Anti-inflammatory and immunoregulatory effects of pinolenic acid in rheumatoid arthritis

Rabaa Takala\textsuperscript{1,2}, Dipak P. Ramji\textsuperscript{2}, Robert Andrews\textsuperscript{1}, You Zhou\textsuperscript{1,3}, James Burston\textsuperscript{1}, Ernest Choy\textsuperscript{1,4,5}

1. Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, UK
2. College of Biomedical and Life Sciences, School of Biosciences, Cardiff University, Cardiff, UK
3. Systems Immunity University Research Institute, Cardiff University, Cardiff, UK
4. CREATE Centre, Division of infection and immunity, School of Medicine, Cardiff University, Cardiff, UK
5. University Hospital of Wales, Rheumatology, Cardiff, UK

Corresponding author: Rabaa Takala
Institute of Infection and Immunity, Tenovus Building, School of Medicine, University Hospital of Wales, Heath Park, Cardiff, CF14 4XN, UK.
E-mail: takalara@cardiff.ac.uk; takala2007rm3@gmail.com

© The Author(s) 2021. Published by Oxford University Press on behalf of the British Society for Rheumatology. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Abstract

Objectives. In pre-clinical studies, pinolenic acid (PNLA), an omega-6-polyunsaturated fatty acid from pine nuts has shown anti-inflammatory effects. We aimed to investigate the effect of PNLA in human cell lines and peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients and healthy controls (HCs).

Methods. Modified Boyden chamber was used to assess chemokine-induced migration of THP-1 monocytes. Macropinocytosis was assessed using lucifer yellow and ox-LDL uptake using Dil-oxLDL in THP-1 macrophages and human monocyte-derived macrophages (HMDM). IL6, TNFα and PGE2 release by lipopolysaccharide (LPS) stimulated PBMCs from RA patients and HCs were measured by ELISA. The transcriptomic profile of PNLA treated, LPS activated PBMCs was investigated by RNA-sequencing.

Results. PNLA reduced THP-1 cell migration by 55% (p<0.001). Macropinocytosis and Dil-oxLDL uptake were reduced by 50% (p<0.001) and 40% (p<0.01) in THP-1 macrophages and 40% (p<0.01) and 25% (p<0.05) in HMDM, respectively. PNLA reduced IL6 and TNFα release from LPS stimulated PBMCs from RA by 60% (p<0.001) and by 50% and 35% respectively (p<0.01) for HCs. PNLA also reduced PGE2 levels in such PBMCs from RA patients and HCs (p<0.0001). Differentially expressed genes included upregulated expression of pyruvate dehydrogenase kinase-4, plasminogen activator inhibitor-1, fructose bisphosphatase 1 and N-Myc downstream-regulated gene, which have potential roles in regulating immune and metabolic pathways. Pathway analysis predicted upstream activation of nuclear receptors peroxisome proliferator-activated receptors involved in anti-inflammatory processes, and inhibition of NF-κB and STAT1.

Conclusions. PNLA has immune-metabolic effects on monocytes and PBMC which are pathogenic in RA and atherosclerosis. Dietary PNLA supplementation may be beneficial in RA.

Keywords: Rheumatoid arthritis, inflammatory cytokines, PBMCs, lipid uptake, macropinocytosis, polyunsaturated fatty acids, pinolenic acid

Rheumatology key messages:

- Pinolenic acid (PNLA) reduces monocyte migration and macrophage uptake of ox-LDL.
- PNLA reduces TNFα, IL6 and PGE2 release in activated PBMC from patients with RA.
- Dietary supplement of PNLA can be beneficial for articular and vascular disease in patients with RA through its immune-metabolic effects and augment current treatments.
Introduction

RA is a chronic inflammatory disease affecting 0.5-1% of the population (1). Cardiovascular death is the major cause of mortality in RA (2)(3)(4). Monocytes/macrophages play a key role both in synovitis and atherosclerosis. Although biologic and targeted synthetic disease modifying anti rheumatic drugs (DMARDs) are highly effective treatment for RA and have improved outcome, most patients do not achieve clinical remission, residual pain and disability are common (5). Furthermore, many patients have concern over potential toxicity and want to use lifestyle modification to improve disease control without completely relying on DMARDs, which are associated with poor adherence (6).

Previous research on omega (n)-3 and -6 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), dihomo-γ-linolenic acid (DGLA) have demonstrated several anti-inflammatory and anti-atherogenic properties (7)(8), and pinolenic acid (PNLA) shows anti-inflammatory actions (9). Data from large observational studies on cardiovascular diseases support the efficacy of n-3 PUFAs, mainly EPA and DHA to prevent inflammation by lowering the blood levels of IL6, TNFα and C-reactive protein (CRP) (10). Calder and Zurier reported that dietary n-6 PUFAs γ-linolenic acid (GLA) and DGLA suppress inflammation in several animal models of RA, which is accompanied by changes in inflammatory cell fatty-acid composition, decreased production of arachidonic acid (AA)-derived eicosanoids, reduced function of leukocytes, and decreased production of reactive oxygen species (ROS) (11). Furthermore, both GLA and DGLA reduced production of TNFα and IL1β by human monocytes, inhibited the proliferation of lymphocytes, and decreased the production of IL2 by human lymphocytes (11). Supplementation studies using GLA-rich oils to provide 2.4 g/day in healthy human volunteers decreased production of pro-inflammatory cytokines (TNFα, IL1 and IL6) by monocytes, decreased lymphocyte reactivity and decreased chemotaxis of neutrophils (12).

PNLA is a plant-based n-6 PUFA from pine nuts, which has a variety of beneficial actions. Incubation of the murine microglial (BV-2 cells) with 50 µM PNLA decreased the production of nitric oxide (NO), IL6 and TNFα by 41, 74 and 27%, respectively (9). A significant decrease in PGE2 production was also observed (9). The same findings were observed when murine macrophages were used; PNLA caused a dose dependent reduction in the production of NO and PGE2 following LPS-stimulation (9)(13). The upregulation of inducible nitric oxide synthetase (iNOS) and cyclooxygenase-2 (COX2) gene expression in response to LPS often involves the activation of the NF-kB pathway. Chen et al. showed that PNLA downregulated LPS-induced iNOS protein expression (54%). The syntheses of PGE1 from DGLA and PGE2 from AA were also suppressed by presence of PNLA and its metabolites (13). These data suggest that PNLA may have anti-inflammatory effects in RA. In this study, we examine the effect of PNLA on monocyctic cell lines, human macrophages and PBMCs from patients with RA together with HCs.
Materials and Methods

Cell culture
Human THP-1 cell line was cultured in RPMI1640 medium with stable glutamine (Lonza, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum (HI-FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) (called RPMI complete media hereafter), at 37°C in a humidified atmosphere containing 5% (v/v) CO2. THP-1 monocytes were differentiated into macrophages by incubation for 24 h with 0.16 μM of phorbol 12-myristate 13-acetate (PMA). PNLA was dissolved in DMSO, which was therefore used as a vehicle control. Primary HMDM were isolated from monocytes obtained from buffy coats (National Blood Service Wales) using Ficoll-Hypaque purification as described in (Supplementary Data S1). Ethical approval and informed consent for each donor were granted by the Welsh Blood Service. Details of reagents and their manufacturers are described in (Supplementary Data S1) together with methods on cell viability, cell proliferation, phagocytosis, and ROS production (Supplementary Fig. S1 S2).

Migration assay
Migration of THP-1 monocytes (1×10⁵ cells/0.5 ml of RPMI complete media) in response to the chemokine monocyte chemotactic protein-1 (MCP1) was performed using a modified Boyden chamber with 8 μm porous inserts. THP-1 monocytes incubated with vehicle or 25, 50, 75 and 100 μM PNLA for the requisite time were in the upper chamber whereas the lower chamber contained 20 ng/ml MCP1 as a chemoattractant. After 3 h incubation at 37°C in a humidified incubator with 5% CO2, the cells on the underside of the inserts were washed, collected with the migrated cells and counted using a haemocytometer.

DiI-oxLDL and lucifer yellow (LY) uptake assays
THP-1 macrophages and HMDM (1×10⁵ cells/0.5 ml of RPMI complete media) were pre-incubated with DMSO or 25, 50, 75 and 100 μM PNLA for 24 h at 37°C. They were then treated with 100 μg/ml LY or 5 μg/ml DiI-oxLDL, the concentrations reflect those commonly used in the literature (14)(15) in RPMI medium containing 0.2% (v/v) fatty-acid free bovine serum albumin. The incubation was continued for another 24 h. The uptake was analysed on a FACS Canto (BD Biosciences, Oxford, UK) flow cytometer with 10,000 events acquired for each sample. LY uptake and Dil-ox LDL were represented as a percentage with the vehicle-treated control assigned as 100%.

PBMCs from RA Patients and healthy controls
Participants ≥18 years old with RA (n=20) from Rheumatology Department at the University Hospital of Wales and HCs (n=10) were recruited for assessing cytokines and PGE2 release from LPS stimulated PBMCs with/without PNLA. For transcriptome study RA (n=6) mean age = 56 years, and HCs (n=6) mean age = 43 years, were recruited. Signed informed consent was obtained from all participants. Study
was approved by Research Ethics Committee for Wales (reference no. 12/WA/0045). Detailed demographic and laboratory data are described in (Supplementary Table S1 and S2). Blood was taken and PBMCs were isolated by standard Ficoll density gradient centrifugation and methodological details are provided in (Supplementary Data S2).

ELISA
Approximately 200 µl of cell culture supernatants were aliquoted of PNLA or vehicle treatment and LPS activation following centrifuging at RT at 400 x g for 10 min and kept at -80°C. Concentration levels of TNFα, IL6 and PGE2 were measured using ELISA kits. TNFα, IL6 from (R&D Systems, Abingdon, UK) and PGE2 (Cayman Chemicals, USA) in accordance with manufacturer’s instructions.

RNA extraction
Total RNA was isolated using RNeasy mini kit (Qiagen) from PBMCs following 100 ng/ml LPS and 25 µM PNLA or vehicle treatment. RNA was purified using a RNeasy on-column with DNase I digestion (Qiagen) as described in (Supplementary Data S3). The cell lysates were stored at -80°C and then passed through a series of spin columns to first bind genomic DNA, then RNA and finally to elute high-quality RNA. Quality control check for RNA was assessed as described in (Supplementary Data S3).

Library preparation including ribosomal RNA depletion using NEBNext Ultra II RNA Library Prep Kit for Illumina. Described in the (Supplementary Data S3).

RNA sequencing (RNA-seq)
Following library quantification and validation, sequencing was done on Illumina® HiSeq4000 flow cells according to the manufacturer’s instructions. To conform to ENCODE guidelines, libraries were sequenced to have greater than 40 million mapped reads (encodeproject.org/documents/cede0cbe-d324-4ce7-ace4-f0c3eddf5972).

RNA-seq data processing and read mapping strategy
Paired end reads from Illumina sequencing were trimmed with Trim Galore (16) and assessed for quality using FastQC (17), using default parameters. Reads were mapped to the human GRCh38 reference genome using STAR (18) and counts were assigned to transcripts using featureCounts (19) with the GRCh38.96 Ensembl gene build GTF. Both the reference genome and GTF were downloaded from the Ensembl FTP site (20).
Normalization and identification of differentially expressed genes (DEGs)

Differential gene expression analyses used the DESeq2 package (21). Genes were discarded from the analysis if differential expression failed to be significant (significance: adj.pval < 0.05, Benjamini-Hochberg correction for multiple testing) (21). The initial WGT data was assessed from the web-based tool, Morpheus (https://software.broadinstitute.org/morpheus/) using heatmaps to view changes in gene expression. The data was then separated into normal and RA groups for analysis.

Heatmap of DEG and principal component analysis (PCA)

Heatmap and PCA are shown in (Supplementary Fig. S3 and S4). Heatmap was generated using broad Morpheus software and visualizations used fragments per kilobase per million mapped fragments (FPKM) reads and log2 fold change (log2FC) comparing PNLA treated and LPS stimulated PBMCs to those treated with vehicle and LPS from both HCs and RA patients at equivalent time point (Supplementary Fig. S3). Data sets were hierarchically clustered using one minus Pearson correlation coefficient. PCA was performed in R using normalised data from the DESeq2 analysis, data were clustered using the top 50 most DEGs overall comparisons, combined (Supplementary Fig. S4). The plots show the results of the first 2 principal components.

Statistical analysis

Statistics used were dependant on the experiments performed. Data are presented as mean ± SEM on assigned number of independent experiments in graph pad prism (version 5 and 8). Normality of data was tested using the Shapiro-Wilk test confirmed with histograms and Q-Q plots in SPSS (version 23). P values were determined using One-way ANOVA with Tukey’s post hoc analysis or Dunnett’s for multiple groups comparisons. Any data transformations were carried out when needed. A p-value < 0.05 was considered significant. DEGs were selected after genes were discarded from the analysis when differential expression failed to reach the significance after Benjamini-Hochberg correction for multiple testing. A DEGs was defined by adjusted p-value < 0.05.

Results

PNLA reduced monocytic migration, macrophagocytosis and ox-LDL uptake in THP-1 macrophages and HMDM

PNLA significantly (p<0.001) inhibits MCP1-driven migration of THP-1 monocytes by 55%, 50%, 50% and 50% at 25, 50, 75 and 100 μM concentrations respectively compared to the vehicle control (Fig1A). LY uptake by THP-1 macrophages was significantly attenuated (p= 0.0006, 0.0002) with 25 and 100 μm PNLA by 55% in both cases, and (p=0.009, 0.0012) at 50 and 75 μM PNLA by 45% and 50% respectively (Fig1B). LY uptake in PNLA treated HMDM was significantly reduced (p=0.0039, 0.0087 and 0.0025) when compared to cells treated with the vehicle control as shown in (Fig1D) by
50%, 45%, and 50% with 50, 75 and 100 μM PNLA, respectively, and (p=0.031) by 40% with 25 μM PNLA. Dil labelled-ox-LDL uptake was increased by almost 95% in THP-1 macrophages and HMDM. PNLA at 25 μM significantly (p=0.003 and 0.047) reduces ox-LDL uptake by almost 50% and 25% in THP-1 macrophages and HMDM respectively (Fig1C, E).

**PNLA reduced TNFα, IL-6 and PGE2 release by LPS stimulated PBMC from patients with RA and HCs**

In PBMC from RA patients; both 25 and 50 μM PNLA significantly reduced LPS stimulated TNFα, IL6 and PGE2 release by monocytes (Fig2 B, D, and F respectively). The effects were similar in monocytes from HCs (Fig2 A, C, and E). More details on experiment and values obtained are provided in (Supplementary Table S3).

The release of TNFα by LPS stimulated monocytes in HCs were reduced from 608 ± 120 pg/ml with 25 and 50 μM PNLA to 298.49 ± 50.81pg/ml (p=0.049) and 374.14 ± 98.09pg/ml (p=0.07) respectively. In RA, TNFα levels was reduced from 443.7 ± 42.7pg/ml to 270.03 ± 40.34pg/ml (p=0.007) and 250.43 ± 38.88pg/ml (p=0.003) by 25 and 50 μM PNLA, respectively.

The release of IL6 by LPS stimulated monocytes from HC were reduced from 206.5 ± 37.78 pg/ml by 25 and 50 μM PNLA to 122.33 ± 31.65 pg/ml (p=0.042) and 135.68 ± 38.84pg/ml (p=0.031) respectively. Similarly, in RA patients, 25 μM and 50 μM PNLA reduced IL-6 release to 103.94 ± 23.18pg/ml (p=0.006) and 93.84 ± 14.66pg/ml (p=0.006) respectively from 206.3 ± 35.87pg/ml.

PGE2 levels were inhibited by PNLA in both HC and RA monocytes to 295 ± 26.3 pg/ml (p<0.0001) and 288 ± 26.10pg/ml (p<0.0001) respectively by 50 μM PNLA in comparison with the control level at 650 ± 85.4pg/ml for healthy stimulated monocytes, and 503.71 ± 48.6pg/ml for RA monocytes.

**Heatmap of DEG and PCA**

Heatmap (Supplementary Fig. S3) shows main clusters of DEGs of all treatment conditions mentioned. The expression of vehicle treated PBMCs genes were upregulated while PNLA and LPS treatment down regulate those genes. HCs clusters on the left side were more consistent than RA clusters on the right side which show some variability. PCA results (Supplementary Fig. S4) show samples clustering within groups demonstrating that inter-sample variation in gene expression is not greater than the biology we hope to observe. HC samples are less variable in terms of gene expression compared to the separation within clusters shown by the RA samples.
5 DEGs whose expression was most significantly affected by PNLA treatment upon LPS stimulation of PBMCs in comparison with vehicle-treatment (adjusted p-value < 0.05)

HCs and RA patients’ global significant and non-significant genes were plotted as volcano plots, and comparisons between groups were performed as shown in (Fig 3A-C) for HCs and (Fig 3D-F) for RA patients. The significantly regulated genes are shown outside the dotted lines; with log2 FC< -1.5 and >1.5; adjusted p value < 0.05.

The genes that were selected their expression levels were most significantly changed after adjusting for multiple comparisons (with lowest adjusted p value and highest fold increase) among all the DEGs; are 2 genes SERPINE1 (PAI-1) (p=0.002; log2 FC=2.60) and PDK4 (p =9.94E-11; log2 FC=3.784) were upregulated by PNLA treated LPS activated PBMCs in HCs. On the other hand, expression of 3 genes FBP1 (p=1.93E-9; log2 FC=3.477), NDRG2 (p=1.73E-4; Log2 FC=2.377), and PDK4 (p=1.11E-9; Log2FC=2.509) were upregulated by PNLA treated LPS activated PBMCs in RA patients. The networks between those genes and the other molecules, chemokines and cytokines are illustrated in (Supplementary Fig. S5).

Canonical Pathways

Using IPA software, the canonical pathways were reproduced for all the comparisons. We then compared the top canonical pathways for both HCs and RA patients of PNLA treated LPS stimulated PBMCs versus vehicle treated and LPS stimulated PBMCs. (Fig.4) shows the pathways involved in the pathogenesis and treatment of RA and cell metabolism. Pathways affected by PNLA in both HCs and patients with RA included granulocyte and agranulocyte adhesion and diapedesis, complement system, IL8 signalling, acute phase response, dermatan sulphate as well as chondroitin sulphate degradation, and hepatic fibrosis. For pathways related to RA, those involving T and B cells, osteoblasts, osteoclasts, chondrocytes, IL10, macrophages, endothelial cells and integrin signalling were affected. Interestingly, pathways related to atherosclerosis were more affected in HCs than RA. In HCs, metabolic pathways affecting adipogenesis, glucose metabolism, and cholecystokinin signalling were affected more than RA, in addition to ROS and NO production.

Upstream regulators analysis

Upstream regulators were further evaluated to identify the effectors that were up or down regulated by PNLA treatment of PBMCs from HCs (Fig 5A, B), RA patients (Fig 5C, D) and (Supplementary Fig. S6 and S7). Pathway analysis predicted upstream inhibition of NF-κB and STAT1(p=1.82E-07 and 1.39 E-04 respectively) that are involved in the transcription of TNFα, IFNγ and IL6 (Fig 6A, B and Supplementary Tables S4 and S5). Also, PPARα (p=9.22E-05) and PPARδ (p=3.95E-04) along with PPARG1A (p=6.33E-04) were predicted to be activated (Fig 6C-E and Supplementary Table S6 and S7). PPARs are involved in anti-inflammatory processes; inhibition of NF-κB, STAT1 and AP-1 while
activation of STAT6 (32)(33). PPARGC1A is a major factor that regulates muscle fibre type determination, this protein is also involved in controlling blood pressure, regulating cellular cholesterol homeostasis, and the development of obesity. It plays an essential role in metabolic reprogramming in response to dietary availability through coordination of the expression of a wide array of genes involved in glucose and fatty acid metabolism. It is required for coactivation of metabolic genes, such as PDK4 (41). Pathway analysis predicted upstream inhibition of IL1α (p=1.02 E-02), IL1β (p=1.69 E-03), and CCR2 (p=3.06 E-03) as supported by the dataset as shown in (Fig6F-H).

Discussion

Data from this study are the first to suggest that PNLA has potential beneficial effects in patients with RA by reducing pain, synovitis, and atherosclerosis through decreasing inflammatory cytokines, PGE2 production, and ox-LDL uptake by macrophages. In this study, we extended previous observations in murine species by Chuang et al, which showed that PNLA incorporation into phospholipids suppressed the production and expression of pro-inflammatory mediators; PGE2, IL6, TNFα, NF-κβ, and iNOS (9) stimulated by DGLA or AA. This was mediated partially through the alleviation of LPS-activated JNK-MAPK signalling. Our data show that PNLA reduced production of TNFα, IL6, and PGE2 in LPS stimulated PBMCs from patients with RA and HCs. Given TNFα and IL6 are established therapeutic targets in RA and PGE2 has a role in inflammatory pain, PNLA may supplement DMARD treatment and increase percentage of patient achieving remission with low risk of side effect.

Cardiovascular events are more frequent in patients with RA (4). Our data suggest PNLA may reduce cardiovascular disease by reducing ox-LDL uptake by macrophages. Foam cell formation, a critical early event in atherosclerosis (14)(22) is a complex process involving chemokine-driven recruitment of monocytes and their differentiation into macrophages, production of ROS leading to oxidation of LDL, uptake of such ox-LDL by macrophages, and the efflux of cholesterol from foam cells to acceptors such as HDL or its key apolipoprotein ApoA1 and subsequent reverse cholesterol transport (14). PNLA attenuates MCP1 driven migration of monocytes and reduces ox-LDL uptake and macropinocytosis by macrophages, although, PNLA in this study does not affect the ROS production or the cell ability for phagocytosis (Supplementary Data S4 and Supplementary Fig. S1 S2).

Our transcriptome data suggest PNLA has major immunometabolic effects that are important in RA and atherosclerosis. This is consistent with observations by Lee and Han in HepG2 cells showing that PNLA has specific effects in reducing the expression of genes related to fatty acid biosynthesis (SREBP1c, FAS, SCD1), cholesterol synthesis (HMGCR), lipoprotein uptake (LDLr), and acyl-CoA binding to fatty acids (23).
In PBMCs, we identified several genes that are involved in immunity and metabolism as well as potential cross-talks between these systems. NDRG2 gene expression is associated with proliferation, differentiation, migration, cytokine production, intracellular signal transduction and stress responses, negative regulation of ERK1, ERK2 cascade (24). Regulation of PDGF and VEGF production (24). PDK4 is important in cellular metabolism, PDK4 deficiency triggers hepatic apoptosis concomitantly with increased numbers of aberrant mitochondria, ROS production, sustained JNK activation, and reduction of glutathione (GSH). Interestingly, PDK4 can interact with death domain containing proteins such as NF-κB. PDK4 can bind to NF-κB and retain it in the cytoplasm (25). Reduction in NF-κB nuclear translocation will reduce signal transduction by TNF (25). Another DEG that was significantly activated by PNLA treatment is SERPINE1 which inhibits tissue plasminogen activator (tPA) and uroplasminogen activator (uPA) for conversion of plasminogen into plasmin. In RA synovia, uPA expression is increased in proliferative lining areas (26). In animal models of RA, plasminogen deficiency reduces synovial inflammation and joint damage in TNF transgenic mice by reducing pro-inflammatory cytokines and MMP (27).

Interestingly, PPARs were predicted to be activated by PNLA by IPA as shown in Fig 5 and 6. PPARα has been implicated in anti-inflammatory responses, lipid and glucose metabolism, and inhibition of oxidative stress (28)(29). PPARα activation with fenofibrate was first reported in a clinical case study for the treatment of RA (30). In a following experimental study, it was shown that fenofibrate treatment inhibits NF-κB activation, cytokine production, and the development of RA (31). PPARγ activation in macrophages can antagonize NF-κB, AP-1 and STAT1 signalling pathways (32)(33). PPARγ stimulates ox-LDL efflux via LXR pathway within the macrophages which consequently can lead to reduce ox-LDL accumulation. PPARγ is a key regulator of adipocyte differentiation and glucose homeostasis and acts as a critical regulator of gut homeostasis by suppressing NF-kB-mediated proinflammatory responses (34)(35)(36). Low PPARγ reduces the capacity of adipose tissue to store fat, resulting in increased storage of fat in nonadipose tissue (lipotoxicity). PPARγ is jointly required for full adipocyte differentiation and fat deposition with variability influencing plasma leptin levels in obese humans, modulating insulin sensitivity, controlling glucose homeostasis and blood pressure and activation of antidiabetic effect of drugs. In addition, it works as a negative regulator of macrophage activation, inhibiting production of monocyte inflammatory cytokines (35) and increasing PAI-1 expression in endothelial cells (36). Also, PPARγ agonists are potential treatments for cardiovascular disease, diabetes mellitus and hypertension as they have been shown to inhibit atherosclerosis development in animal (28)(37) and human studies (29)(35)(38). Our data suggest PNLA may upregulate PPARs and may improve immunometabolism in RA.
Limitations

Our observations are made in cell lines and ex-vivo experiments. The therapeutic benefit of PNLA will require clinical trials. A healthy and balanced diet is recommended by ACR and EULAR for the management of RA (39)(40). However, there is no specific recommendation on specific type of food due to limited scientific evidence. However, dietary advice is a common query from patients with RA. Our data provide the scientific rationale to conduct clinical trials to assess the therapeutic effect PNLA either by diet or as a supplement in patients with RA.
Acknowledgements

The authors would like to thank all RA patients and healthy volunteers who participated in this study. We acknowledge our colleagues at Wales Gene Park for their insight and expertise that assisted this research, for their technical support in generating the NGS data. We thank our colleagues at D.P.R group Jess Williams, Victoria O’Morain and Yee Hung Chan for technical assistance. We acknowledge Versus Arthritis, Health and Care Research Wales CREATE Centre award grant number (20016) and Cardiff University for their support of this study. We acknowledge the support of the Supercomputing Wales project, which is part-funded by the European Regional Development Fund (ERDF) via Welsh Government.

R.T., E.C., D.P.R, J.B., R.A, Y.Z. and R.T., D.P.R. and E.C. were responsible for study conception, design and data interpretation. E.C. was responsible for patient recruitment, sample and clinical data collection. R.T. conducted laboratory experiments and the analysis. R.T., D.P.R. and E.C. drafted the manuscript. R.T., and J.B prepared the figures. R.A did the bioinformatic work and Y.Z designed the transcriptomic data analysis. All authors critically revised and approved the final manuscript to be published.

Funding: This work was funded by a PhD studentship from the Libyan government grant number (513253) and the financial support of Rank Prize Fund of Nutrition through the COVID-19 period.

Disclosure statement: E.C. has received research grants and/or served as a member of advisory boards and speaker bureaus of Abbvie, Allergan, Amgen, AstraZeneca, Bio-Cancer, Biogen, BMS, Boehringer Ingelheim, Celgene, Chugai Pharma, Daiichi Sankyo, Eli Lilly, Ferring Pharmaceutical, GSK, Hospira, ISIS, Jazz Pharmaceuticals, Janssen, MedImmune, Merrimack Pharmaceutical, MSD, Napp, Novimmune, Novartis, ObsEva, Pfizer, Regeneron, Roche, R-Pharm, Sanofi, SynAct Pharma, Synovate, Tonix and UCB. The other authors have declared no conflicts of interest.

Data availability statement: Data will be available upon reasonable request to the corresponding author by any qualified researchers who engage in independent scientific research and will be provided following review and approval of a research proposal and Statistical Analysis Plan (SAP) and execution of a Data Sharing Agreement (DSA). All data are incorporated into the article and its online supplementary material.
References


Legends to the figures

Fig. 1 PNLA reduced monocytes migration, macropinocytosis, and lipid uptake in THP-1 cells and HMDMs. (A) PNLA inhibits MCP1 induced migration of THP-1 monocytes. Migration of THP-1 monocytes in response to MCP-1 stimulus (20 ng/ml) was assessed in the presence of indicated concentrations of PNLA or DMSO vehicle for 3 h. Monocyte migration was then determined as described in the methods. Data were normalized to the percentage of cells that migrated from the apical compartment of the modified Boyden chamber into the basolateral compartment in response to MCP1 only treatment. Migration in presence of MCP-1 and vehicle -treated cells has been assigned as 100% with the others represented to this. (B and C) LY and Dil-oxLDL uptake are attenuated following incubation of THP-1 macrophages with PNLA. (D and E) LY and Dil-oxLDL uptake are attenuated following incubation of HMDM with PNLA. THP-1 macrophages and HMDM were incubated with the indicated concentrations of PNLA for 24 h followed by 100 μg/ml (LY) or 5 μg/ml Dil-oxLDL for another 24 h in RPMI medium supplemented with 0.2% bovine serum albumin. After incubation, cells were collected and subjected to centrifugation at 9,000 x g for 5 min. The supernatants were discarded, and the pellet was resuspended in 2% paraformaldehyde. Uptake in presence of LY or Dil-oxLDL and vehicle assigned as 100% with the others represented to this. The data are presented as mean ± SEM performed from four independent experiments. Statistical analysis was performed using a One-way ANOVA followed by Turkey's post hoc analysis. (*, P≤ 0.05; **, P≤ 0.01; ***, P≤ 0.001).

Fig. 2 PNLA reduced levels of TNFα, IL-6 and PGE2 of activated monocytes from HCs and RA patients. (A and B) PNLA reduces the levels of TNFα, (C and D) IL-6, and (E and F) PGE2 in activated monocytes from HCs and RA patients. Purified monocytes obtained from RA patients (n=20), and HCs (n=10), respectively, were incubated with 25, 50 μM PNLA or DMSO for 24 h followed by 100 ng/ml LPS stimulation for another 16 h. Then, the supernatants were collected and assayed for levels of TNFα, IL6 and PGE2 using ELISA kits, all samples and standards were anlaysed in duplicate. The data are presented as mean ± SEM, each • represents average of one participant. Statistical analysis was performed using One-way ANOVA and a Dunnett’s test. (*, P< 0.05; **, P≤ 0.01; ***, P≤ 0.001, N.S = non-significant).

Fig. 3 Volcano plots of global gene expression profile of HCs and RA patients. Dotted lines identify genes whose expression was significantly regulated (log2 FC < -1.5 and >1.5, p < 0.05) after 24 h of 25 μM PNLA or vehicle treatment and 16 h post 100 ng/ml LPS stimulation. (A and D) show unstimulated versus LPS stimulated PBMCs; (B and E) show PNLA treated, LPS stimulated versus unstimulated PBMCs; (C and F) show PNLA treated, LPS stimulated versus vehicle treated, LPS
stimulated PBMCs from HCs and RAs, respectively. Plots were performed on (Graph pad prism version 8) with highly expressed genes marked as adjusted p value < 0.05.

Fig. 4 Canonical pathways of PBMCs from HCs and RA patients. The comparison analysis of the top canonical pathways following 24 h 25 µM PNLA or vehicle treatment and 16 h post 100 ng/ml LPS stimulation of PBMCs per participants was performed in IPA (Log 2 FC>1.2, P< 0.05). The pathways shown mainly involved in the pathogenesis of RA and atherosclerosis.

Fig. 5 Heatmaps of differentially expressed upstream regulators for comparison analysis from HCs and RA patients. IPA analysis of genes associated with upstream regulators that predicted activated state (red) and the predicted inhibited state (blue) are shown from PBMCs of HCs (A and B) and RA patients (C and D). Relative expression heat maps of the differentially expressed genes regulated by 1. unstimulated (vehicle) versus LPS stimulated PBMCs 2. PNLA treated LPS stimulated versus vehicle treated LPS stimulated PBMCs 3. PNLA treated LPS stimulated versus unstimulated (vehicle) treated PBMCs (Log2 FC>1.2, P<0.05) are shown. The genes shown here are mainly involved in cytokine production, lipid metabolism, drugs used for treating RA or reducing the hyperlipidaemia and transcription regulators as in (Supplementary Fig. 6 and 7)

Fig. 6 PNLA inhibited inflammatory transcription factors, activated anti-inflammatory transcription factors and inhibited inflammatory cytokines. (A and B) NF-κB and STAT1 as upstream regulators of PBMCs from RA patients and HCs are predicted to be inhibited following 25 µM PNLA treatment of 100 ng/ml LPS stimulated PBMCs. (C, D and E) PPARα, PPAR-β/ δ and γ as upstream regulators predicted to be activated following 25 µM PNLA treatment of 100 ng/ml LPS stimulated PBMCs from HCs and RA (F, G, and H) IL1α, IL1β, and CCR2 are also inhibited by 25 µM PNLA treatment and 100 ng/ml LPS stimulation.
Fig. 1 PNLA reduced monocytes migration, macropinocytosis, and lipid uptake in THP-1 cells and HMDMs.
Fig. 2 PNLA reduced levels of TNFα, IL-6 and PGE2 of activated monocytes from HCs and RA patients.
Fig. 3 Volcano plots of global gene expression profile of HCs and RA patients.
Fig. 4 Canonical pathways of PBMCs from HCs and RA patients.
Fig. 5 Heatmaps of differentially expressed upstream regulators for comparison analysis from HCs and RA patients.
Fig. 6 PNLA inhibited inflammatory transcription factors, activated anti-inflammatory transcription factors and inhibited inflammatory cytokines.