | −4.378 | 0.000 |
| P02679 | Fibrinogen gamma chain | | 4608 | 50.0 | 51.5 | −2.460 | 0.038 |
| Q15084 | Protein disulfide isomerase A6 | Protein processing in ER [hsa04141]; (3) | 4403 | 33.9 | 48.1 | 2.339 | 0.010 |
| P00488 | Coagulation factor XIII A chain | Complement and coagulation cascades [hsa04610]; (3) | 7501 | 44.2 | 83.3 | 2.367 | 0.049 |
| P27797 | Calreticulin | Protein processing in ER [hsa04141]; (3)<br>Phagosome [hsa04145]; (2)<br>Antigen processing and presentation [hsa04612]; (2) | 401 | 39.6 | 48.1 | 2.576 | 0.011 |
| *(C) Cell signalling* | | | | | | | |
| Q9ULV4 | Coronin 1C | | 6509 | 49.1 | 53.2 | 1.836 | 0.046 |
| Q04917 | 14-3-3 protein eta | Neurotrophin signaling pathway [hsa04722]; (3)<br>Cell cycle [hsa04110]<br>Oocyte meiosis [hsa04114] | 4203 | 21.3 | 28.2 | 2.745 | 0.010 |
| P52565 | Rho GDP-dissociation inhibitor 1 | Neurotrophin signaling pathway [hsa04722]; (3)<br>Vasopressin-regulated water reabsorption [hsa04962] | 2208 | 26.5 | 23.2 | 1.984 | 0.025 |
| P60953 | GTP-binding protein CDC42hs | Focal adhesion [hsa04510]; (7)<br>Regulation of actin cytoskeleton [hsa04810]; (7)<br>Tight junction [hsa04530]; (5)<br>Leukocyte transendothelial migration [hsa04670]; (4)<br>Neurotrophin signaling pathway [hsa04722]; (3)<br>Adherens junction [hsa04520]; (3)<br>Fc gamma R-mediated phagocytosis [hsa04666]; (2)<br>Pathogenic *Escherichia coli* infection [hsa05130]; (2)<br>Shigellosis [hsa05131]; (2)<br>Bacterial invasion of epithelial cells [hsa05100]; (2)<br>MAPK signaling pathway [hsa04010]; (2)<br>Epithelial cell signaling in *Helicobacter pylori* infection [hsa05120]<br>T-cell receptor signaling pathway [hsa04660]<br>Renal cell carcinoma [hsa05211]<br>VEGF signaling pathway [hsa04370]<br>Axon guidance [hsa04360]<br>Pancreatic cancer [hsa05212]<br>Endocytosis [hsa04144]<br>GnRH signaling pathway [hsa04912]<br>Chemokine signaling pathway [hsa04062] | 3107 | 16.9 | 21.7 | −2.011 | 0.007 |

 

**Table 2.** Continued.

| Accession no. | Protein name | Kegg pathway | SSP | Mr exp [kDa] | Mr theor [kDa] | Change | *p*-value |
|---|---|---|---|---|---|---|---|
| *(D) Chaperone proteins* | | | | | | | |
| P11021 | 78 kDa glucose-regulated protein (HSPA5) | Protein processing in ER [hsa04141]; (3)<br>Antigen processing and presentation [hsa04612]; (2)<br>Prion diseases [hsa05020]<br>Protein export [hsa03060] | 2703 | 76.6 | 72.3 | −2.397 | 0.035 |
| | | | 2704 | 76.4 | 72.3 | −2.931 | 0.015 |
| P48643 | T-complex protein 1 subunit epsilon | | 5602 | 59.4 | 59.7 | −3.243 | 0.001 |
| (E) OXIDATIVE STRESS | | | | | | | |
| P07203 | Glutathione peroxidase | Huntington's disease [hsa05016]; (2)<br>Arachidonic acid metabolism [hsa00590]<br>Amyotrophic lateral sclerosis [hsa05014]<br>Glutathione metabolism [hsa00480] | 6201 | 21.8 | 21.9 | 2.499 | 0.050 |
| P30048 | Peroxiredoxin-3 | | 7201 | 25.3 | 27.7 | 3.862 | 0.000 |
| | | | 7207 | 24.9 | 27.7 | −3.846 | 0.002 |
| *(F) Glucose/energy metabolism* | | | | | | | |
| P11177 | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | Glycolysis/gluconeogenesis [hsa00010]; (2)<br>Pyruvate metabolism [hsa00620]; (2)<br>Butanoate metabolism [hsa00650]<br>Valine, leucine, and isoleucine biosynthesis [hsa00290]<br>Citrate cycle (TCA cycle) [hsa00020] | 5316 | 32.9 | 39.2 | −2.194 | 0.047 |
| P14618 | Pyruvate kinase isozymes M1/M2 | Glycolysis/gluconeogenesis [hsa00010]; (2)<br>Pyruvate metabolism [hsa00620]; (2)<br>Type 2 diabetes mellitus [hsa04930]<br>Purine metabolism [hsa00230] | 7406 | 35.7 | 57.94 | −2.247 | 0.011 |
| P31930 | Cytochrome b-c1 complex subunit 1, mitochondrial | Huntington's disease [hsa05016]; (2)<br>Cardiac muscle contraction [hsa04260]; (2)<br>Alzheimer's disease [hsa05010]<br>Parkinson's disease [hsa05012]<br>Oxidative phosphorylation [hsa00190] | 5502 | 46.6 | 52.6 | −1.537 | 0.041 |
| *(G) Other* | | | | | | | |
| P28070 | Proteasome subunit beta type-4 | Protein processing in ER [hsa04141]; (3) | 5211 | 25.4 | 29.2 | −4.187 | 0.000 |
| CAD33454 | Sequence 181 from patent WO0218424 | | 4302 | 30.5 | 33.3 | 2.074 | 0.017 |
| | | | 5304 | 30.4 | 33.3 | 1.244 | 0.048 |
| Q96KP4 | Cytosolic nonspecific dipeptidase | | 6604 | 50.7 | 52.9 | −2.243 | 0.022 |
| *(H) Not identified* | | | | | | | |
| | Not identified | | 2004 | 12.2 | | 2.142 | 0.045 |
| | Not identified | | 3212 | 21.4 | | −1.155 | 0.044 |
| | Not identified | | 4104 | 14.8 | | 1.848 | 0.028 |
| | Not identified | | 5001 | 11.2 | | 1.884 | 0.031 |
| | Not identified | | 5309 | 29.2 | | −1.991 | 0.020 |
| | Not identified | | 6409 | 36.3 | | −1.720 | 0.010 |

a) SSP, spot number; Mr exp, experimental molecular weight; Mr theor, theoretical molecular weight; Accession no. from Uniprot Database; [hsa04810], KEGG pathway entry; (7) total number of proteins regulated by 80:20 *cis*9,*trans*11 CLA blend annotated in pathway.

 

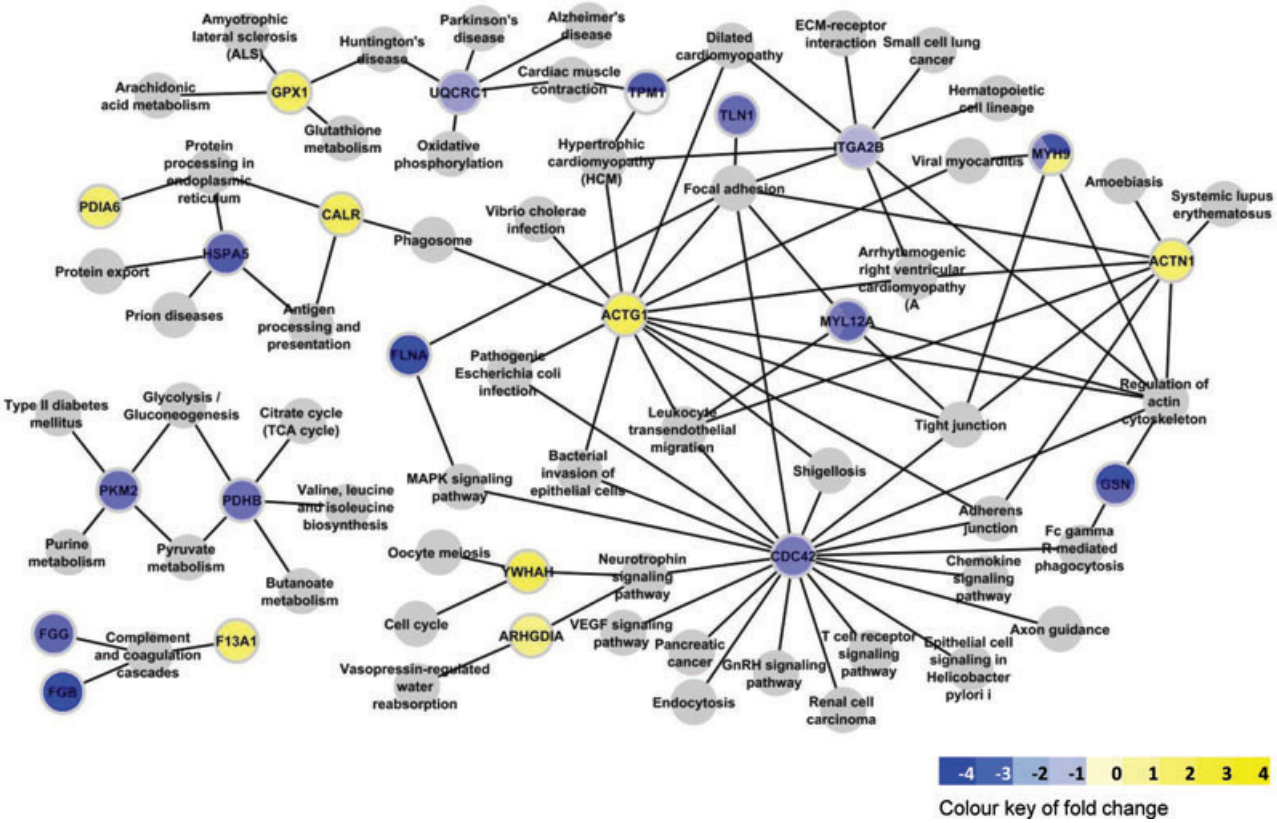


**Figure 1.** Cytoscape pathway plot of regulated proteins. Protein regulation is illustrated with a color scheme from blue (decreased) to yellow (increased). In the case of more than one regulated protein spot per gene identifier, the protein node is segmented in different colors according to up- or down-regulation. A line (edge) is drawn between a protein and pathway node (in gray) when the protein plays a role in that pathway. Proteins are labeled with their gene identifiers: ACTG1, hypothetical protein (belongs to actin family); ACTN1, alpha-actinin-1; ARHGDIA, Rho GDP-dissociation inhibitor 1; CALR, calreticulin; CDC42, GTP-binding protein CDC42hs; F13A1, coagulation factor XIII A chain; FGB, fibrinogen beta chain; FGG, fibrinogen gamma chain; FLNA, filamin-A; GPX1, glutathione peroxidase; GSN, gelsolin precursor; HSPA5, 78 kDa glucose-regulated protein; ITGA2B, integrin alpha-IIb precursor; MYH9, FLJ00279 protein fragment of myosin-9; MYL12A, myosin regulatory light chain 12A; PDHB, pyruvate dehydrogenase E1 component subunit beta; PDIA6, protein disulfide isomerase A6; PKM2, pyruvate kinase isozymes M1/M2; PSMB4, proteasome subunit beta type-4; TLN1, talin-1; TPM1, tropomyosin 1 chain; UQCRC1, cytochrome b-c1 complex subunit 1; YWHAH, 14-3-3 protein eta.

Although platelets are enucleate cells, they do contain stable pre-mRNA that is spliced into mature mRNA upon platelet stimulation, leading to regulation of proteins. Thus, platelets are able to respond to environmental change directly by altering their protein composition [36]. Moreover, the duration of the intervention in this study would allow regulation of gene expression in megakaryocytes. In this way, various relevant pathways could be triggered such as, e.g., modulation of prostaglandin synthesis [37–39]. Also, CLA may compete with arachidonic acid and linoleic acid for cyclo-oxygenase (COX1) once it is incorporated into platelet membranes [39]. CLA could also act as an agonist of peroxisome proliferator activated receptors (PPARs) [40–42]. These nuclear receptors are present on platelets and involved in agonist-induced platelet function [43, 44].

Mapping of proteomics results is an elegant way to handle a more extensive dataset in a partly objective manner. Though care should be taken because the selection of the database used for the mapping may not be complete or based on the most recent evidence. This can be overcome by the simultaneous use of several databases. Network visualization of our proteomics dataset revealed that supplementation with the 80:20 *cis*9,*trans*11 CLA blend regulated the central protein hubs, GTP-binding protein CDC42hs, alpha-actinin-1, and integrin alpha-IIb precursor. We found that the 80:20 *cis*9,*trans*11 CLA blend decreased protein levels of CDC42hs in platelets. CDC42hs has been implicated as an important mediator of filopodia formation, which plays an important role in platelet adhesion [45], although a recent study found that platelets from a murine knock out of CDC42hs were able to form filopodia and spread fully on fibrinogen upon activation [46]. However, CDC42hs was also shown to play important roles in the regulation of platelet activation, granule organization, degranulation, and GPIb signaling [46]. CDC42hs belongs to the Rho GTPase family involved in changes in cell morphology. These signaling proteins are tightly

 

regulated by both activation state and subcellular location [47]. Activated Rho GTPase proteins are GTP bound and associated with their target membrane proteins [47, 48], which could explain why we found lower levels of this protein on the 2D gels. However, this seems unlikely because levels of GDI-dissociation inhibitor protein 1, which is an inhibitory regulator for Rho family GTPases through binding and extracting Rho family proteins from plasma and intracellular membranes, were actually increased in platelets after supplementation with the 80:20 *cis*9,*trans*11 CLA blend. Rho GDP-dissociation inhibitor protein has, on the other hand, been shown to inhibit thrombin-induced aggregation in vitro [49]. Supplementation with the 80:20 *cis*9,*trans*11 CLA blend also affected proteins of the actin family. Actins are highly conserved structural proteins and the most abundant proteins in platelets. They are responsible for cell motility, e.g., the extension of F-actin filaments in the cytoskeleton to form filopodia in activated platelets [45]. The exact mechanism by which up-regulation of actin protein levels by the 80:20 *cis*9,*trans*11 CLA blend may affect platelet function is not known, but may involve the promotion of cytoskeletal stability of the resting platelet, and reducing the incidence of shape change in response to external stimuli.

The precursor for the alpha-IIb subunit of the platelet-specific integrin αIIbβ3, was another protein hub regulated by the 80:20 *cis*9,*trans*11 CLA blend. In addition, CLA supplementation was also associated with down-regulated protein levels of fibrinogen β and γ chains, and up-regulated protein levels of coagulation factor XIII (FXIII) A chain (Table 2). The αIIbβ3 receptor contains the binding site for fibrinogen, the major ligand for platelet aggregation [50]. Fibrinogen is also the precursor of the fibrin clot, which is cross-linked and stabilized by FXIII. Both fibrinogen and FXIII are key proteins in the final step of the coagulation cascade in plasma [51], but they are also present in platelets where their role is much less understood. Previous human intervention studies have revealed inconsistent effects on plasma fibrinogen levels upon CLA supplementation, perhaps relating to differences in the health status of the volunteers and the type of isomeric mixture used [32, 33, 52].

The pathway, as mapped in KEGG, that contained a high amount of regulated proteins in our dataset, was the focal adhesion pathway with seven altered proteins (Fig. 1). Adhesion to an extracellular matrix is a critical step in the platelet aggregation [53] and also a complex process involving a diversity of proteins such as transmembrane receptors of the integrin family, actins and filamins [54]. Filamin-A is a 280 kDa actin-binding protein, but it also binds GTPases of the Ras superfamily as well as CDC42hs in a GTP-independent way [55]. The binding of filamin to the GPIbα-IX-V complex is critical to form a link to von Willebrand factor. When the filamin binding is disturbed, platelets are less likely to aggregate by shear stress although they will respond to thrombin or ADP [56]. In our study, the 80:20 *cis*9,*trans*11 CLA blend lowered protein levels of filamin-A. In addition, we observed that CLA decreased levels of the cytoskeletal platelet protein talin-1. Talin consists of N-terminal globular head of around 50 kDa and a C-terminal rod-like tail of around 220 kDa. The N-terminal head contains a FERM domain, which binds the cytosolic domains of integrin β whereas the C-terminal tail provides a second integrin binding site and additional binding sites for F-actin and vinculin [57, 58]. The FERM domain is essential for integrin activation and platelet aggregation [59] and needs to interact with the β3 subunit of the integrin αIIbβ3 so that the recruited talin can activate αIIbβ3 [60]. Talin-1 deficient fibroblast-like cells showed delayed initiation and stabilization of focal complexes slowing down focal adhesion [61]. Overexpression of the FERM domain leads to integrin activation, e.g., αIIbβ3 [62, 63], whereas talin knock-down in megakaryocytes and mouse models impaired agonist-induced αIIbβ3 activation [64–66]. This indicates that the 80:20 *cis*9,*trans*11 CLA blend may be able to modulate platelet adhesion through down-regulation or shift of post-translationally modified isoforms of talin-1 in platelets.

In conclusion, this comprehensive study of platelet proteomics in a dietary trial of a 80:20 *cis*9,*trans*11 CLA blend revealed many intriguing findings worthy of further investigation. The focal adhesion pathway appeared an important mechanism of action which could contribute to the previously reported antiatherogenic effects of dietary *cis*9,*trans*11 CLA. Furthermore, the proteins CDC42hs, alpha-actinin-1 and integrin alpha-IIb precursor represent important protein hubs that are regulated by the 80:20 *cis*9,*trans*11 CLA blend. These proteins, or indeed downstream proteins or metabolites, are likely candidate biomarkers that could be used in future nutritional intervention studies to measure the efficacy of fatty acids on platelet function.

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