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AVALIAÇÃO DOS MECANISMOS DE AÇÃO DO DERIVADO TIAZOLIDÍNICO LPSF/GQ-02 SOBRE A RESISTÊNCIA À INSULINA, ESTEATOSE HEPÁTICA E ATEROSCLEROSE

AMANDA KAROLINA SOARES E SILVA

Recife, 2015

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas, da Universidade Federal de Pernambuco – UFPE, para obtenção do Título de Doutor em Ciências.

Orientadora: Dra. Christina Alvas Peixoto

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Tese apresentada dia 30/06/2015, na Pós-graduação do CCB-UFPE, às 14:00h

Orientadora: Dra. Christina Alves Peixoto

Recife, 2015

Parecer da comissão examinadora da tese de:

Amanda Karolina Soares e Silva

Intitulada: Avaliação dos mecanismos de ação do derivado tiazolidínico LPSF/GQ-02 sobre a resistência à insulina, esteatose hepática e aterosclerose

A comissão examinadora considera o seguinte trabalho:

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Banca Examinadora

Titulares:

_____

Prof. Dr. Eduardo Isidoro Carneiro Beltrão - UFPE

_____

Prof. Dra. Karla Patrícia Sousa Barbosa - UFPE

_____

Prof. Dra. Maira Galdino da Rocha Pitta - UFPE

_____

Prof. Dra. Ana Célia Oliveira dos Santos - UPE

_____

Prof. Dra. Christina Alves Peixoto – CpqAM

Suplentes:

Prof. Dra. Maria Tereza dos Santos Correia

Prof. Dra. Mariana Aragão Matos Donato

Recife, 30 de junho de 2015.

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RESUMO

A doença do fígado gorduroso não-alcoólico (NAFLD) refere-se a um amplo aspectro de doenças hepáticas causadas pelo depósito de gordura nas células do fígado na ausência do consumo de álcool. Provas evidentes demonstram que a NAFLD desempenha um papel relevante na patogênese da aterosclerose. As tiazolidinadionas (TZDs) agem como meléculas sensibilizadoras da ação da insulina e têm sido utilizadas no tratamento de pacientes com diabetes do tipo 2 e outras condições de resistência à insulina, incluindo a NAFLD. Uma vez que a aterosclerose e NAFLD podem compartilhar mecanismos comuns, estratégias terapêuticas similares poderiam ser empregadas no tratamento de ambas as doenças. No presente estudo, foi avaliado a atividade biológica de LPSF/GQ-02 na patogênese da NAFLD e aterosclerose. Os resultados obtidos com um modelo murino de NAFLD indicaram que a LPSF/GQ-02 foi eficaz em melhorar a arquitetura hepática, diminuindo a acumulação de gordura, reduzindo a quantidade de colágeno, diminuindo a inflamação através da redução da IL-6, iNOS, COX-2 e F4/80, e aumento da expressão de proteínas de IκBα citoplasmática, NFkB-65, eNOS e IRS-1 em camundongos deficientes do receptor de LDL (LDLr-/-). Além disso, ambos os tratamentos (15 e 30 dias) com LPSF/GQ-02 resultou em uma redução de colágeno nas lesões ateroscleróticas. Adicionalmente, o tratamento durante 15 dias também diminuiu os níveis de mRNA de CD40, MCP-1, ABCG1, e aumentou os níveis do PPARα, uma vez que o tratamento com 30 dias reduziu os níveis das proteínas LOX-1, p-IκBα e p-NFkB. Estes resultados sugerem uma ação direta da LPSF/GQ-02 sobre os fatores que afetam a inflamação, resistência à insulina e acumulação de gordura no fígado, bem como uma ação sobre a composição e crescimento das lesões ateroscleróticas nos camundongos LDLr-/-. Sendo assim, nossos dados apoiam os resultados anteriores, que mostraram as propriedades anti-inflamatórias de LPSF/GQ-02 e reforça o potencial terapêutico desta TZD para o tratamento da aterosclerose e desordens relacionadas a inflamação.

Palavras Chave: Aterosclerose. NAFLD. Thiazolidinadionas. LPSF/GQ-02. Anti-inflamatório.

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver diseases caused by fat deposit in the liver cells in the absence of excess alcohol consumption. Compelling evidence has demonstrated that NAFLD plays a relevant role in the pathogenesis of atherosclerosis. Thiazolidinediones (TZDs) act as an insulin sensitizer and have been used in the treatment of patients with type 2 diabetes and other insulin-resistant conditions, including NAFLD. Since atherosclerosis and NAFLD might share common mechanisms, similar therapeutic strategies could be employed in the treatment of both disorders. In the present study, we evaluated the biological activity of LPSF/GQ-02 on the NAFLD and atherosclerosis pathogenesis. The results obtained with an NAFLD murine model indicated that LPSF/GQ-02 was effective in improving the hepatic architecture, decreasing fat accumulation, reducing the amount of collagen, decreasing inflammation by reducing IL-6, iNOS, COX-2 and F4 / 80, and increasing the protein expression of IκBα, cytoplasmic NFκB-65, eNOS and IRS-1 in mice deficient in the LDL receptor (LDLr -/-). In addition, both treatment protocols (15 and 30 days) with LPSF/GQ-02 resulted in lower collagen density in the atherosclerotic lesions. Moreover, the treatment for 15 days also decreased mRNA levels of CD40, MCP-1, ABCG1 and upregulated PPARα, whereas the 30-days treatment reduced the protein levels of LOX-1, p-IκBα and p-NFκB. These results suggest a direct action by LPSF/GQ-02 on the factors that affect inflammation, insulin resistance and fat accumulation in the liver of these animals, and affects the composition and growth of atherosclerotic lesions in LDLr $^{-/-}$ mice. Our data also support previous findings showing anti-inflammatory properties of LPSF/GQ-02 and reinforce the therapeutic potential of this TZD for treating atherosclerosis and inflammation-related disorders.

Keywords: Atherosclerosis. NAFLD. Thiazolidinediones. LPSF/GQ-02. anti-inflammatory.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

ABCA1	Transportadores cassete ligados ao ATP A1
ABCG1	Transportadores cassete ligados ao ATP G1
ALT	Alanina Aminotransferase
AST	Aspartato aminotranferase
AP-1	Proteína ativadora 1
aP2	Proteína de ligação a ácido graxo
ApoA-1	Apolipoproteína A-1
ApoB	Apolipoproteína B
ApoC-III	Apolipoproteína C-III
ApoE	Apolipoproteína E
CCR2	Receptor para MCP-1
CD-36	Receptor scavenger de classe B
CD-40L	Ligante solúvel de CD-40
CD-68	Receptor scavenger de classe D
CETP	Proteína de transferência de ésteres de colesterol
CT	Colesterol Total
CXCL16	Quimiocina pertencente à subfamília CXC
DBD	Domínio de ligação ao DNA
DC	Célula dendritica
DM2	Diabetes mellitus tipo 2
eNOS	Sintase de óxido nítrico endotelial
FA	Ácidos graxos
FATP-1	Proteína transportadora de ácido graxo 1
FFA	Ácidos graxos livres
G-CSF	Fator estimulador de colónias de granulócitos
GM-CSF	Fator estimulador de colónias granulócito-macrófago
HCC	Carcinoma hepatocecular
HDL-C	Colesterol lipoproteína de alta densidade
HSC	Células estreladas Hepáticas

ICAM-1	Molécula de adesão Intercelular 1
iNOS	Sintase de oxido nítrico induzível
IL-1	Interleucina-1
IL-6	Interleucina-6
IL-12	Interleucina-12
IL-18	Interleucina-18
KC	Células de Kupffer
LBD	Domínio de ligação ao ligante
LCAT	Lecitina colesterol aciltransferase
LDL-C	Colesterol lipoproteína de baixa densidade
LDLr	Receptor de LDL
LPL	Lipoproteína lipase
LXRα	Receptor X do fígado α
M-CSF	Fator estimulador de colônia de macrófagos
MCP-1	Proteína quimiotática de monócito-*1*
MMP-9	Matriz metaloproteinase-9
MMPs	Matriz metaloproteinases
NASH	Esteatohepatite não-alcoólica
NFAT	Fator nuclear de células T ativadas
NF-κB	Fator nuclear kapa B
NK	Natural Killer
NO	Óxido nítrico
oxLDL	Lipoproteína de baixa densidade oxidada
PAI-1	Inibidor do ativador de plasminogênio tipo 1
PCR	Proteína C Reativa
PEPKC	Fosfoenolpiruvato *carboxiquinase*
PLTP	Proteína de transferência de fosfolipídios
PPRE	Elementos responsivos aos proliferadores de peroxissomos
PPAR	Receptor ativado por proliferadores de peroxissomos
PPAR-α	Receptor-α ativado por proliferadores de peroxissomos
PPAR-δ/β	Receptor- δ/β ativado por proliferadores de peroxissomos
PPAR- γ	Receptor- γ ativado por proliferadores de peroxissomos
RAR	Receptor do Ácido Retinóico
RI	Resistência à insulina

RN	Receptor nuclear
ROS	Espécies reativas de oxigênio
RXR	Receptor X retinóide
TG	Triglicerídeos
TGF-β	Fator de crescimento transformador β
TNFα	Fator de necrose tumoral α *(Tumor necrosis fator α)*
VLDL	Lipoproteína de muito baixa densidade *(Very low density lipoprotein)*
VCAM-1	Molécula de adesão celular vascular *(Vascular cell adhesion molecule 1)*

SUMÁRIO

1 **INTRODUÇÃO**

A obesidade resulta de um desequilíbrio entre a ingestão calórica e gasto de energia, levando a um excesso de energia, que é armazenado como gordura, principalmente no tecido adiposo branco (CHUGH, SHARMA, 2012; MCKENNEY, SHORT, 2011). De forma importante, a obesidade aumenta ou agrava vários problemas de saúde incluindo doenças cardiovasculares e diabetes tipo 2 (KOPELMAN, 2000). As alterações metabólicas associadas à obesidade têm sido reconhecidas e agrupadas para definir atualmente a síndrome metabólica (SM). SM é uma das principais causas de mortalidade e morbidade em países industrializados (SIMONS et al., 2011) e se caracteriza pela combinação de várias doenças, incluindo a obesidade, dislipidemia, aumento da pressão sanguínea, resistência à insulina e um estado pró-inflamatório (REAVEN, 2002). A prevalência de síndrome metabólica se correlaciona com a epidemia global de obesidade e está crescendo a um ritmo alarmante, afetando mais de 20% da população mundial adulta (ONAT, 2011). A crescente epidemia de síndrome metabólica e suas complicações tais como doenças cardiovasculares, tem sido acompanhada por um aumento em alterações hepáticas, incluindo a doença do fígado gorduroso não alcoólico (NAFLD).

A NAFLD caracteriza-se pelo acúmulo de gordura no fígado, sendo considerada a manifestação hepática da síndrome metabólica e estudos recentes têm sugerido que a NAFLD influencia o aumento do risco cardiovascular de forma independente (BAOU et al., 2007). De acordo com Fracanzani et al. (2005) pacientes com NAFLD têm significativamente maior espessura da parede da artéria carótida, que está fortemente associada com o desenvolvimento de acidente vascular cerebral e infarto.

Embora os mecanismos associados com a NAFLD e o risco para a doença cardiovascular não seja ainda muito compreendido, é possível que a NAFLD na sua forma mais avançada atue como um estímulo para aumentar a resistência à insulina e a dislipidemia, ocasionando aceleração da aterosclerose (TARGHER, 2007). A inflamação e o aumento do estresse oxidativo relacionam-se diretamente com a NAFLD e a aterosclerose (BAHCECIOGLU et al., 2005; MARCHESINI et al., 2005). Finalmente, a NAFLD pode contribuir para a progressão da aterosclerose, diminuindo os níveis de adiponectina (MUSSO et al., 2005).

Durante o desenvolvimento da aterosclerose, a parede arterial se espessa gradualmente para formar a placa aterosclerótica, resultando na diminuição do lúmen da

artéria com consequente redução da quantidade de sangue fornecido aos órgãos, afetando, geralmente, o coração e o cérebro. Ocasionalmente, as placas podem se romper produzindo um coágulo, que por sua vez poderá levar ao infarto do miocárdio ou derrame (STARY, 2000).

Evidências sugerem que a LDL modificada por oxidação ou glicação provoca uma resposta inflamatória na parede da artéria, desencadeando muitos dos processos biológicos que participam do início, progressão, e complicação da aterosclerose (GLASS; WITZTUM, 2001). Desta forma, a LDL oxidada (oxLDL) tem sido relacionada a vários processos que participam da aterogênese como a disfunção endotelial, a migração de macrófagos e células musculares lisas e a produção de citocinas inflamatórias (ISHIGAKI; OKA; KATAGIRI, 2009).

A investigação dos mecanismos da aterosclerose tem indicado que a inflamação desempenha um papel central no desenvolvimento, progressão e letalidade dessa doença (LIBBY, 2002; ROCHA; LIBBY, 2009). E tem estimulado a descoberta e adoção de biomarcadores inflamatórios para o prognóstico de risco cardiovascular (PACKARD; LIBBY, 2008). Alguns desses biomarcadores incluem: Moléculas de adesão como VCAM-1; citocinas tais como TNF-α, interleucina-1 (IL-1), interleucina-18 (IL-18) e interleucina-6 (IL-6); proteases como a metaloproteinase de matriz-9 (MMP-9); produtos de plaquetas incluindo CD40 ligante solúvel (CD40L); adipocinas como adiponectina e reagentes de fase aguda tais como Proteína C Reativa (PCR), inibidor do ativador de plasminogênio tipo 1 (PAI-1) e fibrinogênio (PACKARD; LIBBY, 2008).

Os receptores ativados por proliferadores de peroxissomos (PPARs) são receptores nucleares ativados por ligantes que estão envolvidos na regulação transcricional do metabolismo lipídico, balanço energético, inflamação e aterosclerose. Atualmente são conhecidos três isotipos PPAR-α, PPARδ/β e PPAR-γ (AHMED et al., 2007).

O PPAR- α, é predominantemente expresso no fígado e em menor quantidade no coração, músculo esquelético, intestino e rim onde exerce um papel importante em controlar a oxidação dos ácidos graxos (LEFEBVRE et al., 2006). A ativação do PPAR-α está relacionada com a transcrição de aproximadamente 80-100 genes, atuando sobre diversas vias metabólicas (AHMED et al., 2007). O PPAR-α atua no processo inflamatório, em geral, inibindo a produção de citocinas pró-inflamatórias como a IL-6, bem como reprimindo a expressão de VCAM-1 (STAELS et al., 1998). Os fibratos são

os ligantes sintéticos do PPAR-α e são utilizados para tratar dislipidemias em humanos (FORMAN et al., 1997), sua administração diminui os níveis de triglicérides e estudos clínicos têm mostrado que esses fármacos são capazes de reduzir a incidência de eventos cardiovasculares e aterosclerose (BENSINGER; TONTONOZ, 2008).

O PPAR-δ/β é o menos estudado entre os isotipos de PPARs, embora possua expressão significativa em tecidos responsáveis por controlar o metabolismo lipídico, como os adipócitos, intestino delgado, coração, músculo esquelético e macrófagos (GROSS; STAELS, 2007). A ativação do PPAR- δ/β reduziu lesões ateroscleróticas em cerca de 50% em camundongos (LDLR $^{-/-}$). Essa redução na área da lesão não foi acompanhada por mudanças nos níveis de HDL e LDL, mas sim pela diminuição na expressão de MCP-1 e ICAM-1 na aorta dos camundongos tratados (GRAHAM et al., 2005). Esses dados sugerem ativação farmacológica desse isotipo pode ter ações positivas sobre aterosclerose.

O receptor- γ ativado por proliferadores de peroxissomos (PPAR- γ) é um fator de transcrição presente em adipócitos, macrófagos, monócitos, hepatócitos, células musculares e endoteliais, sendo considerado o isotipo mais estudado. Ele pertencente à superfamília de receptores nucleares que se ligam a agonistas específicos, também conhecidos como ligantes ou ativadores de PPARs (HEIKKINEN et al., 2007). As tiazolidinadionas (TZDs), são agonistas sintéticos do PPAR- γ utilizada clinicamente para tratar pacientes com diabetes tipo 2 atuando como sensibilizadora da ação da insulina (DAY, 1999).

Além da expressão no tecido adiposo, o PPAR- γ é expresso em células que compõem as lesões ateroscleróticas, como células endoteliais, musculares lisas e monócitos/macrófagos (COLLINS et al., 2001). Diante disso, evidências têm indicado que em adição à sua atividade antidiabética, as TZDs apresentam também atividade antiaterosclerótica. O potencial antiaterogênico dos agonistas de PPAR- γ têm sido sugeridos em estudos com modelos animais de aterosclerose (LI et al., 2000; COLLINS et al., 2001) e em ensaios clínicos utilizando pacientes com diabetes tipo 2 (MINAMIKAWA et al., 1998; LANGENFELD et al., 2005).

A utilização de agonistas de PPAR- γ também exerce efeitos favoráveis sobre a NAFLD. De acordo com Uto et al. (2005), doses clínicas de pioglitazona foram capazes de reduzir efetivamente a gordura no fígado de inibir o desenvolvimento de cirrose em ratos. Similarmente, a utilização da pioglitazona foi eficaz em melhorar a histologia do

fígado e normalizar os níveis das aminotransferases em pacientes com Esteato-hepatite Não-Alcoólica (NASH) (PROMRAT et al., 2004).

Apesar de existirem diversos estudos mostrando os efeitos vasculares favoráveis dos agonistas de PPAR- γ, os efeitos cardiovasculares das TZDs tornaram-se alvo de intensa discussão. Em um estudo, a rosiglitazona foi associado ao aumento dos riscos de infarto do miocárdio e mortalidade por causas cardiovasculares (NISSEN; WOLSKI, 2007). De forma controversa, no estudo PROactive, o tratamento com pioglitazona foi associado à redução do risco combinado de infarto agudo do miocárdio, acidente vascular cerebral e mortalidade em 16%, entre pacientes diabéticos de alto risco cardiovascular (DORMANDY et al., 2005). É possível que os efeitos danosos apresentados pela rosiglitazona sejam específicos dessa molécula, não representando um efeito de classe. Entretanto, os mecanismos determinantes do aumento de risco cardiovascular associado às TZDs ainda não são conhecidos.

De forma semelhante, a deleção do PPAR-γ hepático em alguns modelos animais diminui consideravelmente a esteatose independente da presença de hiperinsulinemia ou hiperglicemia (GAVRILOVA et al., 2003; MATSUSUE et al., 2003), indicando, portanto, que a atuação do PPAR- γ pode ser prejudicial para o fígado.

Silva et al (2013), demonstraram que o derivado tiazolidínico LPSF/GQ-02 (5-(4-Cloro-benzilideno)-3-(4-metil-benzil)-thiazolidina-2,4-diona) foi eficaz em melhorar a resistência à insulina, reduzir a área de lesões ateroscleróticas oferecendo um efeito protetor ao endotélio, além de exercer efeitos anti-inflamatórios aumentando a expressão da sintase de óxido nítrico endotelial (eNOS) e reduzindo a expressão da matriz metaloproteinase 9 (MMP-9). Diante dos resultados encontrados com a utilização da LPSF/GQ-02, nosso trabalho se propõe a avaliar as possíveis vias de ação desse candidato a fármaco sobre o processo aterosclerótico e a NAFLD em animais deficientes do receptor de LDL (LDLr-/-).

2 JUSTIFICATIVA

Linhas de pesquisa têm investido na busca por novas moléculas a fim de se obter alternativas terapêuticas mais seletivas e menos danosas contra a patogênese da aterosclerose e seus fatores de risco associados, como a NAFLD. O laboratório de ultraestrutura participa da avaliação biológica de tiazolidinadionas com potencial atividade hipoglicêmica e hipolipidêmica sintetizados pelo Laboratório de Planejamento e Síntese de Fármacos da Universidade Federal de Pernambuco. Resultados preliminares da caracterização da atividade anti-aterogênica do derivado tiazolidínico LPSF/GQ-02, utilizando análises bioquímicas, morfométricas e utraestruturais indicam relevantes benefícios quanto à diminuição de resistência à insulina e dos triglicerídeos hepáticos, além de uma redução expressiva da placa aterosclerótica em camundongos deficientes do receptor de LDL (LDLr-/-). Tais evidências indicam que este novo fármaco apresenta além de uma atividade PPAR-γ, uma possível atividade PPAR-α. Além disso, o derivado tiazolidínico LPSF/GQ-02 também parece estar envolvido na ativação de fatores anti-inflamatórios endoteliais. Sendo assim, faz-se necessário esclarecer as ações da LPSF/GQ-02 sobre o tecido hepático, bem como o seu possível mecanismo de ação sobre o processo aterosclerótico em camundongos LDLr-/- submetidos a uma dieta hiperlipidêmica.

3 OBJETIVOS

3.1 – Objetivo Geral

Analisar a ação do derivado tiazolidínico LPSF/GQ-02, comparativamente com glitazonas comerciais, sobre o desenvolvimento da inflamação endotelial e formação de placas de ateroma, acúmulo de triglicérides no fígado e resistência à insulina em modelo murino deficiente de LDL (LDL-/-).

3.2 – Objetivos Específicos

- Avaliar o perfil lipídico, glicemia, insulinemia, marcadores inflamatórios após o tratamento com LPSF/GQ-02 em camundongos (LDL-/-);

- Avaliar as modificações histopatológicas do endotélio da aorta e fígado de camundongos LDLr-/-;

- Caracterizar imunohistologicamente marcadores inflamatórios e fatores de transcrição envolvidos no processo aterosclerótico e acúmulo de gordura no fígado em camundongos (LDL-/-);

- Detectar a expressão de receptores nucleares e marcadores envolvidos na patogênese da aterosclerose e acúmulo de gordura no fígado em camundongos LDLr-/-, submetidos ao tratamento com a LPSF/GQ-02.

Capítulo I

4. REVISÃO BIBLIOGRÁFICA

4.1 ATEROSCLEROSE

4.1.1 Lipídios e risco cardiovascular

Os lipídios constituem um grupo de compostos hidrofóbicos com muitas funções biológicas, como componentes estruturais de membranas celulares, fonte de energia e complexos sistemas de sinalização intracelular (HEGELE, 2009).

Um adulto ingere cerca de 60 a 150g de lipídeos diariamente, dos quais normalmente mais de 90% são constituídos por triglicérides (TG), formados por uma molécula de glicerol esterificada a três ácidos graxos denominados saturados, monoinsaturados, poliinsaturados e *trans*. O restante dos lipídeos da dieta consiste principalmente de colesterol, ésteres de colesterol, fosfolipídeos e ácidos graxos não-esterificados (“livres”) (CHAMPE et al., 2006). Dentre os lipídios existentes, o TG e colesterol são considerados os mais importantes clinicamente (HEGELE, 2009).

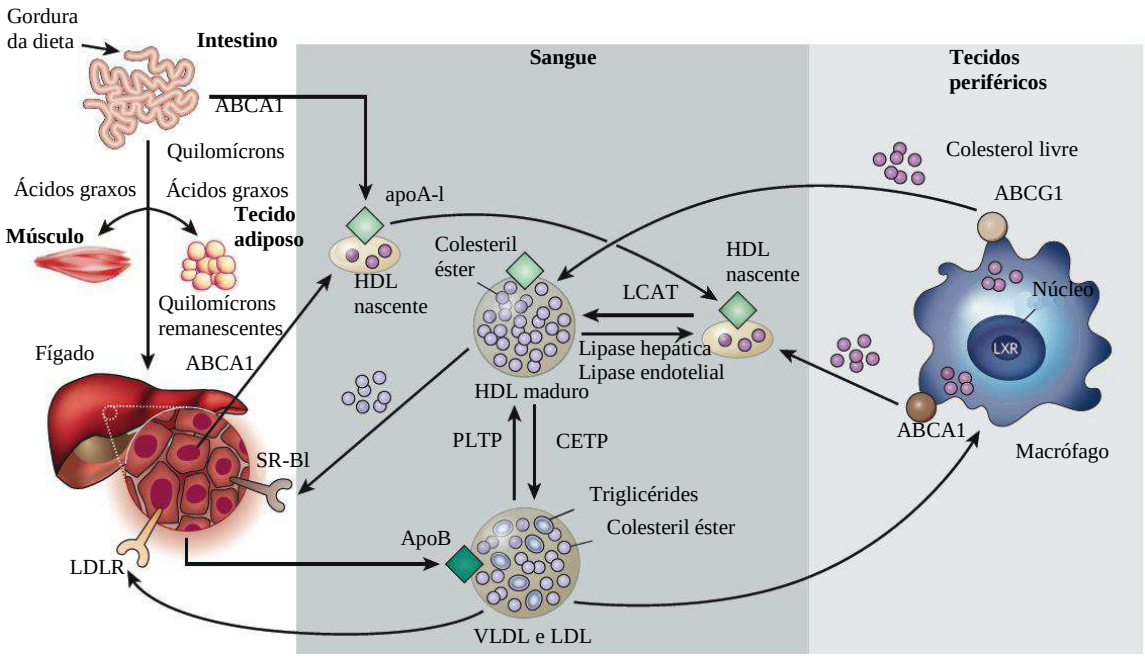
A insolubilidade do colesterol e TG no plasma exige que eles sejam transportados em macromoléculas esferoidais chamadas lipoproteínas, que têm um núcleo hidrofóbico contendo fosfolipídio, antioxidantes solúveis em gordura, vitaminas, ester de colesterol e um revestimento hidrofílico que contém colesterol livre, fosfolipídio e proteínas denominadas apolipoproteínas. As principais lipoproteínas transportadoras de TG são quilomícrons e lipoproteínas de densidade muito baixa (VLDL). As principais lipoproteínas transportadoras de colesterol são lipoproteína de densidade baixa (LDL) e lipoproteína de densidade alta (HDL). Lipoproteínas são distinguidas uma das outras pelo tamanho, densidade, mobilidade eletroforética, composição e função. (HEGELE, 2009)

A função das lipoproteínas é manter os lipídios em solução durante seu transporte entre os tecidos. O intestino absorve a gordura da dieta e a empacota em quilomicrons (Lipoproteínas grandes, ricas em triglicérides) que são transportadas para os tecidos periféricos através do sangue. Ao chegar ao tecido muscular e adiposo, os quilimicrons são digeridos pela lipase lipoproteica, liberando ácidos graxos livres que se inserem nestes tecidos. Os quilimicrons remanescentes são posteriormente removidos pelo fígado. O fígado então sintetiza VLDLs a partir de lipídios e apolipoproteína B

(ApoB), que posteriormente, na circulação sanguínea, sofrem lipólise pela lipase lipoproteica para formar LDLs. Estas últimas são, em seguida, removidas pelo fígado através da ligação ao receptor de LDL (LDLR), bem como através de outras vias (Figura 1) (RADER; DAUGHERTY, 2008).

A apolipoproteína A-I (ApoA-I), sintetizada pelas células intestinais e hepáticas, recruta colesterol desses órgãos através da ação dos transportadores cassete ligados ao ATP AI (ABCA-1), formando as HDLs nascente. Nos tecidos periféricos, as HDLs nascentes promovem o efluxo do colesterol dos tecidos, inclusive de macrófagos, através da ação do transportados ABCA-1. Similarmente, as HDLs maduras também promovem esse efluxo, mas através da ação dos transportadores ABCG1. O colesterol livre é esterificado em ésteres de colesterol nas HDLs nascentes pela enzima lecitina colesterol aciltransferase (LCAT), formando HDLs maduras. O colesterol presente nas HDLs retorna para o fígado, quer diretamente, através de sua absorção pelo receptor SR-BI, ou indiretamente, pela sua transferência para LDLs e VLDLs, que é realizado pela proteína de transferência de ésteres de colesterol (CETP). O conteúdo lipídico de HDLs é alterado pelas enzimas lipase hepática e lipase endotelial e pelas proteínas de transferência CETP e proteína de transferência de fosfolipídios (PLTP) (Figura 1) (RADER; DAUGHERTY, 2008).

Figura 1 – Metabolismo das lipoproteínas



Fonte: Rader; Daugherty (2008).

Legenda – Metabolismo das lipoproteínas. ApoB, apolipoproteína B; VLDLs, lipoproteínas de muito baixa densidade; LDLs, lipoproteínas de baixa densidade; LDLR, receptor de LDL; SR-

B1, receptor *scavenger* classe B tipo 1; HDLs, lipoproteínas de alta densidade; ApoA-I, apolipoproteína A-I, transportador ABCA-1, transportador ABCG1, LXR, receptor nuclear hepático; LCAT, lecitina colesterol aciltransferase, CETP, proteína de transferência de ésteres de colesterol, PLTP, proteína de transferência de fosfolipídeos. Fonte: Rader; Daugherty (2008).

A participação de lipoproteínas que contêm Apo-B, como as LDLs estão associadas ao desenvolvimento da aterosclerose (RADER; DAUGHERTY, 2008). O acúmulo de LDL no compartimento plasmático pode ocorrer em virtude de uma dieta rica em gordura, da síntese endógena de colesterol ou até mesmo pela diminuição do catabolismo da LDL pelo fígado, causado por um defeito gênico que promove a deficiência na expressão ou função dos seus receptores, resultando em hipercolesterolemia (THOMPSON et al., 1981).

Evidências sugerem que a LDL modificada por oxidação ou glicação provoca uma resposta inflamatória na parede da artéria, desencadeando muitos dos processos biológicos que participam do início, progressão, e complicação da aterosclerose (GLASS; WITZTUM, 2001). Desta forma, a LDL oxidada (oxLDL) tem sido implicada em muitos processos aterogênicos como disfunção endotelial, migração de macrófagos e células musculares lisas e produção de citocinas inflamatórias. De modo geral, a oxLDL é endocitada pelos macrófagos, transformando esses macrófagos em células espumosas e a formação de placas ateroscleróticas (ISHIGAKI; OKA; KATAGIRI, 2009).

Em contraste com o colesterol LDL, concentrações plasmáticas de colesterol HDL são inversamente associadas com doença aterosclerótica. O mais bem estabelecido mecanismo pelo qual HDLs protegem contra a aterosclerose é através da promoção do efluxo de colesterol de macrófagos e transportando o colesterol para o fígado para a excreção na bile e fezes (RADER; DAUGHERTY, 2008). HDLs têm várias outras propriedades que poderiam contribuir para a sua propriedade antiaterogênica. Por exemplo, HDLs podem estimular a atividade da óxido nítrico sintase 3 (NOS3; também conhecida como eNOS) e assim aumentar a biodisponibilidade de óxido nítrico (NO) (MINEO et al., 2006). Além disso, HDLs têm efeitos anti-inflamatórios igualmente *in vitro* e *in vivo*, e estes têm sido o objetivo de intensivos estudos (BARTER et al., 2004).

As LDLs e HDLs são as lipoproteínas mais abundantes no plasma e desenvolvem, de forma diferente, ações pro e antiaterogênicas, respectivamente. Dessa forma, o metabolismo dessas lipoproteínas é alvo das principais intervenções para

prevenir e tratar a doença cardiovascular aterosclerótica (RADER; DAUGHERTY, 2008).

4.1.2 Patogênese da Aterosclerose

O endotélio, uma vez considerado uma barreira simples e seletivamente permeável entre a circulação sanguínea e a parede vascular externa, é agora reconhecido como um órgão homeostático, fundamental para a estrutura e regulação do tônus vascular. Sob condições fisiológicas normais, as células endoteliais induzem a produção e liberação de NO, que se difunde em volta de tecidos e células e exerce seu papel protetor cardiovascular, relaxando as células musculares lisas, impedindo a migração e adesão de leucócitos na parede arterial, a proliferação das células musculares lisas, adesão e agregação plaquetária e expressão de moléculas de adesão (LUSCHER; VANHOUTTE, 1990).

Em condições patológicas, incluindo a presença de fatores de risco cardiovascular, o endotélio sofre alterações funcionais e estruturais, perdendo assim o seu papel de proteção, tornando-se uma estrutura pró-aterosclerótica (LUSCHER; VANHOUTTE, 1990). Essa alteração inicial no endotélio é conhecida como "disfunção endotelial e evidências sugerem que esse processo inicia o desenvolvimento aterosclerótico e pode ser detectada antes de mudanças estruturais na parede do vaso (DAVIGNON; GANZ, 2004). Condições comuns que predispõem a aterosclerose, tais como hipercolesterolemia, hipertensão, diabetes e tabagismo, estão associadas à disfunção endotelial (LANDMESSER; HORNING; DREXLER, 2004).

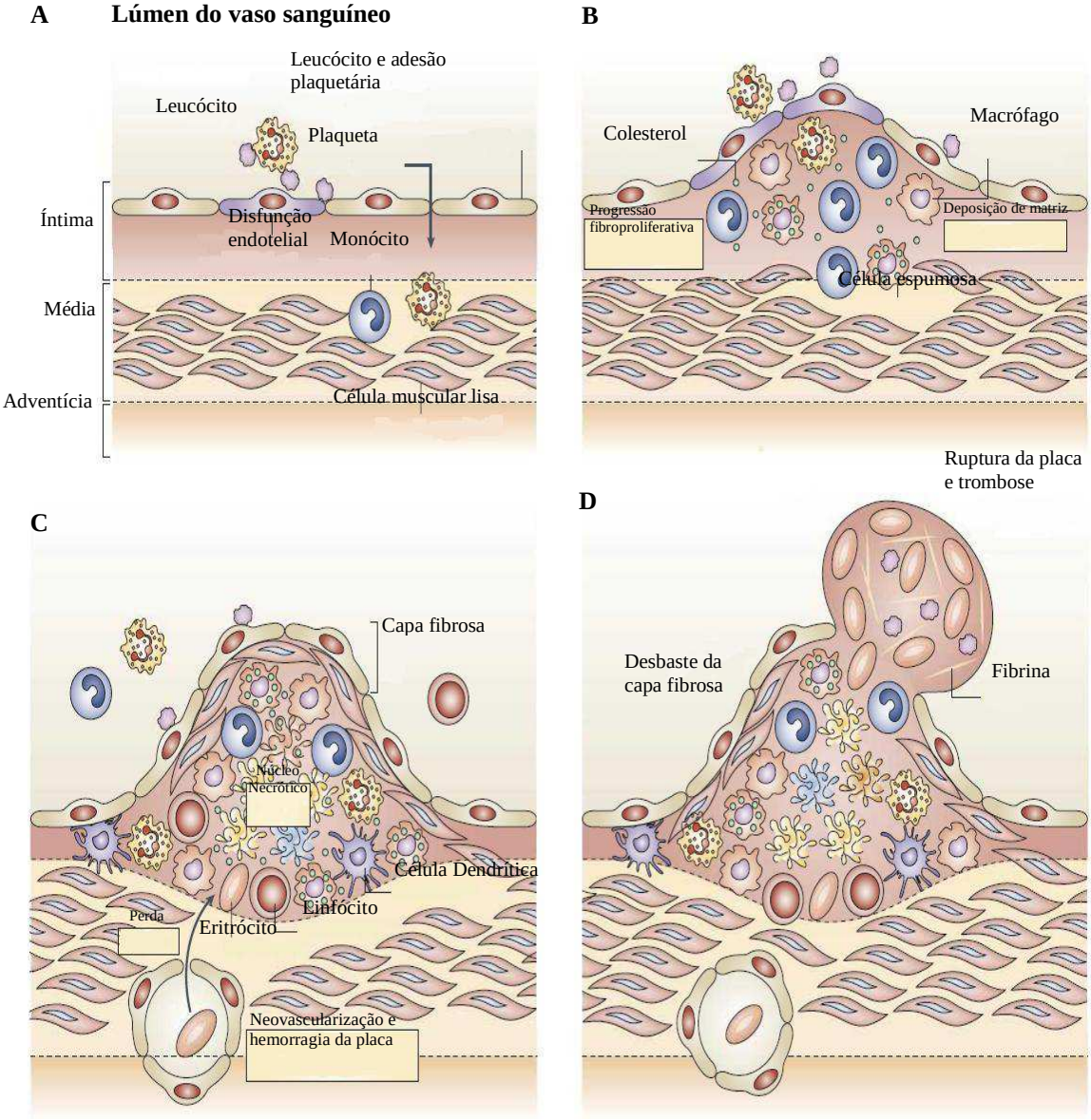
A disfunção endotelial caracteriza-se por ser um dos primeiros estágios da aterosclerose. O endotélio lesado, expressa em sua superfície moléculas de adesão que atraem monócitos, linfócitos e plaquetas, acarretando um aumento na permeabilidade do vaso sanguíneo para os componentes lipídicos presentes no plasma, principalmente a LDL (HANSSON, 2005). Uma vez dentro da íntima, as lipoproteínas aterogênicas são modificadas por oxidação ou atividade enzimática agregando-se dentro do espaço íntimal (RADER; DAUGHERTY, 2008). Os monócitos migram para dentro da íntima e se transformam em macrófagos, onde começam a fagocitar a oxLDL e outros lipídios, transformando-se em células espumosas, constituindo o primeiro estágio da lesão aterosclerótica (conhecido como estrias gordurosas) na íntima (figura 2) (WEBER; ZERNECKE; LIBBY, 2008). Estrias gordurosas são frequentemente presentes em

aorta de crianças, em artérias coronárias de adolescentes e em vasos periféricos de adultos jovens. Mesmo não causando nenhuma patologia clínica, essas estrias gordurosas são consideradas lesões iniciais que levam ao desenvolvimento de complexas lesões ateroscleróticas (RADER; DAUGHERTY, 2008).

Em um segundo estágio, as estrias gordurosas progridem formando uma placa aterosclerótica madura que acumula subpopulações de células inflamatórias e lipídios extracelulares, essas mudanças formam um núcleo que é rodeado por uma capa de células musculares lisas (SMCs) e matriz rica em colágeno. A secreção de citocinas e fatores de crescimento pelas células da placa e mais deposição de componentes da matriz extracelular, contribui para a progressão da lesão e causa um estreitamento do lumen arterial (estenose). O núcleo central da lesão pode tornar-se necrótico, e o desenvolvimento de neovascularização na placa pode permitir perda dos componentes do sangue e hemorragia. (Figura 2) (WEBER; ZERNECKE; LIBBY, 2008).

Ao longo do tempo, a secreção de proteases que degradam a matriz e a produção de citocinas pelas células da placa pode causar um desbaste da capa fibrosa, o que impede o contato entre o sangue e o material pró-trombótico na placa. Finalmente, a lesão pode se desintegrar, causando a erosão ou ruptura da placa. A liberação de detritos resultantes da placa e o contato do fator tecidual com o sangue acionam uma cascata da coagulação e ocorre a formação de um trombo, este pode obstruir a artéria e resultar em infarto do miocárdio ou acidente vascular cerebral. O alargamento e a remodelação da artéria para acomodar a expansão da íntima pode levar à formação de um aneurisma. (Figura 2) (WEBER; ZERNECKE; LIBBY, 2008).

Figura 2 – Evolução da aterosclerose



Fonte: Weber; Zernecke; Libby (2008).

Legenda – Evolução da aterosclerose. A – adesão de leucócitos e plaquetas. B – formação das estrias gordurosas. C – Formação de um núcleo necrótico, capa fibrosa e neovascularização. D – Ruptura da placa com consequente ativação do sistema de coagulação e formação do trombo.

4.1.3 Inflamação e Aterosclerose

A aterosclerose é considerada uma doença inflamatória crônica que envolve a participação de componentes da imunidade inata e adaptativa que juntos medeiam à iniciação, progressão e complicações trombóticas da aterosclerose (WEBER; ZERNECKE; LIBBY, 2008).

Células endoteliais normalmente resistem à adesão de leucócitos. Estímulos pró-inflamatórios, incluindo uma dieta rica em gordura saturada, hipercolesterolemia, obesidade, hiperglicemia, resistência à insulina, hipertensão e tabagismo, desencadeiam a expressão endotelial de moléculas de adesão como a P-selectina e molécula de adesão celular vascular (VCAM-1), que medeiam à ligação de monócitos e leucócitos circulante (CYBULSKY et al., 2001). Fatores quimiotáticos, incluindo a proteína-1 quimiotática de monócitos (MCP-1), produzida por células da parede vascular em resposta a lipoproteínas modificadas, direcionam a migração e diapedese de monócitos aderentes. MCP-1 se liga ao seu receptor específico, o CCR2, sobre a superfície de monócitos circulantes para exercer os seus efeitos (GU et al., 1998).

Dentro da íntima, os monócitos se transformam em macrófagos sobre a influência do fator estimulador de colônia de macrófagos (M-CSF), molécula que é expressa de forma abundante na íntima inflamada (CLINTON et al., 1992). O M-CSF também favorece a uma maior expressão de “receptores scavenger” nos macrófagos. Esses receptores estão envolvidos na formação das células espumosas, através da captação desregulada de lipoproteínas modificadas pelos macrófagos. Os principais “receptores scavenger” incluem o CD-36, CD-68, CXCL16, SR-A e SR-BI (HANSSON; LIBBY, 2006)

Os macrófagos proliferam dentro da lesão e ampliam a resposta inflamatória através de secreção de numerosos fatores de crescimento e citocinas, tais como o fator de necrose tumoral α (TNF- α) e interleucina - 1β (IL-1β). Essas duas citocinas são importantes uma vez que aceleram a resposta inflamatória e são capazes de induzir a expressão de VCAM-1, MCP-1, M-CSF e Matriz Metaloproteinases (MMPs) (HANSSON; LIBBY, 2006).

No estágio avançado da placa, mediadores inflamatórios podem inibir a síntese de colágeno e influenciar a expressão de colagenases como as metaloproteinases, pelas células espumosas dentro da íntima. Como consequência, ocorre a diminuição do

conteúdo de colágeno da capa fibrosa, tornando a lesão frágil e propensa à ruptura (PACKARD; LIBBY, 2008).

A importância do envolvimento da inflamação na aterosclerose tem estimulado a descoberta e adoção de biomarcadores inflamatórios para o prognóstico de risco cardiovascular. Alguns desses biomarcadores incluem: Moléculas de adesão como VCAM-1; citocinas tais como TNF-α, interleucina-1 (IL-1), interleucina-18 (IL-18) e interleucina-6 (IL-6); proteases como a metaloproteinase de matriz-9 (MMP-9); produtos de plaquetas incluindo CD40 ligante solúvel (CD40L); adipocinas como adiponectina e reagentes de fase aguda tais como Proteína C Reativa (PCR), inibidor do ativador de plasminogênio tipo 1 (PAI-1) e fibrinogênio (PACKARD; LIBBY, 2008).

Dessa forma, a prevenção eficaz da aterosclerose inclui o tratamento dos mais importantes fatores de risco cardiovascular, como hipertensão, diabetes, hipercolesterolemia e obesidade. Entretanto, a ausência dos chamados “tradicionais” fatores de risco não completamente protege contra a doença e novos “fatores emergentes” têm sido identificados, incluindo marcadores de inflamação (CORRADO; NOVO, 2005).

4.1.4 Receptores Ativados por Proliferadores de Peroxissomos (PPARs)

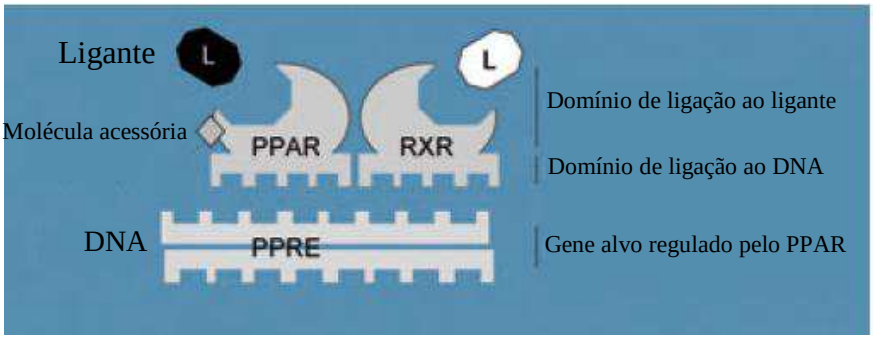
Peroxissomos são organelas subcelulares, que tem como função clássica remover o hidrogênio por meio do uso de oxigênio molecular através de uma série de enzimas oxidase e catalase. Eles também desempenham um papel crucial em diversos processos metabólicos celulares, incluindo o catabolismo de colesterol para bile e β-oxidação dos ácidos graxos. Em condições fisiológicas normais, o metabolismo nos peroxissomos ocorre de forma secundária ao sistema mitocondrial (VAMECQ; DREY, 1989), e envolve principalmente a oxidação de ácidos graxos de cadeia longa que não podem ser metabolizados de outra forma. Em hepatócitos de ratos a ativação dos peroxissomos por vários estímulos farmacológicos mostrou induzir o aumento dos peroxissomos em tamanho e número (LOCK et al., 1989) e esse aumento foi associado a uma maior expressão de genes relacionados a oxidação dos ácidos graxos. Em humanos, agentes farmacológicos também atuam aumentando a expressão de genes peroxissomais, mas não se observa um aumento no tamanho e número de peroxissomos tal como visto em roedores (KLIEWER et al., 2001).

Em 1990, a clonagem de um gene de roedor ligado a proliferação de peroxissomos foi descrita pela primeira vez (ISSEMAN; GREEN, 1990). Posteriormente, foi descoberto que a proliferação de peroxissomos atuava através da estimulação de um receptor de hormônio nuclear órfão que foi nomeado de receptor ativado por proliferadores de peroxissomos (PPAR). O receptor original é conhecido como PPARα. Subsequentemente, foram descobertos mais dois isotipos, o PPARδ/β e PPAR-γ (ROBINSON; GRIEVE, 2009), considerados importantes reguladores do metabolismo de lipídios e carboidratos.

Os PPARs possuem organização estrutural semelhante a outros membros da superfamília de receptores nucleares, como o receptor do ácido retinóico e vitamina D. De modo geral, contém uma região N-terminal exibindo um ligante independente, um domínio de trans-ativação fraco chamado AF-1, domínio de ligação ao DNA (LBD) e uma região C – terminal que é responsável pela dimerização com o receptor X retinóide (RXR) (FEIGE et al., 2006).

Em resposta a ligação de um determinado ligante o PPAR promove uma mudança conformacional que facilita a formação de um complexo heterodimérico com outro receptor nuclear ativado por ligantes, o receptor X retinoide (RXR) (WILLSON et al., 2000). Essa mudança conformacional induzida por ligante também facilita a ligação e liberação de pequenas moléculas acessórias que são importantes para realização do complexo transcricional (DIRENZO et al., 1997). Essas moléculas acessórias incluem proteínas co-repressoras, como a N-Cor , liberada após a ativação do PPAR e proteínas co-ativadoras, como a PPAR- γ co-ativador 1 (PGC-1), recrutada para a ativação, por exemplo, do receptor nuclear PPAR-γ (YANG et al., 2000). O complexo heterodimérico formado pelo PPAR/RXR pode então interagir via domínio de ligação ao DNA com elementos responsivos do PPAR (PPREs), situados em sítios regulatórios de cada gene e então induzir a expressão de genes alvos (Figura 3) (WILLSON et al., 2000).

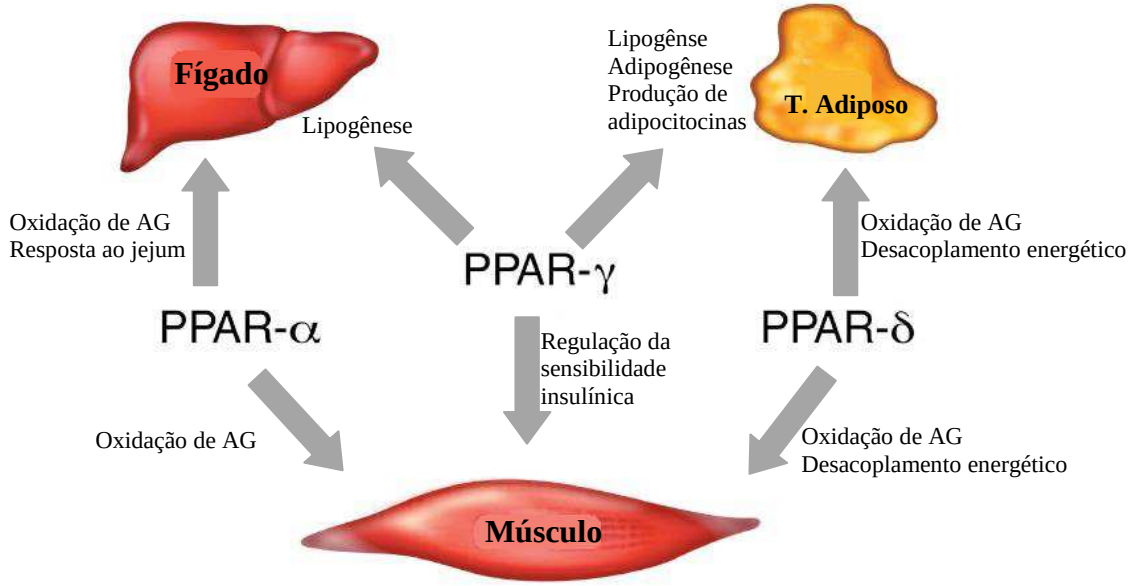
Figura 3 – Representação esquemática mostrando a ativação dos PPARs



Fonte: Willson et al., 2000.

Os PPARs podem não somente induzir, mas também reprimir a transcrição de genes e esta ação ocorre através da interação com outros fatores de transcrição como o fator nuclear Kapa B (NF-kB), Transdutores de sinal e ativadores de transcrição (STAT), Proteina ativadora-1 (AP-1) e fator nuclear de células T ativadas (NFAT) (CHINETTI et al., 2000). Apesar desses elementos múltiplos comuns aos PPARs, cada isotipo mantém papéis biológicos distintos que integrados, garantem a coordenação do metabolismo energético (Figura 4) (EVANS et al., 2004).

Figura 4 – Integracão metabólica dos PPARs



Fonte: Evans et al. (2004).
Legenda- As três isoformas de PPAR regulam a homeostase de lipídios e glicose através de suas atividades coordenadas no fígado, músculo esquelético e tecido adiposo.

O PPAR- α, primeiro isotipo do PPAR conhecido é predominantemente expresso no fígado e em menor intensidade no coração, músculo esquelético, intestino e rim onde exerce um papel importante em controlar a oxidação dos ácidos graxos (LEFEBVRE et al., 2006). A ativação do PPAR- α está relacionada com a transcrição de aproximadamente 80-100 genes, atuando sobre a oxidação dos ácidos graxos, metabolismo lipídico e inflamação (AHMED et al., 2007). O PPAR- α atua também sobre o metabolismo das lipoproteínas, estando envolvido na regulação da síntese de lipoproteínas ricas em triglicérides e HDL-C. Nesses aspectos suas ações principais são induzir o aumento da apolipoproteína A1, um elemento chave na formação de partículas de HDL-C. Atua também sobre a síntese lipoproteína lipase (LPL), uma enzima central

no metabolismo dos triglicérides e reprime a apolipoproteína C-III (apoC-III), que é inibidora endógena da LPL (LEE et al., 2003)

Como foi mencionado anteriormente o PPAR-α também participa de eventos relacionados ao processo inflamatório (LEFEBVRE et al., 2006). Em geral, inibindo a produção de citocinas pró-inflamatórias como a IL-6, bem como reprimindo a expressão de VCAM-1 (STAELS et al., 1998). Consistente com esses achados, camundongos deficientes do PPAR- α manifestam um aumento basal no seu estado inflamatório com resposta prolongada a estímulos pró-inflamatórios (DEVCHAND et al., 1996). Os fibratos são os ligantes sintéticos do PPAR- α e são utilizados para tratar dislipidemias em humanos (FORMAN et al., 1997). A administração dos fibratos diminui os níveis de triglicérides e estudos clínicos têm mostrado que esses fármacos são capazes de reduzir a incidência de eventos cardiovasculares e aterosclerose (BENSINGER; TONTONOZ, 2008).

O PPAR-δ/β é o menos estudado entre os isotipos de PPARs, embora possua expressão significativa em tecidos responsáveis por controlar o metabolismo lipídico, como os adipócitos, intestino delgado, coração, músculo esquelético e macrófagos (GROSS; STAELS, 2007). A retirada do gene que codifica a isoforma PPAR- δ/β se torna letal para o desenvolvimento inicial de quase todos os embriões, devido a um defeito na formação da placenta (NADRA et al., 2006) e por esse motivo, essa isoforma desempenha um papel importante não só na regulação do metabolismo, mas também no desenvolvimento do organismo (GRIMALDI, 2007). Existem fortes indícios que relacionam os ácidos graxos, triglicérides e prostaciclina como ativadores endógenos do PPAR- δ/β. Na linha de ligantes sintéticos, uma série de compostos têm sido desenvolvidos, mas ainda não existe nenhum agonista do PPAR- δ/β aprovado para uso clinico, embora a molécula GW501516 esteja na fase II de desenvolvimento para tratar dislipidemias (BISHOP-BAILEY; BYSTROM, 2009). Evidências experimentais sugerem que a ativação do PPAR- δ/β pode ter um valor terapêutico no tratamento da síndrome metabólica. A utilização de agonistas do PPAR- δ/β no tratamento de primatas e camundongos obesos aumentou os níveis de HDL e diminuiu os níveis de partículas de LDL pequenas e densas (LEIBOWITZ et al., 2000; OLIVER et al., 2001). De acordo com Oliver et al. (2001) o tratamento com GW1516 elevou o conteúdo plasmático de apolipoproteína AI, A-II e CIII, também foi observado uma maior expressão de ABCA-1 com consequente aumento do efluxo do colesterol nos macrófagos, fibroblastos e células intestinais. Outro estudo mostrou que a ativação do PPAR- δ/β reduziu lesões

ateroscleróticas em cerca de 50% em camundongos LDLR $^{-/-}$. Essa redução na área da lesão não foi acompanhada por mudanças nos níveis de HDL e LDL, mas sim pela diminuição na expressão de MCP-1 e ICAM-1 na aorta dos camundongos tratados (GRAHAM et al., 2005). Esses dados indicam que o PPAR- δ/β encontra-se envolvido em muitos mecanismos biológicos que regulam o metabolismo lipídico e algumas observações sugerem que a ativação farmacológica desse isotipo pode ter ações benéficas sobre aterosclerose.

O PPAR-γ, isotipo mais estudado dentre as isoformas de PPAR será abordado de forma mais especifica a seguir.

4.1.4.1 Receptor γ ativado por proliferadores de peroxissomos (PPAR- γ)

O gene PPARG foi clonado a partir de vertebrados, incluindo camundongos (ZHU et al., 1993) e humanos (GREENE et al., 1995). A análise filogenética dessa estrutura revelou que ela é bem conservada entre humanos e camundongos (99% de similaridade e 95% de identidade) (ZHU et al., 1995; FAJAS et al., 1997). Em humanos, a transcrição do gene PPARG dá origem a quatro RNAs mensageiros (mRNA) diferentes: PPAR-γ 1, PPAR-γ 2, PPAR-γ3 e PPAR-γ 4 que são transcritos a partir de quatro promotores distintos (FAJAS et al.,1997, FAJAS; FRUCHART; AUVERX, 1998, SUNDVOLD; LIEN, 2001). Em camundongos somente dois subtipos de mRNA são encontrados, o PPAR-γ1 e PPAR-γ 2 (ZHU et al., 1995).

Apesar do PPAR-γ apresentar quatro subtipos de mRNA em humanos, só existe duas isoformas de proteínas conhecidas (hPPAR-γ 1 e hPPAR-γ 2) que diferem em sua extremidade 5', como consequência de diferentes promotores e a *splicing* alternativos. O mRNA do PPAR-γ1, PPAR-γ3 e PPAR-γ 4 dão origem a proteína hPPAR-γ 1. O mRNA do PPAR-γ 2 codifica a proteína hPPAR-γ 2 que tem 30 aminoácidos adicionais na sua região N-terminal (ZIELENIAK; WÓJCIK; WOZNIAK, 2008). Em condições fisiológicas a proteína hPPAR-γ 2 é quase exclusivamente produzida pelo tecido adiposo branco e marrom, enquanto que a proteína hPPAR-γ 1 é detectada em outros tecidos incluindo células imunes, intestino, rim e fígado (VIDAL-PUIG et al., 1997).

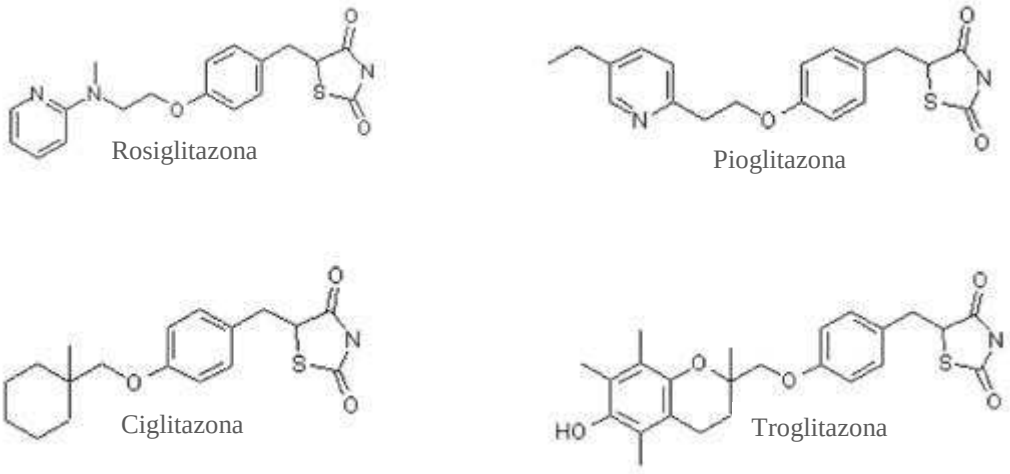
O PPAR-γ exerce papel crucial na diferenciação de adipócitos, induzindo a expressão de importantes marcadores envolvidos no metabolismo de lipídios como a

proteína de ligação a ácido graxo (aP2) (TONTONOZ et al., 1994a), fosfoenolpiruvato carboxiquinase (PEPKC) (TONTONOZ et al., 1995) e LPL (SCHOONJANS et al., 1996). Também controla a expressão da proteína transportadora de ácidos graxos 1 (FATP-1) e CD-36 (SFEIR; IBRAHIMI; AMRI, 1997), ambas envolvidas na captação de lipídios pelos adipócitos. A importância do PPAR-γ na adipogênese tem sido demonstrada por vários trabalhos. Por exemplo, PPAR-γ é induzido durante a diferenciação de pré-adipocitos *in vitro* e sua expressão ectópica em fibroblastos não adipogênicos estimula a adipogênese na presença de ligantes de PPAR-y (TONTONOZ; HU; SPIEGELMAN, 1994b).

Uma serie de ligantes naturais podem ativar o PPAR-γ, incluindo ácidos graxos insaturados, eicosanóides e componentes de LDLs oxidadas. Entretanto, a afinidade dos receptores para muitos desses ligantes é baixa e, em alguns casos, a relevância fisiológica do ligante ainda não foi determinada (BENSINGER; TONTONOZ, 2008).

O PPAR-γ é o alvo molecular de uma classe de ligantes sintéticos conhecidos como Thiazolidinadionas (TZDs). Essas moléculas possuem como característica principal a presença de um anel diona (figura 5). A Ciglitazona, o primeiro agonista do PPAR-γ apresentou bons resultados em melhorar a glicemia em modelo de resistência à insulina, entretanto não foi comercializada por causar hepatotoxicidade. Troglitazona, foi a primeira agonista de PPAR-γ aprovada pela *US food and drug Administration* (FDA*)* para o tratamento da diabetes tipo 2 em 1997, mas após encontrado casos de hepatotoxidade (WATKINS; WHITCOMB, 1998), foi retirada do mercado pela confirmação de grave hepatotoxicidade e morte (ISLEY, 2003). Rosiglitazona e pioglitazona, aprovadas para uso pela FDA em 1999, são consideradas a segunda geração de agonistas do PPAR-γ.

Figura 5 – Estrutura química das tiazolidinadionas



Fonte: (GUO et al., 2006)

Primariamente as TZDs exercem ações sensibilizadoras da ação da insulina diretamente sobre os adipócitos e indiretamente por alterar a liberação de adipocitocinas. De acordo com o efeito direto, as TZDs promovem a captação e estoque de ácidos graxos no tecido adiposo, aumentando a massa desse tecido e poupando outros tecidos sensíveis à insulina, como o músculo esquelético e fígado (YKI-JARVINEN, 2004). Em outras palavras, as TZDs promovem a distribuição de gordura das células do fígado e músculo esquelético para os adipócitos.

Os efeitos indiretos das TZDs sobre o tecido adiposo e melhora da sensibilização da insulina está relacionado com a adiponectina. Essa proteína é secretada exclusivamente pelo tecido adiposo e possui propriedades sensibilizadoras da insulina, promovendo uma maior captação de ácidos graxos, maior captação e utilização da glicose no tecido adiposo e muscular e a menor produção hepática de glicose, promovendo, assim, um melhor controle dos níveis séricos de glicose, de ácidos graxos livres e triglicérides (FEIGE et al., 2006). De acordo com Bajaj et al. (2004), a utilização da pioglitazona em pacientes com diabetes tipo 2 resultou em um aumento significativo nos níveis plasmáticos de adiponectina que foi associada com a melhora da resistência à insulina e diminuição no conteúdo de lipídio hepático.

Além da expressão no tecido adiposo, o PPAR-γ é expresso em células que compõem as lesões ateroscleróticas, como células endoteliais, musculares lisas e monócitos/macrófagos (COLLINS et al., 2001). Diante disso, evidências têm indicado que em adição à sua atividade antidiabética, as TZDs apresentam também atividade

antiaterosclerótica e o potencial antiaterogênico dos agonistas de PPAR-γ tem sido sugerido em estudos com modelos animais de aterosclerose (LI et al., 2000; COLLINS et al., 2001) e em ensaios clínicos utilizando pacientes com diabetes tipo 2 (MINAMIKAWA et al., 1998; LANGENFELD et al., 2005).

De acordo com Li et al. (2000), agonistas específicos de PPAR-γ foram capazes de inibir o desenvolvimento de lesões ateroscleróticas em camundongos LDLR-/- , apesar de aumentar a expressão do receptor scavenger CD36 na parede arterial. Os efeitos antiaterogênicos foram associados a uma melhora na sensibilização da insulina e diminuição da expressão tecidual do fator de necrose tumoral α (TNF-α) e MMP-9, indicando ações coordenadas locais e sistêmicas. Similarmente, em pacientes com diabetes *mellitus* tipo 2 (DM2), a pioglitazona conseguiu diminuir a espessura da carótida, a qual está fortemente associada com o desenvolvimento de acidente vascular cerebral e infarto (LANGENFELD et al., 2005).

Um número de genes-alvo do PPAR-γ tem sido documentado estar relacionado com as ações biológicas locais mediadas por esse receptor nuclear no sistema cardiovascular. Por exemplo, ligantes de PPAR-γ reduziram a produção de citocinas inflamatórias, como IL-1β, IL-6, sintase de óxido nítrico induzível (iNOS) e fator de necrose tumoral-α (TNF-α), por inibir a atividade de fatores de transcrição como AP-1, STAT e NF-κB em monócitos / macrófagos (JIANG; TING; SEED, 1998). Esses dados sugerem que a ativação do PPAR-γ pode ter efeitos benéficos na modulação da resposta inflamatória na aterosclerose.

A expressão de moléculas de adesão pelas células endoteliais leva à adesão de leucócitos, que é considerado um passo crítico no início da lesão aterosclerótica. Evidências comprovam o papel importante dos ligantes de PPAR-γ em inibir a expressão de VCAM-1 e ICAM-1 e também diminuir a produção de quimiocinas, como IL-8 e MCP-1, através de supressão do AP-1 e NF-kβ em células endoteliais (PASCERI et al., 2000). As células musculares lisas também desempenham um papel importante na progressão da aterosclerose, através da sua proliferação e migração dentro das lesões ateroscleróticas e as TZDs inibem esses dois eventos celulares (MARX et al., 1998).

Por outro lado, a ativação do PPAR-γ nos macrófagos desencadearia efeitos proaterogênicos, uma vez que ele regula positivamente a expressão do receptor “scavenger” CD-36, responsável pela captação de oxLDL e consequente formação de células espumosas (TONTONOZ et al., 1998). Entretanto, estudos comprovam que a

ativação de PPAR-γ não induz a formação das células espumosas, isto porque eles também são capazes de induzir a expressão do gene que codifica o transportador ABCA-1, responsável por controlar o efluxo de colesterol dos macrófagos mediado pela apoAI. Esses efeitos são provavelmente devido ao aumento na expressão do receptor X do fígado (subtipo α) (LXR α), um receptor nuclear que é ativado por oxisteróis e que tem a capacidade de induzir a transcrição do transportador ABCA-1 (CHINETTI et al., 2001). Dessa forma, a ativação farmacológica do PPAR-γ pode reprimir vários eventos patológicos importantes que são necessários para o desenvolvimento e progressão da aterosclerose.

Apesar de existirem diversos estudos mostrando os efeitos vasculares favoráveis dos agonistas de PPAR-γ, os efeitos cardiovasculares das TZDs tornam-se alvo de intensa discussão. Em uma recente metanálise, o tratamento com rosiglitazona foi associado a um aumento dos riscos de infarto do miocárdio e mortalidade por causas cardiovasculares (NISSEN; WOLSKI, 2007). De forma controversa, no estudo PROactive, o tratamento com pioglitazona foi associado a redução do risco combinado de infarto agudo do miocárdio, acidente vascular cerebral e mortalidade em 16%, entre pacientes diabéticos de alto risco cardiovascular (DORMANDY et al., 2005). É possível que os efeitos danosos apresentados pela rosiglitazona sejam específicos dessa molécula, não representando um efeito de classe. Entretanto, os mecanismos determinantes do aumento de risco cardiovascular associado às TZDs ainda não são conhecidos.

4.1.4.2 Novos Ligantes do PPAR-γ

O envolvimento do PPAR-γ em diversos processos biológicos, principalmente na modulação do metabolismo lipídico e resposta inflamatória torna esse receptor nuclear um importante alvo para o desenvolvimento de novas moléculas. As TZDs, utilizada clinicamente para tratar pacientes com DM2, são moléculas que possuem interação específica com o PPAR-γ e por essa razão são consideradas potentes agonistas desse receptor nuclear. Entretanto, a utilização das TZDs está relacionada com diversos efeitos colaterais como ganho de peso, retenção de fluido e hepatotoxicidade (YKI-JARVINEN, 2004), os quais podem ser responsáveis pelos riscos cardíacos associados com a utilização desses fármacos (NISSEN; WOLSKI, 2007). Dessa forma, diversas

pesquisas estão sendo realizadas para encontrar bons candidatos a fármacos, que possibilite o tratamento dessas patologias sem causar efeitos colaterais tão severos.

4.2 DOENÇA DO FÍGADO GORDUROSO NÃO-ALCOÓLICO (NAFLD)

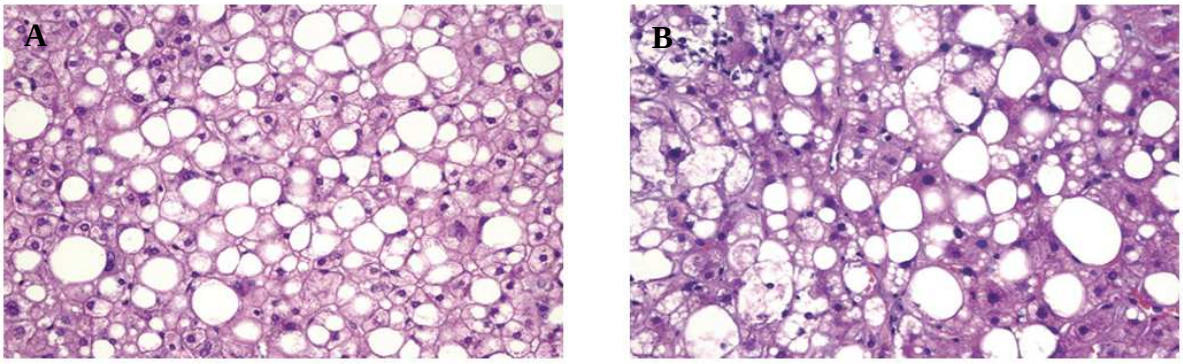
4.2.1 Patogênese da NAFLD

Hipernutrição é a causa mais comum de acúmulo de lipídios em excesso nos hepatócitos e estima-se que mais de seiscentos milhões de pessoas em todo o mundo com excesso de peso irá desenvolver doença hepática gordurosa (STEPHEN et al., 2012). Na prática clínica, o diagnóstico inicial da esteatose hepática é geralmente estabelecido com técnicas de imagens radiológicas, pela presença da acumulação de gordura hepática ⩾5 %, na ausência de outras causas reconhecidas de gordura no fígado, como por exemplo, álcool, vírus e medicamentos (BYRNE; TARGHER, 2015).

A NAFLD é uma condição clínico-patológica caracterizada pela significativa deposição de lipídios nos hepatócitos do fígado com alterações persistentes nas enzimas hepáticas como AST e ALT. (SONSUZ et al., 2000). Embora o quadro histológico se assemelhe ao de lesão hepática induzida pelo álcool, a NAFLD ocorre em pacientes que não abusam do álcool (EL-KADER; ASHMAWY, 2015). Essa patologia compreende um amplo espectro de danos no fígado, variando de esteatose macrovesicular simples para esteatohepatite não-alcoólica (Figura 6), fibrose avançada e cirrose (ÂNGULO, 2002). Estudos têm mostrado que cada vez mais, a NAFLD é reconhecida como uma causa do estágio final da doença hepática e está associada com aumento das taxas de

carcinoma hepatocelular (HCC), transplante de fígado e morte (CHARLTON et al., 1997; MCCULLOUGH, 2002; SASS et al., 2005)

Figura 6– (A) características histológicas de esteatose simples e da esteatohepatite não-alcoólica

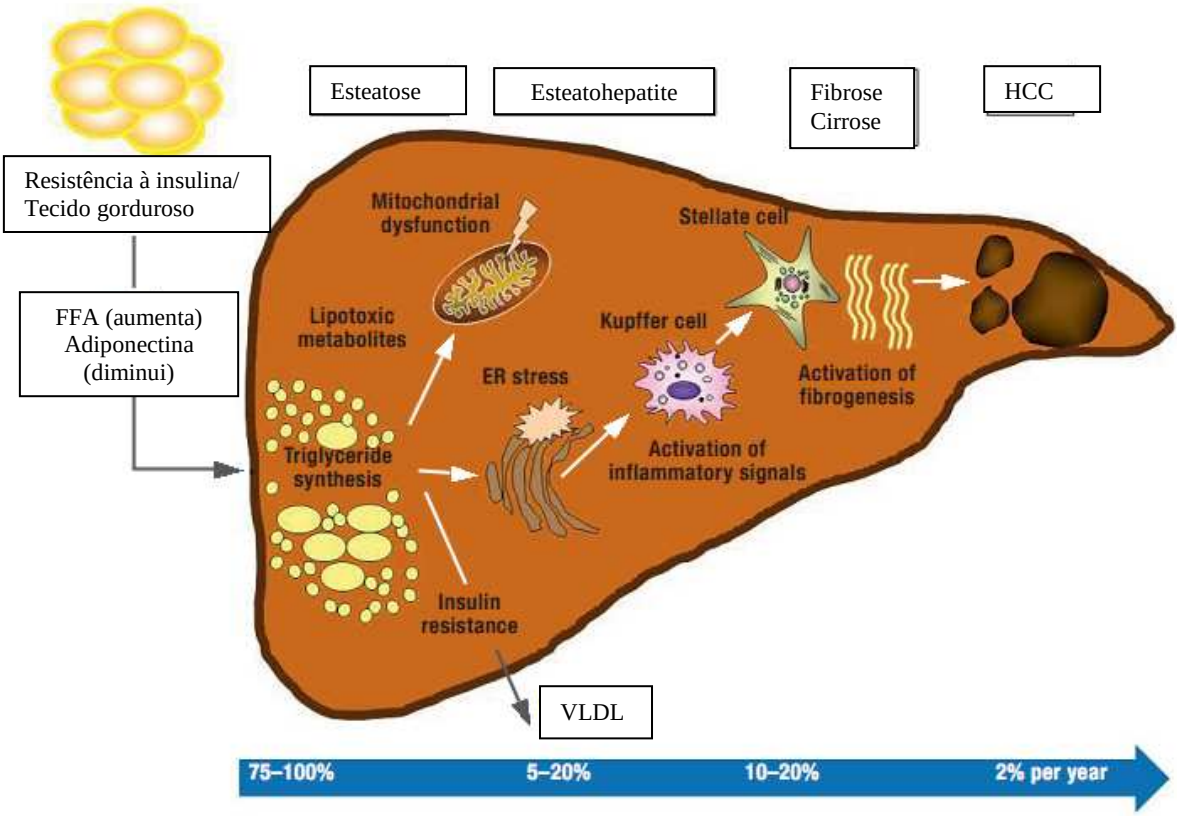


Fonte: (EL-KADER; ASHMAWY, 2015).

Mesmo não sendo ainda muito compreendida, é reconhecido que a resistência à insulina (RI) desempenha um papel importante na patogênese da NAFLD. Em indivíduos saudáveis, a insulina estimula a absorção hepática bem como a absorção de glicose periférica e suprime a produção de glicose hepática (EDENS et al., 2009). Por outro lado, no estado de jejum, o fígado torna-se o local principal de produção de glicose mediada por glicogenólise e gliconeogênese (DOWMAN et al., 2010; TARGHER et al., 2010). Em pacientes com resistencia à insulina (RI), a auto-regulação hepática é interrompida e, por conseguinte, tanto a gliconeogênese e a glicogenólise são aumentadas resultando no desenvolvimento de hiperglicemia (GAGGINI et al., 2013).

Como mostrado na figura 7, uma parte importante na patogênese da NAFLD é desempenhada pela resistência à insulina, o stress oxidativo, e a cascata inflamatória. De acordo com a teoria do " múltiplo hit ", a hiperinsulinemia no âmbito da resistência à insulina conduz, numa primeira fase, a um aumento da libertação de ácidos graxos livres a partir de adipócitos e que são então absorvidos pelo fígado, onde se acumulam e resultam na esteatose. Este passo inicial é seguido por uma série de interarações complexas entre os hepatócitos, células de Kupffer, adipócitos, mediadores inflamatórios, e radicais de oxigénio. O resultado é a esteatohepatite não-alcoólica. A inflamação crônica contribui para a progressão da lesão hepática e, a longo prazo, para o desenvolvimento de fibrose e cirrose (WEIß et al., 2014).

Figura 7 – Patogênese da doença do fígado gorduroso não-alcoólico (NAFLD)



(WEIß et al., 2014).

4.2.2 Metabolismo Lipídico e NAFLD

O fígado desempenha um papel importante no metabolismo dos lípidos, importando e produzindo ácidos graxos livres (FFAs), estocando e exportando lipídios. Sendo assim, alterações em qualquer um destes processos pode levar ao desenvolvimento de NAFLD (MUSSO et al., 2009). Ácidos graxos são envolvidos em eventos celulares importantes, como por exemplo, síntese de membranas celulares, estoque de energia e vias de sinalização celular. Entretanto, a elevação crônica dos níveis de FFAs pode desrregular diversas vias metabólicas e induzir a resistência à insulina (RI) em muitos órgãos.

No fígado, o acúmulo de gordura tem sido associado de forma bastante clara a resistência à insulina (PETTA et al., 2009; UTZSCHNEIDER; KAHN, 2006). A presença de resistência à insulina no tecido adiposo, aumenta a lipólise e a liberação de FFAs do tecido adiposo para o fígado. Em particular, a obesidade aumenta a produção

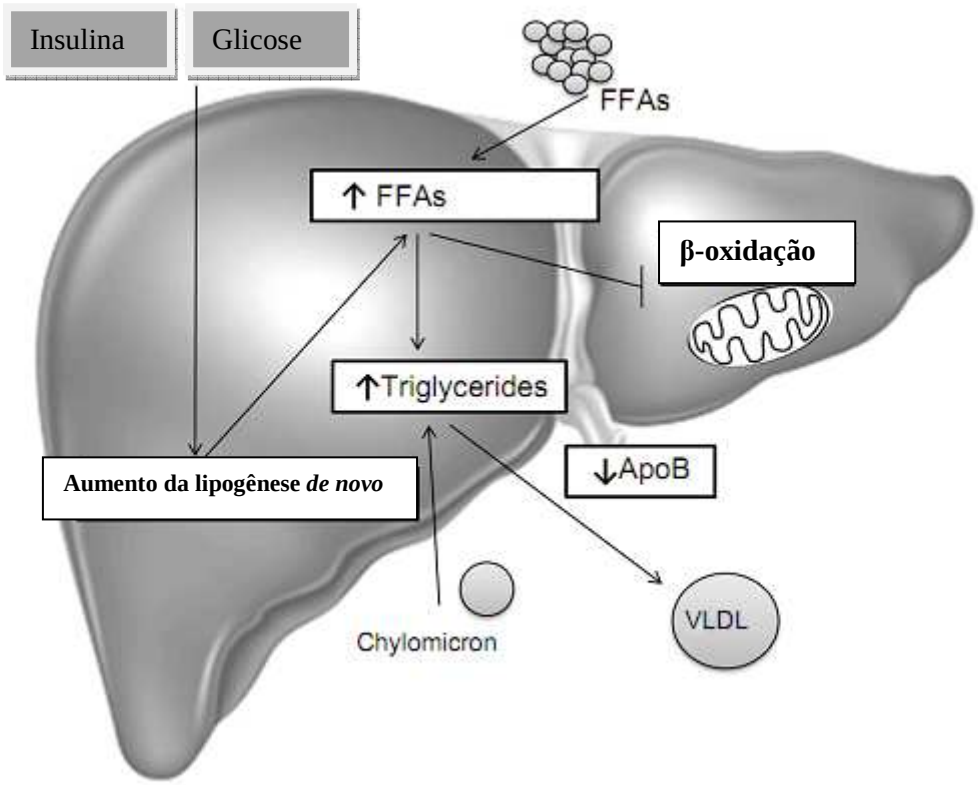
de TNFα nos adipócitos, facilitando a resistência a insulina nesse tecido e aumentando a taxa de lipólise (HOTAMISLIGIL, 2006). Assim, os FFAs circulantes são elevados em indivíduos obesos e são os lipídios mais presentes no processo de NAFLD (SAVAGE; SEMPLE, 2010).

Em condições fisiológicas, a síntese de trigliceridios (TG) é estimulada pelo excesso de FFAs. Os TGs podem então ser armazenados na forma de gotículas de lípidos dentro dos hepatócitos ou secretados para o sangue em forma de lipoproteína de muito baixa densidade (VLDLs) (POSTIC; GIRARD, 2008). Estudos em roedores têm mostrado que os mecanismos que levam ao acúmulo excessivo de TGs no fígado estão associados com o aumento da oferta de FFAs do tecido adiposo para o fígado, bem como um aumento na síntese de lipídios através da via lipogênica. Por outro lado, a via da β-oxidação e exportação de VLDLs são moderadamento afetadas (LEWIS et al., 2002).

Ao nível celular, defeitos nas vias de sinalização da insulina contribuem para o aumento de fluxo de FFAs no fígado, o que, por sua vez, ativa uma série de cascatas de sinalização e leva à fosforilação de vários substratos (TILG; MOSCHEN, 2010). FFAs e colesterol, especialmente quando acumulados na mitocondria, são considerados lipídios "agressivos" levando a lesão hepática mediada por TNFα e a formação de espécies reativas de oxigênio (ROS) (FELDSTEIN et al., 2004; MARI et al., 2006). Como já é conhecido, o excesso de FAs causa lipotoxicidade através da indução e produção de ROS, causando inflamação, apoptose, e assim, a progressão para a NASH e fibrogênese (CHEUNG; SANYAL, 2008; MALHI; GORES, 2008).

Em resumo, o acúmulo de TGs no citoplasma dos hepatócitos é considerado como um marcador chave da NAFLD, surgindo a partir de um desequilíbrio entre a aquisição de lipídios (captação de FAs e lipogênese de novo) e remoção (oxidação mitocondrial de FA e exportação de partículas de VLDL), bem como a participação de multiplos mecanismos fisiopatológicos da NASH (figura 8). Sendo assim, a fim de controlar a progressão da NAFLD, é importante compreender os mecanismos de regulação do acúmulo de lipídios hepático (BERLANGA et al., 2014)

Figura 8 – esteatose Hepática



Fonte: (BERLANGA et al., 2014)
Legenda - A marca da NAFLD é a acumulação de triglicéridos no citoplasma de hepatócitos, como resultado de um desequilíbrio entre a entrada e a saída de lípidios: 1- aumento da absorção de FFAs derivado da circulação devido a um aumento da lipólise no tecido adiposo e / ou a partir da dieta em forma de quilomicrons; 2- um aumento nos níveis de glicose e insulina em resposta à ingestão de carboidratos que promove a lipogênese novo; 3- uma diminuição na oxidação dos FAs mitocondriais; 4- uma diminuição na secreção de triglicerídeos hepáticos através do empacotamento de ApoB em partículas de VLDL.

4.2.3 Participação da inflamação na NAFLD

A inflamação é o conteúdo patogênico de várias doenças hepáticas agudas e crônicas, contribuindo para a progressão do dano hepático e evolução para estágios mais graves como a fibrose e carcinoma hepatocelular (HCC) (SZABO et al., 2007). Além dos hepatócitos, o fígado também contém um complexo repertório de células linfoides e não linfoides, que possuem papel chave na imunorregulação hepática e defesa (BOGDANOS et al., 2013).

As células que fazem parte da imunidade inata no fígado incluem células de Kupffer, monócitos, neutrófilos, células dendríticas (DCs), células natural killer (NK), e natural killer T (NKT). Essas células iniciam e mantêm a inflamação hepática através

da produção de citocinas (LIASKOU et al., 2012). A produção desrregulada de citocinas após lesão hepática pode resultar em morte excessiva dos hepatócitos, um evento chave encontrando em várias doenças hepáticas agudas e crônicas (SCHATTENBERG et al., 2011) Abaixo segue um resumo das principais células e citocinas importantes no processo inflamatório hepático.

4.2.3.1 Células de Kupffer

Céulas de Kupffer (residentes ou derivadas de monócitos) tem a capacidade de fagocitar e, depois da sua ativação, elas liberam citocinas como TNF-α, IL-1 e IL-6, bem como as quimiocinas CXCL1-3, CXCL-8, CCL2-4, onde inicia a resposta de fase aguda (ex: proteína C reativa, componentes do complemento) e inflamação. As citocinas pró-inflamatórias liberadas das células de kupffer ativadas podem promover a apoptose dos hepatócitos, esteatose e inflamação, mas também podem interagir diretamente com as células estreladas hepáticas (HSCs) via TGF-β, contribuindo para a diferenciação dessas células em miofibroblastos e produção de colágeno (TACKE, 2012). Além disso, estas citocinas pró-inflamatórias podem também ativar as células endoteliais sinusoidais hepáticas que regulam positivamente as moléculas de adesão como ICAM 1 e 2 e VCAM-1, estimulando o recrutamento de neutrófilos, monócitos, e células NKT para o fígado (SILVA, 2010).

4.2.3.2 Neutrófilos

Após sua ativação, os neutrófilos alteram o seu fenótipo e liberam sinais citotóxicos como ROS, oxidantes, defensinas e quimiocinas para atrair mais monocitos e neutrofilos para o local da inflamação. Esses monocitos podem se diferenciar em macrofagos teciduais e liberar TNF-α, IL-1β, fator estimulador de colónias de granulócitos (G-CSF) e fator estimulador de colónias granulócito-macrófago (GM-CSF), extendendo a vida útil dos neutrófilos, assim sustentando sua presença no sitio de inflamação (SOEHNLEIN; LINDBOM, 2010)

4.2.3.3 Natural Killer (NK) e Natural Killer derivadas de células T (NKT)

O fígado é também rico em células NK e NKT, que apresenta função de defesa do patógeno, recrutamento de células T e modulação de lesão no fígado . As células NK são reguladas por citocinas liberadas pelas células de Kupffer (por exemplo, IL - 12 e IL -18), bem como as células NKT são reguladas pela IL - 4. As células NK

modulam as respostas das células T no fígado, promovem alterações intracelulares em células endoteliais e hepatócitos, e pode até mesmo diretamente promover a morte ou lise dos hepatócitos . Além disso, demonstrou-se que as células NK pode matar células estreladas ativadas, melhorando assim a fibrose hepática (CLARIA, 2012).

4.2.3.4 Células dendriticas hepáticas

Células dendriticas hepáticas são as células apresentadoras de antígeno no fígado. Em condições inflamatórias, essas células produzem citocinas como IL-12 e TNF-α e expressam moléculas co-estimulatórias como CD-40, CD-80 e CD86 (DE CREUS et al., 2005)

4.2.4 Receptores Ativados por Proliferadores de Peroxissomos (PPARs) e NAFLD

Os três isotipos de PPARs (PPAR-α, PPAR-γ e PPAR-δ/β) possuem papéis importantes na patogênese da NAFLD como é exemplificado na figura 9.

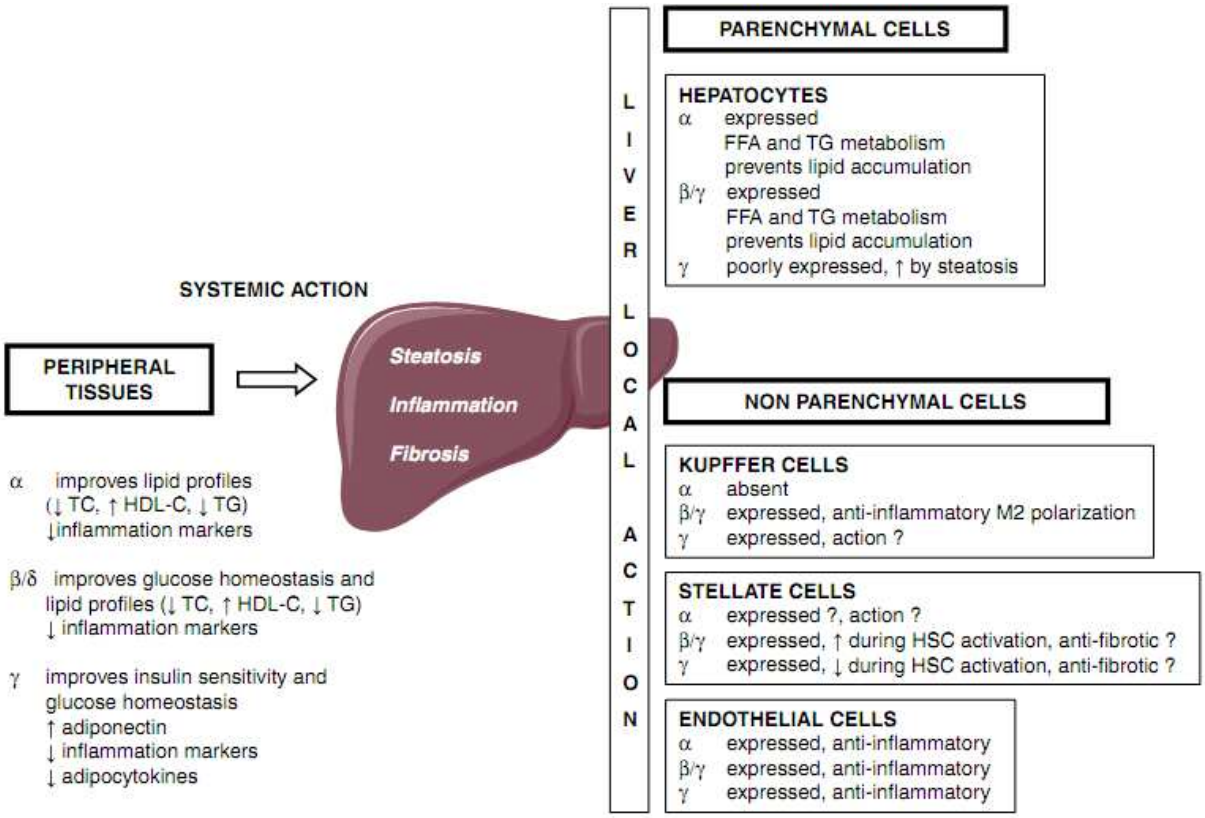
O PPAR-α é bastante expresso no fígado e especialmente nos hepatócitos, o PPAR-α é responsável pelo transporte de ácidos graxos e β-oxidação para diminuir o estoque de lipídios. O PPAR-α também modula a gliconeogênese e o metabolismo dos aminoácidos (LEFEBVRE et al., 2006; STAELS et al., 2008). Além da atuação sobre o metabolismo, essa isoforma é responsável por controlar a inflamação no fígado e em outros tecidos. O PPAR-α inibe diretamente genes inflamatórios ativados pelo NF-κB (STAELS et al., 1998) e reduz a expressão da IL-1 induzida pela proteína C reativa (PCR) em hepatócitos primários de humanos (KLEEMANN et al., 2003). Os efeitos anti-inflamatórios do PPAR-α no fígado podem também estar relacionados com a habilidade dessa isoforma de aumentar a expressão de genes como o IκB-α, um inibidor citoplasmático do NF-κB (KLEEMANN et al., 2003)

O PPAR-γ é altamente expresso em tecido adiposo, onde ele controla a diferenciação de adipócitos e sua ativação desempenha um papel importante no aumento a sensibilidade à insulina, bem como em promover a absorção de ácidos graxos nos adipócitos. O efeito líquido deste processo reduz a entrega de ácidos graxos para o fígado (LALLOYER; STAELS, 2010; FIEVET; STAELS, 2009). Existem vários trabalhos na literatura que apontam que a ativação farmacológica ou não do PPAR-γ exerce efeitos favoráveis sobre o fígado. A superexpressão do PPAR-γ mediada por adenovírus reduziu a esteatose hepática, inflamação e fibrose em um modelo de

esteatohepatite (NAN et al., 2011). Similarmente, a utilização da rosiglitazona, impediu o desenvolvimento de NASH em um modelo animal induzido com dieta deficiente de colina-metionina (NAN et al., 2011). Entretanto, o aumento da expressão de PPAR-γ é uma característica do fígado esteatótico e vários estudos atribuem um papel causal do PPAR-γ no desenvolvimento da esteatose por mecanismos que envolvem a ativação de genes das vias da lipogênese e da lipogênese *de novo* (MATSUSUE et al., 2003; GAVRILOVA et al., 2003) Corroborando com esses dados, a deleção do PPAR-γ nos hepatócitos e macrófagos protegem os camundongos contra a esteatose hepática induzida por dieta (MORAN-SALVADOR et al., 2011), sugerindo um papel pro–esteatótico do PPAR-γ tanto nas células do parênquima bem como nas células não-parenquimatosas. Adicionalmente, o tratamento de camundongos ob/ob (um modelo animal que reproduz muitos dos distúrbios metabólicos presentes em pacientes com NAFLD, mas não progressão espontânea para NASH) com rosiglitazona não reverteu as alterações histológicas da NAFLD, mas aumentou o estresse oxidativo e a esteatose nesses camundongos (GARCIA-RUIZ et al., 2007).

Em contraste com os papéis farmacológicos de PPAR-α e PPARγ que são relativamente bem definidos, o papel do PPAR-δ/β como um alvo farmacológico permanece menos claro. No entanto, torna-se cada vez mais evidente que PPAR-δ/β é um regulador metabólico importante, com ações no músculo esquelético, gordura, intestino, fígado e coração. Quando ativado, ele aumenta o transporte de ácidos graxos e a oxidação, melhora homeostase de glicose através da melhoria da sensibilidade à insulina e inibição da produção de glicose, atenua a resposta inflamatória dos macrófagos e aumenta as concentrações plasmáticas de HDL (GROSS et al., 2005). Especificamente no fígado, o tratamento farmacológico com GW501516, um agonista do PPAR-δ/β, melhorou a esteatose hepática e a inflamação em um modelo de camundongo induzido por dieta (NAGASAWA et al., 2006). Os efeitos benéficos do PPAR-δ/β sobre a doença hepática gordurosa pode ser explicado pelo papel dessa isoforma sobre os diferentes tipos celulares do fígado. O PPAR-δ/β é expresso em células do parênquima hepático que são responsáveis pela diminuição do conteúdo de lipídios, evento esse que acontece pelo aumento da β-oxidação dos ácidos graxos hepáticos (NAGASAWA et al., 2006).

Figura 9 – Localização celular específica das três isoformas de PPAR e seus potencias efeitos sobre a NAFLD



Fonte: (TAILLEUX et al., 2012)

4.2.5 NAFLD e Aterosclerose

A NAFLD está relacionada com obesidade, resistência à insulina, hipertensão e dislipidemia, sendo considerada componente da síndrome metabólica. O aumento da mortalidade de pacientes com NAFLD está associado mais com doenças cardiovasculares do que com as próprias complicações referentes a doença hepática gordurosa (POLIMENI et al., 2015).

A NAFLD e aterosclerose são patologias caracterizadas pelo acúmulo de lipídios e inflamação crônica (MA et al., 2008; WOOLLARD; GEISSMANN, 2010) e numerosos estudos têm mostrado a NAFLD como um importante fator de risco cardiovascular (TARGHER et al., 2008; BHATIA et al., 2012). Kleemann et al, demonstraram que após 10 semanas de uma dieta rica em colesterol (1% de colesterol), camundongos fêmeas APOE*3, apresentaram lesões ateroscleróticas correlacionadas

com níveis plasmáticos da proteína A amiloide, sugerindo que a resposta inflamatória hepática está envolvida na formação de lesões ateroscleróticas inicias (KLEEMANN et al., 2007). Devido ao papel proeminente do fígado na captação de lipídios, a resposta inflamatória hepática em modelos hiperlipidêmicos precede a formação da placa. Evidências sugerem que exista um compartilhamento metabólico e fatores inflamatórios associados com a ativação de macrófagos na aterosclerose e NAFLD (MAINA det al., 2012; LINGREL et al., 2012). Todas essas informações apoiam a proposta de que a NAFLD e aterosclerose são, na verdade, dois aspectos de uma mesma patologia.

A progressão da aterosclerose e NAFLD são acompanhadas pelo desenvolvimento de inflamação, onde diversos fatores são expressos, incluindo várias quimiocinas (proteína quimiotática de monócitos 1; MCP-1), citocinas (factor de necrose tumoral-α; TNF-α), interleucina-6 (IL-6) e proteína C-reactiva (CRP) (HANLEY et al., 2005; SERINO et al., 2007; WUNDERLICH et al., 2010; BAECK et al., 2012). Na aterosclerose, os macrófagos são capazes de acumular grandes quantidades de lípidos, se transformar em células espumosas e conduzir a aterogênese (GUI et al., 2012; TRUMAN et al., 2012). O mesmo processo de acumulação de macrófagos carregados com lípidos foi observado na NAFLD, onde camundongos hiperlipidêmicos apresentaram células de Kupffer (KCs) espumosas (repletas de lipídios) após a administração de uma dieta rica em gordura (WOUTERS et al., 2008).

Sendo assim, fármacos de possam atuar regulando o metabolismo lipidico e controlando a inflamação, se tormam terapais importantes para melhorar a NAFLD e reduzir o desenvolvimento da aterosclerose.

5. REFERÊNCIAS

AHMED, W.; ZIOUZENKOVA, O.; BROWN, J. et al. PPARs and their metabolic modulation: new mechanisms for transcriptional regulation? **Journal of Internal Medicine.** v. 269, p.184-198, 2007.

ANGULO P. Nonalcoholic fatty liver disease. **N Engl J Med.** v. 346, p. 1221-1231, 2002.

BAECK C; WEHR A; KARLMARK KR, et al: Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. **Gut**. v. 61, p. 416-426, 2012.

BAHCECIOGLU, I. H.; YALNIZ, M.; ATASEVEN, H. et al. Levels of serum hyaluronic acid, TNF- α and IL-8 in patients with non-alcoholic steatohepatitis. **Hepatogastroenterol**. v. 52, p. 1549–1553, 2005.

BAJAJ, M.; SURAAMORNKUL, S.; PIPER, P. et al. Decrease plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients. **J Clin Endocrinol Metab**. v. 89, p. 200-6, 2004.

BAOU, K.; VLACHOPOULOS, C.; MANESIS, E. et al. Non-Alcoholic Fatty Liver and Cardiovascular Disease: An Emerging Relationship. **Hellenic J Cardiol.** v. 48, p. 37-41, 2007.

BARTER, P. J.; NICHOLLS, S.; RYE, K. et al. Antiinflammatory properties of HDL. **Circ. Res**. v. 95, p. 764-772, 2004.

BENSINGER, S. J.; TONTONOZ, P. Integration of metabolism and inflammation by lipid-activated nuclear receptors. **Nature**. v. 454, p. 470-477, 2008.

BERLANGA, A; GUIU-JURADO, E; PORRAS, JA; AUGUET, T. Molecular pathways in non-alcoholic fatty liver disease. **Clinical and Experimental Gastroenterology.** v. 7, p. 221–239, 2014.

BHATIA LS; CURZEN NP; CALDER PC; BYRNE CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? **Eur Heart J**. v. 33, p. 1190-1200, 2012.

BISHOP-BAILEY, D.; BYSTROM, J. Emerging roles of peroxisome proliferator-activated receptor-β/δ in inflammation. **Pharmacology & Therapeutics**. v. 124, p. 141–150, 2009.

BOGDANOS DP, GAO B, GERSHWIN ME. Liver immunology. **Compr Physiol**. v. 3, p. 567–98, 2013.

BYRNE, CD; GIOVANNI TARGHER. NAFLD: A multisystem disease. **Journal of Hepatology** vol. 62 S47–S64, 2015.

CHAMPE, P. C.; HARVERY, R. A.; FERRIER, D. R. Bioquímica Ilustrada. 3ª ed. Porto Alegre: Ed. **Artmed**, 2006.

CHARLTON MR, KONDO M, ROBERTS SK, STEERS JL, KROM RA, WIESNER RH. Liver transplantation for cryptogenic cirrhosis. **Liver Transpl Surg**. v. 3, p. 359-364, 1997.

CHEUNG O, SANYAL AJ. Abnormalities of lipid metabolism in nonalcoholic fatty liver disease**. Semin Liver Dis.** v. 28(4), p. 351–359, 2008.

CHINETTI, G.; FRUCHART, J. C.; STAELS, B. peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. **Inflamm Res.** v. 49, p. 497-505, 2000.

CHINETTI, G.; LESTAVEL, S.; BOCHER, V. et al. PPARalpha and PPARgamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA-1 pathway. **Nature Medicine**. v. 7 (1) p. 53-58, 2001.

CHUGH, P.K; SHARMA, S. Recent advances in the pathophysiology and pharmacological treatment of obesity. **J. Clin. Pharm. Ther**. v. 37, p. 525–535, 2012.

CLARIA, J. Natural killer cell recognition and killing of activated hepatic stellate cells. **Gut**. v. 61, p. 792–793, 2012.

CLINTON, S. K.; UNDERWOOD, R.; HAYES, L. et al. Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. **Am J Pathol**. v. 140, p. 301-316, 1992.

COLLINS, A. R.; MEEHAN, W. P.; KINTSCHER, U. et al. Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice. **Arterioscler Throm Vasc Biol.** v. 21, p. 365-371, 2001.

CORRADO, E.; NOVO, S. Role of inflammation and infection in vascular disease. **Acta Chir Belg**. v. 105, p. 567-579, 2005

CYBULSKY, M. I.; IIYAMA, K.; LI, H. et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis**. J Clin Invest**. v. 107, p. 1255-1262, 2001.

DAVIGNON, J.; GANZ, P. Role of endothelial dysfunction in atherosclerosis. **Circulation.** v. 109. suppl III, p. III-27-III-32, 2004.
DAY, C. Thiazolidinediones: a new class of antidiabetic drugs. **Diabet Med.** v. 16 (3), p. 179-92, 1999.

DE CREUS A, ABE M, LAU AH, HACKSTEIN H, RAIMONDI G, THOMSON AW. Low TLR4 expression by liver dendritic cells correlateswith reduced capacity to activate allogeneicTcells in response to endotoxin. **J Immunol.** v. 174, p. 2037–45, 2005.

DEVCHAND, P.; KELLER, H., PETERS, J. et al. The PPARalpha-leukotriene B4 pathway to inflammation control. **Nature**. v. 384, p. 39-43, 1996.

DIRENZO, J.; SODERSTROM, M.; KUROKAWA, R. et al. Peroxisome proliferator-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptor heterodimers with ligands, coactivators, and corepressors. **Molecular cell biology.** v. 17, p. 2166-2176, 1997.

DORMANDY, J. A.; CHARBONNEL, B.; ECKLAND, D. J. et al. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. **Lancet.** v. 366, p. 1279-1289, 2005.

DOWMAN, JK; J.W. TOMLINSON, P.N. Newsome Pathogenesis of non-alcoholic fatty liver disease. **Qjm**. v.103 (2), p. 71–83, 2010.

EDENS, MA; F. KUIPERS, R.P. StolkNon-alcoholic fatty liver disease is associated with cardiovascular disease risk markers. **Obes. Rev**. v. 10 (4), p. 412–419, 2009.

EL-KADER SHEHAB M ABD, EMAN M SALAH EL-DEN ASHMAWY. Non-alcoholic fatty liver disease: The diagnosis and management. **World J Hepatol.** v. 7(6), p. 846-858, 2015.

EVANS, R. M.; BARISH, G. D.; WANG, Y. X. PPARs and the complex journey to obesity. **Nature Medicine**. v. 10 (4), p. 3551-361, 2004.

FAJAS L., AUBOEUF D., RASPÉ E., et al. The organization, promoter analysis,and expression of the human PPAR-gamma gene. **J Biol Chem**. v. 272, p. 18779–18789, 1997.

FAJAS L.; FRUCHART J. C.; AUVERX J. PPAR-gamma3 mRNA: a distinct PPAR-gamma mRNA subtypetranscribed from an independent promoter. **FEBS Lett.** v. 438, p. 55–60, 1998.

FEIGE, J. N.; GELMAN, L.; MICHALIN, L. et al. From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. **Prog Lipid Res**. v. 45 (2), p. 120-159, 2006.

FELDSTEIN AE, WERNEBURG NW, CANBAY A, et al. Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression via a lysosomal pathway. **Hepatology**. v. 40(1), p. 185–194, 2004.

FIEVET, C; STAELS, B. Efficacy of peroxisome proliferator-activated receptor agonists in diabetes and coronary artery disease, **Curr. Atheroscler. Rep**. v.11, p. 281–288, 2009.

FORMAN, B. M.; CHEN, J.; EVANS, R. M. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. **Proc. Natl Acad. Sci**. v. 94, p. 4312–4317, 1997.

FRACANZANI, A.L.; BURDICK, L.; RASSELLI, S. et al.. Risk of early atherosclerosis evaluated by carotid artery intima-media thickness in patients with non-alcoholic fatty liver disease: a case-control study. **Hepatology.** v. 42, p. 610–611A, 2005.

GAGGINI, M et al. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. **Nutrients.** v. 5 (5), p. 1544–1560, 2013.

GARCIA-RUIZ, I; RODRIGUEZ-JUAN, C; DIAZ-SANJUAN,T; MARTINEZ, M.A et al. Effects of rosiglitazone on the liver histology and mito- chondrial function in ob/ob mice. **Hepatology**. v. 46, p. 414–423, 2007.

GAVRILOVA, O.; HALUZIK, M.; MATSUSUE, K. et al. Liver peroxisome proliferator- activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. **J. Biol. Chem**. v. 278, p.34268–34276, 2003.

GAVRILOVA, O; HALUZIK, M; MATSUSUE, K et al. Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass, **J. Biol. Chem.** v. 278, p. 34268–34276, 2003.

GLASS, C. K; WITZTUM, J. L. Atherosclerosis: the road ahead. **Cell**. v. 04, p. 503–16, 2001.

GRAHAM, T. L.; MOOKHERJEE, C.; SUCKLING, K. E. et al. The PPARdelta agonist GW0742X reduces atherosclerosis in LDLR(−/−) mice. **Atherosclerosis.** v. 181. p. 29–37, 2005.

GREENE M. E.; BLUMBERG B.; MCBRIDE O. W. et al. Isolation of the human peroxisome proliferator activated receptor gamma cDNA: expression in hematopoietic cells and chromosomal mapping. **Gene Expr.** v. 4, p. 281–299, 1995.

GRIMALDI, P. A. Regulatory functions of PPARβ in metabolism: implications for the treatment of metabolic syndrome. **Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids** v. 1771, p. 983-990, 2007

GROSS, B.; STAELS, B. PPAR agonists: multimodal drugs for the treatment of type-2 diabetes. **Best Practice & Research Clinical Endocrinology & Metabolism** v. 21 (4), p. 687–710, 2007.

GROSS, BS; FRUCHART, JC; STAELS, B. Peroxisome proliferator-activated receptor [beta]/[delta]: a novel target for the reduction of atherosclerosis, **Drug Discov. Today: Ther. Strat.** v. 2, p. 237–243, 2005.

GU, L.; OKADA, Y.; CLINTON, S. K. et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. **Mol Cell.** v. 2, p. 275-281, 1998.

GUI T; SHIMOKADO A; SUN Y, et al: Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. **Mediators Inflamm**. v. 2012, p. 693083, 2012.

HANLEY AJ, WILLIAMS K, FESTA A, et al: Liver markers and development of the metabolic syndrome: the insulin resistance atherosclerosis study. **Diabetes**. v. 54, p. 3140-3147, 2005.

HANSSON, G. K.; LIBBY, P. The immune response in atherosclerosis: a Double-edged sword. **Nat Rev Immunol**. v. 6, p. 508-519, 2006.

HEGELE R.A. Plasma lipoproteins: genetic influences and clinical implications. **Nature reviews/Genetics**, v.10, p. 109-119, 2009.

HEIKKINEN, S.; AUWERX, J.; ARGMANN, C. A. PPAR-γ in human and mouse physiology. **Biochimica et Biophysyca Acta**. v. 1771, p. 999-1013, 2007.

HOTAMISLIGIL GS. Inflammation and metabolic disorders. **Nature**. v. 444(7121), p. 860–867, 2006.

ISHIBASHI, S.; BROWN, M. S.; GOLDSTEIN, J.L. et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. **The Journal of Clinical Investigation.** v. 92, p. 883-893, 1993.

ISHIGAKI, Y; OKA, Y; KATAGIRI, H. Circulating oxidized LDL: a biomarker and a pathogenic factor. **Curr Opin Lipidol.** v. 20 (5), p. 363-9, 2009.

ISLEY, W.L., Hepatotoxicity of thiazolidinediones, **Expert. Opin. Drug Saf**. v. 2, p. 581–586, 2003.

ISSEMAN, I.; GREEN, S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. **Nature**. v. 347, p. 645-9, 1990.

JAWIEN, J.; NASTALEK, P.; KORBUT R. Mouse models of experimental atherosclerosis. **Journal of Physiology and Pharmacology,** v.55, p. 503-517, 2004.

JIANG C, TING AT, SEED B. PPAR-γ agonists inhibit production of monocytes inflammatory cytokines. **Nature**. v. 391, p. 82 – 86, 1998.

KLEEMANN R, GERVOIS PP, VERSCHUREN L, STAELS B, PRINCEN HM, KOOISTRA T. Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFkappa B-C/EBP-beta complex formation. **Blood**. v. 101, p. 545–551, 2003

KLEEMANN R; VERSCHUREN L; VAN ERK MJ, et al: Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. **Genome Biol.** v. 8, p. R200, 2007.

KLEEMANN, R; GERVOIS, PP; VERSCHUREN, L; STAELS, B et al. Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing

nuclear p50-NFkappa B-C/EBP-beta complex formation. **Blood.** v. 101, p. 545–551, 2003.

KLIEWER, S. A., XU, H. E., LAMBERT, M. H., & WILLSON, T. M. Peroxisome proliferatoractivated receptors: from genes to physiology. **Recent Prog Horm Res. V.** 56, p. 239−263, 2001.

KOPELMAN, P.G. Obesity as a medical problem. **Nature**. v. 6;404(6778), p.635-43. 2000.

LALLOYER, F; STAELS, B. Fibrates, glitazones, and peroxisome proliferator-activated receptors. **Arterioscler. Thromb. Vasc. Biol.** v.30, p. 894–899, 2010.

LANDMESSER, U.; HORNIG, B.; DREXLER, H. Endothelial Function: A critical determinant in atherosclerosis? **Circulation**. v. 109[suppl II], p. II-27-II-33, 2004.

LANGENFELD, M. R.; FORST, T.; HOHBERG, C. et al. Pioglitazone decreases carotid íntima-media thickness independently of glycemic control in patients with type 2 diabetes mellitus: results from a controlled randomized study. Circulation. v. 111(19), p. 2525-31, 2005.

LEE, C. H.; OLSON, P.; EVANS, R. M. Lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology. v.144, p. 2201-7, 2003.

LEFEBVRE, P; CHINETTI, G; FRUCHART, JC; STAELS, B. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis, **J. Clin. Invest.** v. 116, p. 571–580, 2006.

LEIBOWITZ, M. D.; FIEVET, C.; HENNUYER, N. et al. Activation of PPARdelta alters lipid metabolism in db/db mice. **FEBS Lett.** v. 473, p. 333–336, 2000.

LEWIS GF, CARPENTIER A, ADELI K, GIACCA A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. **Endocr Rev.** v. 23(2), p. 201–229, 2002.

LI, A. C. ; BROWN, K. K.; SILVESTRE, M. J. et al. Peroxisome proliferator-activated receptor ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. **The Journal of Clinical Investigation**. v. 106, p. 523-531, 2000.

LIASKOU, E. ET AL. Innate immune cells in liver inflammation. **Mediators Inflamm.** 2012, p. 949157, 2012.

LIBBY, P. Inflammation in atherosclerosis. **Nature**. v. 420, p. 868-874, 2002.

LICIA POLIMENI; MARIA DEL BEM; FRANCESCO BARATTA; LUDOVICA PERRI et al. Oxidative stress: New insights on the association of non-alcoholic fatty liver disease and atherosclerosis. **World J Hepatol**. v. 7(10), p. 1325-1336, 2015

LINGREL JB, PILCHER-ROBERTS R, BASFORD JE, et al: Myeloid-specific Krüppel-like factor 2 inactivation increases macrophage and neutrophil adhesion and promotes atherosclerosis. **Circ Res.** V. 110, p. 1294-1302, 2012.

LOCK, E. A.; MITCHELL, A. M.; ELCOMBE, C. R. Biochemical mechanisms of induction of hepatic peroxisome proliferation. **Annu Rev Pharmacol Toxicol. V.** 29, p.145−163, 1989.

LUSCHER, T. F, VANHOUTTE, P. M. The Endothelium: Modulator of Cardiovascular Function. Boca Raton. FL. CRC Press. 1990.

MA KL; RUAN XZ; POWIS SH et al: Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. **Hepatology**. v. 48, p. 770-781, 2008.

MAINA V; SUTTI S; LOCATELLI I, et al: Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. **Clin Sci (Lond).** v. 122, p 545-553, 2012.

MALHI H, GORES GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. **Semin Liver Dis**. v. 28(4), p. 360–369, 2008.

MARCHESINI, G.; MARZOCCHI, R.; AGOSTINI, F. et al. Non-alcoholic fatty liver disease and the metabolic syndrome. **Curr Opin Lipidol**. v. 16, p. 421–427, 2005.

MARÍ M, CABALLERO F, COLELL A, et al. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. **Cell Metab.** v. 4(3), p. 185–198, 2006.

MARX N, SCHONBECK U, LAZAR MA. et al. Peroxisome proliferator-activated receptor gamma activators inhibit gene expression and migration in human vascular smooth muscle cells. **Circ Res**. v. 83, p. 1097– 1103, 1998.

MATSUSUE, K.; HALUZIK, M.; LAMBERT, G. et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. **J. Clin. Invest**.v.111, p. 737–747, 2003.

MCCULLOUGH AJ. Update on nonalcoholic fatty liver disease. **J Clin Gastroenterol.** v.34, p. 255-262, 2002.

MCKENNEY, R.L; SHORT, D.K. Tipping the balance: the pathophysiology of obesity and type 2 diabetes mellitus. **Surg. Clin. North Am**. v.91, p. 1139–1148, 2011.

MINAMIKAWA, J.; TANAKA, S.; YAMAUCHI, M. et al. Potent inhibitory effects of troglitazone on carotid arterial wall thickness in type 2 diabetes. **J Clin Endocrinol Metab.** v. 83, p. 1818-20, 1998.

MINEO, C.; DEGUCHI, H.; GRIFFIN, J. H. et al. Endothelial and antithrombotic actions of HDL. **Circ. Res.** v. 98, p. 1352-1364, 2006.

MORÁN-SALVADOR, E; LÓPEZ-PARRA, M; GARCÍA-ALONSO, V et al. Role for PPARγ in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage- specific conditional knockouts, **FASEB J**. v. 25, p. 2538–2550, 2011.

MUSSO G, GAMBINO R, CASSADER M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). **Prog Lipid Res**. v. 48(1), p. 1–26, 2009.

MUSSO, G.; GAMBINO, R.; DURAZZO, M. et al. Adipokines in NASH. postprandial lipid metabolism as a link between adiponectin and liver disease. **Hepatology**. v. 42, p. 1175–1183, 2005.

NADRA, K.; ANGHEL, S.I.; JOYE, E. et al. Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta. **Mol. Cell. Biol.** v. 26, p. 3266–3281, 2006.

NAN, Y.M; HAN, F; KONG, L.B; ZHAO, S.X et al. Adenovirus-mediated peroxisome proliferator activated receptor gamma overexpression prevents nutritional fibrotic steatohepatitis in mice. **Scand. J. Gastroenterol**. v. 46, p. 358–369, 2011.

NISSEN, S. E.; WOLSKI, K. Effect of Rosiglitazone on the Risk of Myocardial Infarction and Death from Cardiovascular Causes. **N. Engl. J. Med**. v. 356 (24), p. 2457-2471, 2007.

OLIVER, W. R.; SHENK, J. L.; SNAIT, M. R. et al. A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. **Proc. Natl. Acad. Sci. U. S. A.** v. 98, p. 5306–5311, 2001.

ONAT, A. Metabolic syndrome: nature, therapeutic solutions and options. **Expert Opin.**
Pharmacother. v.12, p.1887–1900. 2011.

PACKARD, R. R. S.; LIBBY, P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. **Clinical Chemistry.** v. 54 (1), p. 24-38, 2008.

PASCERI, V.; WU, H. D.; WILLERSON, J. T. Et al. Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-γ activators. **Circulation.** v. 101, p. 235 – 238, 2000.

PETTA S, MURATORE C, CRAXÌ A. Non-alcoholic fatty liver disease pathogenesis: the present and the future. **Dig Liver Dis**. v. 41(9), p. 615–625, 2009.

POSTIC C, GIRARD J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. **J Clin Invest**. v. 118(3), p. 829–838, 2008.

PROMRAT, K.; LUTCHMAN, G.; UWAIFO, G. I. et al. A pilot study of pioglitazone treatment for non-alcoholic steatohepatitis. **Hepatology.** v. 39, p. 188-96, 2004.

RADER, D. J.; DAUGHERTY, A. Translating molecular discoveries into new therapies for atherosclerosis. **Nature**. v. 451. p. 904-913, 2008.

REAVEN, G. Metabolic syndrome: pathophysiology and implications for management of cardiovascular disease. **Circulation**. v. 106, p.286–288, 2002.

ROBINSON, E.; GRIEVE, D. J. Significance of peroxisome proliferator-activated receptors in the cardiovascular system in health and disease. **Pharmacology & Therapeutics**. v. 122, p. 246–263, 2009.

ROCHA, V.Z.; LIBBY, P. obesity, inlfammation, and atherosclerosis. **Nature reviews Cardiology**. Advance online publication, www.nature.com/nrcardio. 2009

SASS DA, CHANG P, CHOPRA KB. Nonalcoholic fatty liver disease: a clinical review. **Dig Dis Sci**. v. 50, p 171-180. 2005.

SAVAGE DB, SEMPLE RK. Recent insights into fatty liver, metabolic dyslipidaemia and their links to insulin resistance. **Curr Opin Lipidol.** v. 21(4), p. 329–336, 2010.

SCHATTENBERG, J.M. et al. Cell death and hepatocarcinogenesis: Dysregulation of apoptosis signaling pathways. **J. Gastroenterol. Hepatol**. v. 26(Suppl. 1), p. 213–219, 2011.

SCHOONJANS K.; PEINADO-ONSURBE, J.; LEFEBVRE, A. M. et al. PPARα and PPARγ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. **EMBO J.** v. **15**, p. 5336–5348, 1996.

SERINO M; MENGHINI R; FIORENTINO L et al: Mice heterozygous for tumor necrosis factor-alpha converting enzyme are protected from obesity-induced insulin resistance and diabetes. **Diabetes.** v. 56, p. 2541-2546, 2007.

SFEIR, Z.; IBRAHIMI, A.; AMRI, E. Regulation of FAT/CD36 gene expression: further evidence in support of a role of the protein in fatty acid binding/transport. **Prostaglandins Leukot Essent Fatty Acids**. v. 57, p. 17-21. 1997.

SILVA, A. K. S. et. al. Effect or new thiazolidine derivative LPSF/GQ-02 and LPSF/GQ-16 on atherosclerotic lesion in LDL receptor-deficient mice (LDLR-/-). **Cardiovascular Patology**. v. 22(1), p. 81-90, 2013.

SILVA, M.T. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system**. J. Leukoc. Biol**. v. 87, p. 93–106, 2010.

SIMONS, L.A; SIMONS, J; FRIEDLANDER, Y; MCCALLUM, J. Is prediction of cardiovascular disease and all-cause mortality genuinely driven by the metabolic syndrome, and independently from its component variables? The Dubbo study. **Heart Lung Circ**. V. 20, p. 214–219. 2011.

SOEHNLEIN, O. AND LINDBOM, L. Phagocyte partnership during the onset and resolution of inflammation. **Nat. Rev. Immunol**. v. 10, p. 427–439, 2010.

SONSUZ A, BASARANOGLU M, OZBAY G. Relationship between aminotransferase levels and histopathological findings in patients with nonalcoholic steatohepatitis. **Am J Gastroenterol.** v. 95, p. 1370-1371, 2000.

STAELS, B.; KOENIG, W.; HABIB, A. et al. Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. **Nature.** v. 393, p. 790-793, 1998.

STAELS, B; MAES, M; ZAMBON, A. Fibrates and future PPARalpha agonists in the treatment of cardiovascular disease. **Nat. Clin. Pract. Cardiovasc. Med.** v.5, p. 542–553, 2008.

STARY, H. C. Natural history and histological classification of atherosclerotic lesions: an update. **Arterioscler. Thromb. Vasc. Biol**. v. 20, p. 1177–1178, 2000.

STEPHEN, S; A. BARANOVA, Z.M. YOUNOSSI. Nonalcoholic fatty liver disease and bariatric surgery. **Expert Rev Gastroenterol Hepatol,** v. 6, p. 163–171, 2012.

SUNDVOLD H.; LIEN S. Identification of a novel peroxisome proliferator-activated receptor (PPAR) gamma promoter in man and transactivation by the nuclear receptor RORalpha1**. Biochem. Biophys. Res. Commun**. v. 287. p. 383–390, 2001.

SZABO G; PRANOTI MANDREKAR; ANGELA DOLGANIUC. Innate Immune Response and Hepatic Inflammation. **Seminars in liver disease.** v27, p 4, 2007.

T. NAGASAWA; Y. INADA; S. NAKANO; T. TAMURA; T. TAKAHASHI; K. MARUYAMA; Y. YAMAZAKI; J. KURODA; N. SHIBATA. Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet, **Eur. J. Pharmacol.** v. 536, p. 182–191, 2006.

TACKE, F. Functional role of intrahepatic monocyte subsets for the progression of liver inflammation and liver fibrosis in vivo. **Fibrogenesis Tissue Repair 5** (Suppl. 1), S27, 2012.

TAILLEUX, A; WOUTERS, K; STAELS, B. Roles of PPARs in NAFLD: Potential therapeutic targets. **Biochimica et Biophysica Acta.** v. 1821, p. 809–818, 2012.

TARGHER G; MARRA F; MARCHESINI G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? **Diabetologia.** v. 51, p. 1947-1953, 2008.

TARGHER, G; C.P. DAY, E. BONORA. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. **N. Engl. J. Med**. v. 363 (14), p. 1341–1350, 2010.

THOMPSON, G. R.; SOUTAR, A. K.; SPENGER, F. A. et al. Defects of receptor-mediated low density lipoprotein catabolism in homozygous familial hypercholesterolemia and hypothyroidism in vivo. **Proc Natl Acad Sci USA.** v. 78(4), p. 2591-2595, 1981.

TILG H, MOSCHEN AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. **Hepatology**. v. 52(5), p. 1836–1846, 2010.

TONTONOZ P.; HU E.; DEVINE J. et al. PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase. **gene. Mol. Cell Biol.** v. 15, p. 351–357, 1995.

TONTONOZ P.; HU E.; GRAVES R. A. et al. mPPARγ2: tissue-specific regulator of an adipocyte enhancer. **Genes Dev**. v. 8, p. 1224–1234, 1994a.

TONTONOZ, P.; HU, E.; SPIEGELMAN, B. M. Stimulation of adipogenesis in fibroblasts by PPARy2, a lipid-activated transcription factor. **Cell.** v. 79, p. 1147–1156, 1994b.

TONTONOZ, P.; NAGY, L.; ALVAREZ, J. G. et al. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. **Cell**. v. 93(2), p. 241-52, 1998.

TRUMAN JP; AL GADBAN MM; SMITH KJ, et al: Differential regulation of acid sphingomyelinase in macrophages stimulated with oxidized low-density lipoprotein (LDL) and oxidized LDL immune complexes: role in phagocytosis and cytokine release**. Immunology**. v. 136, p. 30-45, 2012.

UTO H, NAKANISHI C, IDO A, HASUIKE S, KUSUMOTO K, ABE H, NUMATA M, NAGATA K, HAYASHI K, TSUBOUCHI H. The peroxisome proliferator-activated receptor-gamma agonist, pioglitazone, inhibits fat accumulation and fibrosis in the livers of rats fed a choline-deficient, l-amino acid-defined diet. Hepatol Res. v.32(4), p.235-42. 2005.

UTZSCHNEIDER KM, KAHN SE. Review: the role of insulin resistance in nonalcoholic fatty liver disease. **J Clin Endocrinol Metab**. v. 91(12), p. 4753–4761, 2006.

VAMECQ, J.; DRAYE, J. P. Pathophysiology of peroxisomal beta-oxidation. **Essays Biochem**. v. 24, p. 115−225, 1989.

VIDAL-PUIG, A. J.; CONSIDINE, R. V.; JIMENEZ-LIÑAN, M. et al. Peroxisome proliferator-activated receptor gene expression in human tissues: effects of obesity, weight loss, and regulation by insulin and glucocorticoids. Journal of clinical investigation. V. 99 (10), p. 2416-2422, 1997.

WATKINS, P.B.; WHITCOMB, R.W. Hepatic dysfunction associated with troglitazone. **N. Engl. J. Med**. v. 338, p. 916–917, 1998.

WEBER, C.; ZERNECKE, A.; LIBBY, P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. **Nature.** v. 8, p. 802-815, 2008.

WEIß J, RAU M, GEIER A: Non-alcoholic fatty liver disease: epidemiology, clincal course, investigation and treatment. **Dtsch Arztebl Int**. v. 111, p. 447–52, 2014.

WILLSON, T. M.; BROWN, P. J.; STERNBACH, D.D. et al. The PPARs: From Orphan Receptors to Drug Discovery. **Journal of medicinal chemistry.** v. 43, p. 527–550, 2000.

WOOLLARD KJ; GEISSMANN F. Monocytes in atherosclerosis: subsets and functions. **Nat Rev Cardiol**. V. 7, p. 77-86, 2010.

WOUTERS K; VAN GORP PJ; BIEGHS V, et al: Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. **Hepatology**. v. 48, p. 474-486, 2008.

WUNDERLICH FT; STRÖHLE P; KÖNNER AC, et al: Interleukin-6 signaling in liver-parenchymal cells suppresses hepatic inflammation and improves systemic insulin action. **Cell Metab**. v. 12, p. 237-249, 2010.

YANG, W.; RACHEZ, C.; FREEDMAN, L. P. Discrete roles for peroxisome proliferator-activated receptor gamma and retinoid X receptor in recruiting nuclear receptors coactivators. **Molecular cell biology.** v**.** 20, p. 8008-8017, 2000.

YKI-JARVINEN, H. Thiazolidinediones. **N. Engl J Med**. v.351 (11), p. 1106-18, 2004.
YU, S.; REDDY, K. J. Transcription coactivators for peroxisome proliferator-activated receptors . Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. V. 1771 (8), p 936-951, 2007.

YU, S; MATSUSUE, K; KASHIREDDY, P et al. Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression, **J. Biol. Chem**. 278 (2003) 498–505.

ZHU Y.; ALVARES K.; HUANG Q. et al. Cloning of a new member of the peroxisome proliferator- activated receptor gene family from mouse liver**. J. Biol. Chem**. v. 268, p. 26817–26820, 1993.

ZHU, Y.; QI, C.; KORENBERG, J. R. et al. Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR-gamma) gene: Alternative promoter use and different splicing yield two mPPAR-gamma isoforms. **Proc. Natl. Acad. Sci. USA**. v. 92, p. 7921–7925, 1995.

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RESEARCH ARTICLE

LPSF/GQ-02 Inhibits the Development of Hepatic Steatosis and Inflammation in a Mouse Model of Non-Alcoholic Fatty Liver Disease (NAFLD)

Amanda Karolina Soares e Silva[1]*, Dilênia de Oliveira Cipriano Torres[2], Fabiana Oliveira dos Santos Gomes[1], Bruna dos Santos Silva[1], Edlene Lima Ribeiro[1], Amanda Costa Oliveira[1], Laise Aline Martins dos Santos[1], Maria do Carmo Alves de Lima[3], Ivan da Rocha Pitta[3], Christina Alves Peixoto[1]

1 Laboratório de Ultraestrutura, Centro de Pesquisa Aggeu Magalhães (FIOCRUZ), Pernambuco, Recife, Brazil, **2** Laboratório de Análises Clínicas da Universidade de Pernambuco, Pernambuco, Recife, Brazil, **3** Laboratório de planejamento e síntese de fármacos, Universidade federal de Pernambuco, Pernambuco, Recife, Brazil

* kls.amanda@gmail.com

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LPSF/GQ-02 INHIBITS THE DEVELOPMENT OF HEPATIC STEATOSIS AND INFLAMMATION IN A MOUSE MODEL OF NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

Amanda Karolina Soares e Silva[1,*], Dilênia de Oliveira Cipriano Torres[2], Fabiana Oliveira dos Santos Gomes[1], Bruna dos Santos Silva[1], Edlene Lima Ribeiro[1], Amanda Costa Oliveira[1], Laise Aline Martins dos Santos[1], Maria do Carmo Alves de Lima[3], Ivan da Rocha Pitta[3], Christina Alves Peixoto[1,]

1 Laboratório de Ultraestrutura, Centro de Pesquisa Aggeu Magalhães(FIOCRUZ),Pernambuco, Recife, Brazil 2 Laboratório de Análises Clínicas da Universidade de Pernambuco, Pernambuco, Recife, Brazil, 3 Laboratório de planejamento e síntese de fármacos, Universidade federal de Pernambuco, Pernambuco, Recife, Brazil

[*]Corresponding address: MSc Amanda Karolina Soares e Silva, *Laboratório de Ultraestrutura, Centro de Pesquisas Aggeu Magalhães*, FIOCRUZ, Av. Moraes Rego s/n, Recife, Brazil. CEP 50670-420.

Fax number: (55) 81 21012557. Email: kls.amanda@gmail.com

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Abstract

Non-alcoholic fatty liver disease (NAFLD) defines a wide spectrum of liver diseases that extends from simple steatosis to non-alcoholic steatohepatitis. Although the pathogenesis of NAFLD remains undefined, it is recognized that insulin resistance is present in almost all patients who develop this disease. Thiazolidinediones (TZDs) act as an insulin sensitizer and have been used in the treatment of patients with type 2 diabetes and other insulin-resistant conditions, including NAFLD. Hence, therapy of NAFLD with insulin-sensitizing drugs should ideally improve the key hepatic histological changes, while also reducing cardiometabolic and cancer risks. Controversially, TZDs are associated with the development of cardiovascular events and liver problems. Therefore, there is a need for the development of new therapeutic strategies to improve liver function in patients with chronic liver diseases. The aim of the present study was to assess the therapeutic effects of LPSF/GQ-02 on the liver of LDLR-/- mice after a high-fat diet. Eighty male mice were divided into 4 groups and two different experiments: 1-received a standard diet; 2-fed with a high-fat diet (HFD); 3–HFD+pioglitazone; 4–HFD+LPSF/GQ-02. The experiments were conducted for 10 or 12 weeks and in the last two or four weeks respectively, the drugs were administered daily by gavage. The results obtained with an NAFLD murine model indicated that LPSF/GQ-02 was effective in improving the hepatic architecture, decreasing fat accumulation, reducing the amount of collagen, decreasing inflammation by reducing IL-6, iNOS, COX-2 and F4 / 80, and increasing the protein expression of IκBα, cytoplasmic NFκB-65, eNOS and IRS-1 in mice LDLR -/-. These results suggest a direct action by LPSF/GQ-02 on the factors that affect inflammation, insulin resistance and fat accumulation in the liver of these animals. Further studies are being conducted in our laboratory to investigate the possible mechanism of action of LPSF/GQ-02 on hepatic lipid metabolism.

Keywords: LPSF/GQ-02, NAFLD; inflammation; insulin resistance; hepatic steatosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of hepatic disease in western civilization [1] and is considered as a hepatic manifestation of a metabolic syndrome strongly associated with dyslipidemia, obesity, hypertension and insulin resistance[2]. NAFLD covers a spectrum of pathologies that range from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by cell ballooning, inflammation and different degrees of fibrosis [3]. Considering that the inflammation found in steatosis is usually benign, the most advanced state of NASH, and particularly fibrosing NASH, is one of the main causes of cirrhosis and mortality related to the liver [4].

One of the important and unresolved problems in NASH is the pathogenesis of hepatocyte injuries. One hypothesis for the pathogenesis of NAFLD is the “two-hit” hypothesis [5]. According to this paradigm, the primary abnormality (“first hit”) is most likely insulin resistance (IR), which leads to the accumulation of triglycerides within the hepatocytes. Then, a “second hit” induces the hepatocyte injury and inflammation (NASH) [6].

Hepatic inflammation can also induce insulin resistance via an imbalance in the secretion of pro-inflammatory cytokines, subsequent to activation of inflammatory/oxidative transcription factors [7]. A key transcription factor that mediates the inflammatory response in hepatocytes is nuclear factor κB (NF-B) [8, 9]. Activated hepatic NF-κB alone can drive insulin resistance, as evidenced by the finding that transgenic expression of the inhibitor of nuclear factor κB kinase subunit β (IKK-κβ), which increases NF-κB activity, resulted in overt insulin resistance in mice fed with a normal chow diet [10].

NF-κB activation increases the secretion of a number of pro-inflammatory cytokines, including interleukin (IL)-6, TNFα, and IL-1β [7]. NF-κB activation involves a complex series of signaling events that begins with the activation of the inhibitor κB (IκB) kinase complex, which, in turn, phosphorylates IκB [11, 12]. IκB is an inhibitor protein of NF-κB that binds to NF-κB, sequestering it in the cytoplasm [13]. However, once phosphorylated, IκB is targeted for ubiquitination and subsequent degradation, leaving NF-κB free to translocate to the nucleus and initiate the transcription of target genes [14]. These findings provide strong evidence that the liver is a primary site of the inflammatory action that causes insulin resistance, and that NF-κB is a central pathogenic factor underlying inflammation-induced insulin resistance.

Thiazolidinediones (TZDs) are a class of oral anti-diabetic medication that improves insulin resistance by acting as a selective agonist of the peroxisome proliferator activated receptor gamma (PPARγ) [15, 16]. Troglitazone, the first generation TZD, has been withdrawn from the market due to its hepatotoxicity [17], whereas rosiglitazone and pioglitazone are second generation TZDs and are currently available for clinical use [16, 18]. They redistribute fat from muscle and the liver to adipose tissue and thereby improve peripheral (skeletal muscle) and hepatic insulin sensitivity [16].

In general, TZDs improve hepatic histology in patients with NASH, although their favorable effect on steatosis is more striking than on other histological variables such as inflammation, ballooning or fibrosis. Their favorable effect on liver histology and liver biochemistry disappears upon their discontinuation, suggesting that long-term treatment is required to maintain their therapeutic benefits [19]. This is potentially a significant issue as recent studies have questioned the long-term safety of TZDs (especially rosiglitazone) [20]. Since the majority of the participants in these studies

were non-diabetic, it is not clear if TZDs are equally effective in diabetics with NASH. Furthermore, it is possible that TZDs alone, without lifestyle modifications, may not be as effective [21].

Existing treatment for liver diseases is limited and differentiated, depending on the etiology and / or persistence of the stimulus. In general, current therapies try to stop or delay tissue injury, only managing to minimize damage in the cells in order to reduce the complications associated with the disease. When therapeutic measures are not effective, patients may progress to cirrhosis [22]. A liver transplant is the most effective treatment available for patients with chronic liver failure [23]. Therefore, there is a need for the development of new therapeutic strategies to improve the liver function of patients with chronic liver diseases.

In this context, our laboratory has already begun biological assessment studies with thiazolidine derivative LPSF/GQ-02 compound 5-(4-Chloro-benzylidene)-3-(4-methylbenzyl)-thiazolidine-2,4-dione, using biochemical, molecular, morphometric and ultra-structural analysis. The results have demonstrated that LPSF/GQ-02 was effective in decreasing the risk factors associated with the development of atherosclerosis, such as insulin resistance and inflammation, and consequently reducing atherosclerotic plaque in mice without the LDL receptor (LDLR-/-) [24]. Molecular modeling studies using nuclear PPAR-γ as molecular target available on the PDB database as 2PRG, suggested that LPSF/GQ-02 is a PPARγligand [25].

In the present study, mice with NAFLD, induced by a high-fat diet, were treated with LPSF/GQ-02 and assessed in relation to the development of hepatic steatosis and inflammation.

Materials and Methods

Ethics Statement

This study was carried out in accordance with the ethical principles in animal experimentation adopted by the Colégio Brasileiro de Experimentação Animal (COBEA). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Fundação Oswaldo Cruz – FIOCRUZ (Permit Number: L-010/09).

Synthesis of Thiazolidine Derivative LPSF/GQ-02

LPSF/GQ-02 representing the compounds 5-(4-chloro-benzylidene)-3-(4-methyl-benzyl)-thiazolidine- 2,4-dione was synthesized at the Department of Antibiotics of the Universidade Federal de Pernambuco (Brazil) following the methodology described by Mourão et al. [26].

Study Design

Eighty mice were used, divided in 8 groups, all of which were homozygous for the absence of the LDL receptor gene (LDLR-/-), generated in the C57BL6/J background, obtained from Jackson Laboratories (USA) and bred in the vivarium of the Centro de Pesquisas Aggeu Magalhães. The state of the health of the mice was determined and they were acclimated in a laboratory environment with a temperature of 22°C (±1°) and artificial light from fluorescent lamps for a light/dark period of 12/12 hours. After weaning, the animals were submitted to a standard diet for eight days of adaptation. After this period, they received an atherogenic diet for 10 weeks [27] with drugs and they were separated into groups (n=10) and submitted to daily treatment by gavage with pioglitazone and LPSF/GQ-02 for 15 days, as follows:

Control15 – This group received a standard diet throughout the entire experiment.

HFD15 – This group received the atherogenic diet (HFD) consisting of 21% milk fat and 1.25% cholesterol [27].

PIO15 – This group received HFD and was treated with 20mg/kg/day of pioglitazone for 15 days [28].

LPSF/GQ-02–15 - This group received HFD and was treated with 30mg/kg/day of glitazone LPSF/GQ-02 for 15 days [26].

A second experiment assessed the glitazones for a longer period of time. The animals were submitted to the abovementioned criteria, although they received the atherogenic diet for 12 weeks [29] and the drugs were administered during the last four weeks of the experimental diet, totaling 30 days of treatment with glitazones (Controle30/ HFD30/ PIO30/ LPSF/GQ-02–30). The atherogenic diet was acquired commercially (PragSoluções Biociências). The animals had free access to water and were kept in a controlled light cycle of 12 hours light/darkness. At the end of the therapy, the animals were anesthetized (Ketamine/Xylasine) before blood collection by cardiac puncture (without anticoagulant). The serum was separated and stored at -20°C for biochemical measurements. The livers were dissected and fixed for posterior processing (morphological analysis) and frozen at - 80°C for posterior western blotting analysis.

Biochemical Determinations

Serum levels of aspartate aminotransferase (AST), total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TGs), Low-density lipoprotein (LDL) and glucose were determined photometrically in the Cobas Integra 400 automatic analyzer (Roche, Mannheim, Germany), using Roche kits.

Histopathology

Liver fragments were fixed in 10% formalin for 24 hours, before being processed and embedded in paraffin. Sections of 4-5µm were cut and mounted on glass slides. The sections were stained with hematoxylin-eosin (HE) and assessed with an inverted microscope (Observer Z1, Zeiss MicroImaging GmbH), equipped with a camera and 4.7.4 image analysis software (AxionCam MRm Zeiss), at a magnification of 400 x.

Oil Red O Staining

In order to specifically detect lipids, samples of hepatic tissue were fixed in paraformaldehyde at 4% for 2h and embedded O.C.T (Tissue-Tek, Zoeterwoude, Netherlands) in the presence of liquid nitrogen. Afterwards, frozen cuts (8µm thickness) were made on a cryostat and the samples were then fixed with pure formaldehyde solution for 15 minutes. Next, the slides were stained with hematoxylin for 30 seconds to identify the nuclei of the cells. The cells were then washed in distilled water and stained with Oil Red O for 15 minutes. Five images of the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

Determination of Liver Triglyceride Content

Livers were immediately collected and snap frozen in liquid nitrogen. A 50-mg piece of liver was homogenized in PBS. Folch's reagent (CHCl3/MeOH, 2:1) (0.75 ml)

was added to the homogenate. The nonaqueous phase was collected, and 30µl of 200 mg/ml Triton X-100 in CHCl3 was added. Samples were dried and triglycerides level was performed using the Roche kit by photometric method in the analyzer Cobas Integra 400, Roche, Mannheim, Germany.

Picrosirius Red Staining

The cuts were stained with Sirius Red to assess the amount of collagen in the liver. The slides were pre-treated with xylene to remove paraffin and hydrated with a decreasing amount of ethanol. Subsequently, the cuts were stained with 1% Sirius Red solution in saturated picric acid for 2 hours and counter-stained with a solution of Fast green at 0.1% for 30 seconds. After this process, the slides were dehydrated in ethanol at 100%, cleared in xylene and mounted. Five images of the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

Immunohistochemical Assays

Five sections (5 µm in thickness) of each group were cut and adhered to slides treated with 3-amino-propyl-trietoxi-silane (APES [Sigma, USA]). The sections were deparaffinized with xylene and rehydrated in graded ethanol (100 to 70%). To increase epitope exposure, the sections were heated for 30 minutes in a sodium citrate buffer (0.01 M, pH 6.0). To minimize endogenous peroxidase activity, the slides were treated with 0.3% (v/v) H_2O_2 in water for five minutes. The sections were washed with 0.01M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1h at room temperature. The sections were then incubated for 12 hours at 4ºC with antibodies against IL-6 (1:100 eBioscience, San Diego, US), COX-2 (1:400 Abcam, Cambridge,

UK), iNOS (1:100 Abcam, Cambridge, UK) and F4/80 (1:50 Abcam, Cambridge, UK). The antigen-antibody reaction was visualized with avidin-biotin peroxidase (Dako Universal LSAB + Kit, Peroxidase), using 3.3-diaminobenzidine as the chromogen. The slides were counterstained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, but with the omission of the first antibody. Five pictures at the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

Cytosolic and Nuclear Protein Extraction

Cytosolic and nuclear proteins from the liver were isolated using Cayman's Nuclear Extraction kit (Item No. 10009277, Cayman chemical company, Ann Arbor, Michigan, USA). The liver fragments were homogenized in a hypotonic buffer supplemented with DTT and Nonidet P-40 per gram of tissue. The livers were centrifuged and re-suspended by adding specified assay reagents, following the manufacturer's instructions. The cytosolic and nuclear fractions were stored in pre-chilled vials at -80˚C until further analysis. Liver cytosols were used to determine the quantity of IκBα, IRS-1, CD-220, ABCA1, eNOS and NFκB p65 in the immunoblotting, whereas nuclear fractions were used for PPARα and PPARγ immunoblotting.

Measurement of Protein Levels

The total, cytosolic and nuclear extraction protein levels were determined using the Bradford method, with bovine serum albumin as standard [30]. The samples were read in a spectrophotometer at 660nm. All samples were run in duplicate and the mean

of the two absorbency levels was used to determine the protein quantity. The protein concentration per sample amount was determined using the equation from a calibration curve. The curve was generated using the same method as the samples, with the substitution of bovine serum albumin at five concentration levels.

Western Blot Analysis

The proteins (40 µg) were separated to 12% (NF-κB p65, IκBα, ABCA-1, eNOS, PPARα, PPARγ, IRS-1 and CD-220) sodium dodecyl sulfate–polyacrylamide by gel electrophoresis under reduced conditions and were electrophoretically transferred onto a nitrocellulose membranes (Bio Rad, CA, USA, Ref. 162-0115). After overnight blocking at 4ºC with 5% non-fat milk in TBS-T (Tris-buffered saline 0.1% plus 0.05% Tween 20, pH 7.4), the membranes were incubated at room temperature for 3h with antibodies against the following: NF-κB p65 (1:200, Santa Cruz Biotechnology, CA); IκBα (1:500, Santa Cruz Biotechnology, CA); ABCA1 (1:1000, Abcam Cambridge, UK); eNOS (1:1000, Abcam Cambridge, UK); PPARα (1:1000, Abcam Cambridge, UK); PPARγ (1:1000, Abcam Cambridge, UK); IRS-1 (1:1000, Abcam Cambridge, UK) and CD-220 (1:1000, Abcam Cambridge, UK), diluted in TBS-T buffer solution containing 3% non-fat milk. After washing (six times, 10 min each) in TBS-T, the membranes were further reacted with horseradish peroxidase-conjugated anti-rabbit antibody (1:80000, Sigma, USA), diluted in TBS-T with 1% nonfat milk, for 1h 30min at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used to visualize the labeled protein bands and the blots were developed on X-ray film (Fuji Medical, Kodak, Ref. Z358487-50EA). For quantification, the density of pixels of each band was determined by Image J 1.38

software (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD). The results were confirmed in three sets of experiments for each protein investigated. Immunoblotting for β-actin was performed as a control for the above protein blots. After protein blot visualization with enhanced chemiluminescence, the protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-β-actin antibody (1:2000, Sigma, USA), and protein densitometry was performed.

Statistical Analysis

GraphPad Prism software (version 5) was used for the statistical analysis. Data were expressed as mean ± standard deviation. Differences between the control and treated groups were analyzed using analysis of variance (ANOVA), prior to the performance of Tukey's post hoc test or the Student's t-test. Probability values less than 0.05 were considered significant.

Results

Biochemical Determinations

After 15 and 30 days, the high-fat diet group showed a significant increase in total and LDL cholesterol and triglycerides compared to the control group. Similarly, treatment pioglitazone and LPSF/GQ-02 did not reduce total and LDL cholesterol and triglycerides levels (Fig. 1A-F), even treatment with pioglitazone for 30 days significantly increased levels of total and LDL cholesterol compared with HFD group (Fig. 1D)

Treatment with pioglitazone and LPSF/GQ-02 did not alter the HDL levels compared with HFD group after 15 days (Fig. 1G). However, after 30 days of treatment, pioglitazone and LPSF/GQ-02 induced a significant increase in serum HDL, indicating a time-dependent effect (Fig. 1H). Serum glucose levels were evaluated after 15 and 30 days of treatment. There was a significant decrease in serum glucose levels after the use of pioglitazone and LPSF/GQ-02 compared with the HFD group (Fig. 1I-J). Finally, in relation to the serum AST, no significant difference was observed between groups (Fig. 1K-L).

Effects of LPSF/GQ-02 on the Hepatic Architecture

Histological analysis of the hepatic fragments in the control15 group exhibited well-preserved architecture with characteristic hepatocytes distributed homogenously throughout the hepatic parenchyma (Fig. 2A). After induction to the high-fat diet, the HFD15 group exhibited significant alterations, including tissue disorganization with macro and microvesicular steatosis in the cytoplasm of the hepatocytes, as well as the presence of multiple foci of inflammable infiltrates (Fig. 2B). The pioglitazone group was not capable of reversing the alterations caused by the high-fat diet and exhibited severe tissue disorganization, macro and microvesicular steatosis and many inflammatory infiltrates throughout the hepatic tissue (Fig. 2C). The group that received the diet and was treated with LPSF/GQ-02 for 15 days showed a significant improvement in the hepatic architecture, with steatosis reduction as well as inflammation, which was confirmed by the reduction of foci of inflammatory infiltrates (Fig. 2D).

A second experiment, involving the administration of drugs for 30 days, assessed the time-dependant action of both pioglitazone and LPSF/GQ-02. The

histological analysis revealed patterns similar to the groups that received the drug for only 15 days. The control group maintained the same characteristics (Fig. 3A). However, the HFD30 and PIO30 groups exhibited a greater severity in the accumulation of lipids in the cytoplasm of hepatocytes and increased inflammation, confirmed by the greater presence of inflammatory infiltrates (Fig. 3B and 3C). The group that received LPSF/GQ-02 reduced macro and microvesicular steatosis and decreased the quantity of inflammatory infiltrates, reestablishing a hepatic architecture similar to that found in the control group (Fig. 3D).

LPSF/GQ-02 Decreased the Levels of Hepatic Fat After Induction of a High Fat Diet

The accumulation of fat in the liver is the key point in the appearance of NAFLD. Consequently, the quantity of lipids in the liver was analyzed using the specific staining of Oil red O. The control groups from both the 15-day and 30-day experiments exhibited a basal amount of lipids, characterizing a normal pattern (Fig. 2A and 3A). After the induction of the high-fat diet, there was a significant increase in the quantity of lipids, when compared to the control group, in both the 15-day and 30-day experiments (Fig. 2B and 3B). The pioglitazone groups (15 and 30 days) did not decrease the quantity of fat in the liver when compared to the HFD group (Fig. 2C and 3C). However, the use of LPSF/GQ-02 brought about a significant decrease in the quantity of fat in the hepatic tissue when compared to the HFD and pioglitazone groups (Fig. 2D and 3D). Quantitative analysis was performed using ANOVA and Tukey's post hoc tests (Fig. 2M and 3M).

Additionally, liver triglyceride content was determined. The results obtained confirmed the specific staining of Oil red O analyses. Liver triglyceride content was significantly elevated after the high fat diet, and pioglitazone treatment had no effect on liver triglycerides On the other hand, the LPSF/GQ-02 promoted a significant decrease compared to HFD groups and pioglitazone(Fig. 2O and 3O).

Action of LPSF/GQ-02 on Hepatic Fibrosis

Sirius Red staining was used to analyze the evolution of fibrosis since it interacts strongly with the basic amino acids of molecules from different types of collagen [31]. Both the 15-day and 30-day control groups exhibited normal patterns, with collagen deposition in the perivenular region (Fig. 2A and 3A). The HFD groups exhibited significant collagen accumulation when compared to the control group. There was an increase in the amount of collagen in the perivenular and sinusoidal regions, especially in the 30-day group (Fig. 2B and 3B). The group treated with pioglitazone for 15 days was not effective in decreasing the collagen content when compared with the HFD group, exhibiting the same collagen deposition pattern. However, in the group treated with pioglitazone for 30 days, there was a reduction in collagen when compared with the HFD group (Fig. 2C and 3C), indicating that the action of the drug, in terms of decreasing fibrosis, occurs in a time-dependant manner. LPSF/GQ-02 significantly reduced collagen in the 15-day and 30-day groups when compared with the HFD and pioglitazone groups, exhibiting a pattern similar to the control group, with collagen only found in the perivenular region ((Fig. 2D and 3D). Quantitative analysis of the collagen content was performed in all groups using ANOVA and Tukey's post hoc tests (Fig. 2N and 3N).

LPSF/GQ-02 Decreased the Levels of Inflammatory Markers in Mice with NAFLD

IL-6 is a multifunctional cytokine that regulates immune responses, as well as acute phase reactions and hematopoiesis, and can play a central role in inflammation of the host defense and tissue injuries [32]. The expression of IL-6 in immunohistochemistry was analyzed after treatment with pioglitazone and LPSF/GQ-02. The control group exhibited a weak reactivity to IL-6 in both the 15-day and 30-day groups (Fig. 4A and 5A). The use of a high-fat diet significantly increased the expression of this cytokine in the hepatic tissue, exhibiting immunoreactivity in the cytoplasm of the Kupffer cells and in the membranes of the hepatocytes (Fig. 4B and 5B). After the administration of pioglitazone, a staining pattern similar to the HFD group was observed, indicating that the drug was not effective in reducing the expression of this inflammatory cytokine (Fig. 4C and 5C). However, in both the 15 and 30-day groups, LPSF/GQ-02 significantly decreased the expression of IL-6 when compared to the HFD and pioglitazone groups, exhibiting a labelling pattern similar to the control (Fig. 4D and 5D).

After analyzing the expression of iNOS in the hepatic tissue, the 15 and 30-day control groups exhibited a low reactivity to iNOS (fig. 4E and 5E). The HFD group significantly increased reactivity to iNOS, with diffuse marking in the cytoplasm of the hepatocytes of animals treated for 15 and 30 days, indicating an increase in the inflammatory process (fig. 4F and 5F). The groups treated with pioglitazone for 15 and 30 days exhibited a strong reactivity to iNOS, similar to the HFD group, indicating an intense labelling throughout the cytoplasm of the hepatocytes, as well as in the Kupffer

cells, suggesting that pioglitazone increases the inflammatory process in the hepatic tissue (fig. 4 and 5G). After the administration of LPSF/GQ-02 for 15 days, there was a significant decrease in the staining of iNOS in all of the hepatic tissue, when compared with the HFD and pioglitazone groups (fig. 4H). The group treated with LPSF/GQ-02 for 30 days did not exhibit any specific staining for iNOS (fig. 5H), suggesting that LPSF/GQ-02 participates in the inflammatory process in a beneficial manner, decreasing the expression of iNOS and contributing to an improvement in the hepatic tissue.

In an attempt to elucidate the anti-inflammatory mechanisms of LPSF/GQ-02, the expression of COX-2 was assessed through immunohistochemistry after treatment with pioglitazone and LPSF/GQ-02 in LDLR-/- animals submitted to a high-fat diet. Both the 15-day and 30-day control groups exhibited low reactivity to the enzyme COX-2 (Fig. 4I and 5I). After exposure to a high-fat diet for 15 or 30 days, a strong immunoreactivity to the enzyme COX-2 was recorded, especially in the cytoplasm of hepatocytes (Fig. 4Jand 5J), which indicates that the high-fat diet contributed to the hepatic inflammation process. In the pioglitazone group, reactivity to COX-2 was similar to the HFD group, indicating that pioglitazone does not help to reduce inflammation in this animal model (Fig. 4Kand 5K). However, after the use of LPSF/GQ-02, a significant reduction in the immunoreaction for COX-2 was observed when compared to the HFD and pioglitazone groups, both for 15 and 30 days (Fig. 4Land 5L). These findings strengthen the theory that the thiazolidine derivative LPSF/GQ-02 helps to reduce the inflammatory process.

Since the involvement of Kupffer cells is important in the hepatic inflammatory process, F4/80, a specific marker for Kupffer cells, was used to assess their expression in hepatic tissue after treatment with LPSF/GQ-02. Both the 15-day and 30-day control

groups exhibited low positivity to F4/80 (Fig. 4M and 5M) in all of the hepatic tissue. After the administration of the diet, increased immunoreactivity to F4/80 was recorded, especially in the 15-day group, indicating an increase in the population of Kupffer cells in the hepatic tissue (Fig. 4Nand 5N). The administration of pioglitazone led to a reduction in the positivity of cells to F4/80, especially in the 15-day group, although this reactivity was not significant in either group, indicating the presence of Kupffer cells and possibly an inflammatory process (Fig. 4Oand 5O). LPSF/GQ-02 exhibited less immunoreactivity for F4/80. The 15-day and 30-day groups exhibited low reactivity in all of the hepatic tissue, with a significant decrease when compared with the HFD and pioglitazone groups (Fig. 4P and 5P), which corroborates previous results, indicating that LPSF/GQ-02 decreases hepatic inflammation

All of the quantitative analysis was performed using Gimp 2.6 software.

Effects of LPSF/GQ-02 on the Protein Expression of PPARα, PPARγ, IκBα, NFκB p65, eNOS, CD-220, IRS-1 and ABCA1

The protein levels of nuclear PPARα were assessed after treatment with pioglitazone and LPSF/GQ-02. No significant differences were found in the PPARα protein levels in the groups treated for 15 days (Fig. 6Aand 6B). However, in the 30-day experiment, the HFD group exhibited a significant reduction in the expression of PPARα when compared to the control group (Fig. 6Dand 6E). In addition, treatment with LPSF/GQ-02 was not capable of increasing the expression of PPARα, similar to the HFD group (Fig. 6Dand 6E).

After assessing the protein expression of nuclear PPARγ, it is notable that among the animals treated for 15 days, only the pioglitazone group exhibited a

significant difference in relation to all of the other groups (Fig. 6A and 6C), thereby confirming its action as an agonist of PPARγ. Meanwhile, the animals that were treated for 30 days with pioglitazone and LPSF/GQ-02 exhibited the following results: the HFD group significantly decreased the levels of PPARγ when compared to the control and pioglitazone groups, with no alteration in relation to the LPSF/GQ-02 group. Furthermore, the LPSF/GQ-02 group exhibited levels of expression of PPARγ that were similar to the HFD group (Fig. 6D and 6F).

Expression of the protein IκBα was analyzed through western blotting on the hepatic tissue after treatment with pioglitazone and LPSF/GQ-02. As a result, there was no alteration in the levels of this protein after treatment with pioglitazone and LPSF/GQ-02 for 15 days (Fig. 7A and 7B). However, the prolonged treatment (30 days) led to a significant increase of the protein IκBα in the group treated with LPSF/GQ-02 when compared to the HFD group (Fig. 7D and 7E).

It is known that NFκB is involved in the hepatic inflammatory process, activating different cytokines, and consequently, the present study assessed the cytosolic levels of this transcription factor in the hepatic tissue after the administration of pioglitazone and LPSF/GQ-02. The levels of NFκB-p65 in the animals that were treated for 15 days did not alter after the administration of the drug (Fig. 7A and 7C). However, after using the drug for 30 days, it was possible to observe a significant increase in the expression of cytoplasmic NFκB-65 in the pioglitazone and LPSF/GQ-02 groups when compared with the HFD group (Fig. 7D and 7F).

The integrity of the hepatic sinusoidal endothelium is extremely important for the maintenance of hepatic physiology and disturbance of the sinusoidal endothelium function could play an important role in the physiopathology of the liver. The expression of eNOS was assessed by western blotting after treatment with pioglitazone

and LPSF/GQ-02. The HFD, pioglitazone and LPSF/GQ-02 groups exhibited a significant reduction in the expression of eNOS after 15 days of treatment (Fig. 8A and 8B). However, after using the drugs for 30 days, a significant increase in the expression of eNOS was observed when compared to the HFD group (Fig. 8F and 8G), indicating that the increase in eNOS expression was time-dependant, after the administration of the drugs.

Insulin resistance is directly associated with the development of NAFLD. Therefore, markers of the insulin signaling pathway were assessed after treatment with pioglitazone and LPSF/GQ-02. Although the protein levels of the insulin receptor (CD-220) increased after treatment with pioglitazone and LPSF/GQ-02, this increase was not significant after either 15 or 30 days of treatment and there was no significant difference between the groups (Fig. 8Aand 8D/ Fig. 8F and 8I). However, after treatment with pioglitazone and LPSF/GQ-02 for 15 days, there was a significant increase in the protein levels of the insulin receptor substrate (IRS-1) when compared to the HFD group (Fig. 8A and 8E). Furthermore, after 30 days administering these drugs, only the LPSF/GQ-02 group induced a significant increase in the levels of IRS-1 when compared with the HFD (Fig. 8F and 8J), indicating an improvement in the signaling cascade of insulin.

ATP-binding cassette transporter AI (ABCA1) is a member of the ABC family of transporters that are necessary for the formation of HDL plasma. After treatment for 15 days, no significant differences were found in the protein expression of ABCA1 in relation to all of the groups (Fig. 8A and 8C). Similarly, after treatment for 30 days, although there was an increase in the expression of ABCA1 in the LPSF/GQ-02 group, this increase was only significant when compared to the pioglitazone group (Fig. 8F and 8H)

Discussion

Disturbances in the lipid profile, such as an increase in TGs and LDL and a reduction in HDL, are associated with NAFLD [33]. In the present study, Western diet induced significant increases in the TGs, TC and LDL profiles, similar results were achieved after treatment with pioglitazone and LPSF/GQ-02, although pioglitazone induced higher levels of LDL when compared to the HFD group. These results are according with the fact that PPARγ agonists (TZDs) do not have favorable effects on the lipid profile and are not considered a lipid-lowering class of drugs [34, 35]. However, after 30 days of treatment with pioglitazone and LPSF/GQ-02 improved plasmatic HDL was observed, possibly by promoting cholesterol efflux PPARγ dependent mechanism [36].

TZDs are considered blood glucose-lowering drugs [37] by increasing the uptake of insulin-mediated glucose in skeletal muscle, suppress the production of hepatic glucose, and improve the secretion of insulin in β cells of the pancreas [38]. In the present study, the animals treated with pioglitazone and LPSF/GQ-02 promoted an accentuated decrease in glucose levels in comparison to the HFD group. These results confirmed previous studies that demonstrated a beneficial effect on insulin sensibility of LPSF/GQ-02 [24].

According to Yang et al. [39] the main histological characteristic of NAFLD is excessive accumulation of triglycerides in hepatocytes. In addition, obesity and insulin resistance lead to an increase in the hepatic flow of free fatty acids and the accumulation of fat in the liver [40]. In the present study, histological sections were used to analyze the hepatic architecture after the administration of pioglitazone and LPSF/GQ-02. The results showed an improvement in all hepatic tissue after the use of LPSF/GQ-02, for 15

or 30 days, as evidenced by decreased vacuolization and inflammatory infiltrates, as well as better organization in all of the hepatic parenchyma. Controversially, the group that received pioglitazone was not effective in reducing hepatic damage, exhibiting similar characteristics to the HFD group. Pioglitazone is a drug classified as thiazolidinedione and is considered an agonist of PPARγ. It is known that PPARγ is elevated in murine models of diabetes and obesity [41-43]. Furthermore, the deletion of PPARγ from hepatocytes protects mice against hepatic steatosis induced by a high-fat diet [44]. These results suggest a strong association between the accumulation of fat in the liver and elevated levels of PPARγ. Recently, Zhang, et al. [45] observed that the administration of pioglitazone decreased hepatic steatosis in rats with NASH, induced by a high-fat diet. Based on all of these findings, it remains controversial to confirm if PPARγ is the causal factor or a consequence of the accumulation of fat in the liver.

Oil red O, a specific coloration for lipids, and the hepatic triglyceride dosage were used to confirm the reduction of fat in the liver of animals treated with LPSF/GQ-02. As expected, there was a significant reduction in the concentration of lipids in the livers of animals treated with LPSF/GQ-02 in both the 15-day and 30-day groups, indicating that the thiazolidine derivative LPSF/GQ-02 was effective in reducing fat in the livers of animals fed with a high-fat diet. On the other hand, the hepatic tissue of the animals treated with pioglitazone maintained a large accumulation of lipids, confirming the results found in the histology, in which pioglitazone was not effective in decreasing hepatic steatosis. Controversially, Wang et al. [46] showed that rosiglitazone reduced the levels of hepatic lipids evidenced by the reduction in Oil red O staining in rats fed with a high-fat diet. It is possible that the damaging effects exhibited by pioglitazone in the present study are specific to this molecule and do not represent a class effect.

However, the determining mechanisms for the increase in hepatic steatosis associated with TZDs remain unknown.

According to Ciupin´ska-Kajor et al [47], the fibrosis process in the liver is considered the most important step in the progression of NAFLD. Hepatic fibrosis involves the disorganization of the architecture of the hepatic tissue and the accumulation of extracellular matrix in response to pathological insults [48]. For this reason, the quantity of collagen was assessed by means of specific Sirius red staining. Similar to the results found in the histology and the staining of lipids, both the 15-day and 30-day treatment groups with LPSF/GQ-02 significantly decreased the quantity of collagen in comparison to the HFD group. On the other hand, the pioglitazone group exhibited a time-dependant response, with a significant reduction of collagen only found in the 30-day group. These results suggest effective action by LPSF/GQ-02 on the morphological factors that endanger normal liver function, leading to the development of NAFLD.

In the inflammatory profile, IL - 6 is a key element in the acute phase response, mediating the synthesis of different proteins of the acute phase (such as protein C - reactive and serum amyloid A) [49]. Furthermore, IL - 6 is considered as a marker of the prognostic of resistance to insulin and cardiovascular diseases. Elevated serum levels of IL-6 are present in animal models and in patients with NAFLD [50-52]. Similarly, Mas et al [53] demonstrated that diet-induced NASH was reduced in IL-6 knockout mice, indicating that this cytokine has a pro-inflammatory action during hepatic diseases. In the present study, LPSF/GQ-02 significantly reduced the expression of IL-6, indicating a possible hepatic anti-inflammatory action. Controversially, in the present study, pioglitazone increased the levels of IL-6 after 15 and 30 days of treatment. Esterson et al [54] studied biopsies of the adipose tissue of non-obese and

obese individuals with type 2 diabetes and reported that pioglitazone was capable of reducing the levels of IL-6 in obese individuals, although it was not effective in reducing the levels of this cytokine in non-obese individuals. Mohapatra et al. [55] studied female db/db mice and concluded that pioglitazone was effective in reducing the plasma levels of IL-6 in mice that received a sub-therapeutic (3 mg/kg) or therapeutic dose of pioglitazone (30 mg/kg). These different results related to the effect of pioglitazone on the levels of IL-6 could be partly due to the experimental model used.

During inflammation, inducible nitric oxide synthase (iNOS) plays an important role in the exacerbated production of nitric oxide, contributing to tissue damage [56]. Nitric oxide (NO) is produced endogenously by iNOS and leads to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). NO can react rapidly with superoxide anion and produce peroxynitrite, leading to the nitration of proteins [57]. In the present study, the expression of iNOS was significantly reduced after using LPSF/GQ-02 in LDLR-/- mice submitted to a HFD. Similarly, Salamone and collaborators administered silibinin and reported a significant reduction in the expression of iNOS in db/db mice submitted to experimental non-alcoholic steatohepatitis [57].

Cicloxigenase-2 (COX-2) is a key enzyme in the activation of the inflammatory response. Inflammation caused by the activation of COX-2 in the hepatic tissue plays an important role in the development of insulin resistance and NAFLD [58]. According to Yu et al. [59] the use of COX-2 inhibitors could protect against NASH in animal models. In the present study, LPSF/GQ-02 effectively reduced the expression of COX-2 in an animal model of NAFLD, strengthening the hypothesis that this thiazolidine derivative decreases inflammation in the hepatic tissue. Furthermore, these results

corroborate the findings of Silva et al. [24], who reported that LPSF/GQ-02 was effective in reducing insulin resistance.

Kupffer cells mediate the hepatic response in relation to the numerous inflammatory stimuli and can play an important role in the progression from steatosis to NAFLD [60]. In a model of NASH induced by a high-fat diet, Kupffer cells were widely recruited and activated [61]. Rivera et al. [62] used a murine model of NASH and confirmed that the reduction in the number of Kupffer cells can attenuate the histological appearance of hepatic steatosis, inflammation and necrosis, suggesting that this cellular type contributes to the pathogenesis of NASH/NAFLD. For this reason, the expression of F4/80 in the hepatic tissue was assessed after treatment with LPSF/GQ-02. The thiazolidine derivative LPSF/GQ-02 was effective in reducing positive marking for F4/80 in comparison to the HFD group, indicating the presence of a smaller population of Kupffer cells n the hepatic tissue. On the other hand, pioglitazone did not reduce the positive marking for macrophages, as evidenced by the great reactivity of F4/80 in the hepatic tissue. Similar to the results found with LPSF/GQ-02, the use of new agonists to PPARα, such as Wy 14643, was effective in reducing the recruitment of macrophages marked by F4/80, as evidenced by the immunohistochemistry of female foz/foz mice [63]. These results suggest that LPSF/GQ-02 acts directly on the inflammation, reducing the recruitment of Kupffer cells and consequently improving the pathological process triggered by NAFLD.

Interestingly, in the present study, pioglitazone did not exhibit hepatic anti-inflammatory effects when the markers IL-6, COX-2, iNOS and F4/80 were assessed. The anti-inflammatory activity of LPSF/GQ-02 may be due to a possible action in other molecular targets.

In an attempt to understand the possible active mechanism of LPSF/GQ-02 in the improvement of hepatic pathological processes, the expression of IκBα, PPARα, PPARγ, IRS-1, CD-220, ABCA1, eNOS and NFκB p65 was analyzed by western blotting analysis.

PPARα is expressed metabolically in active tissues such as the heart, kidneys, intestinal mucous, skeletal mucous and liver, regulating genes involved in the lipid metabolism, gluconeogenesis and amino acids [64]. As well as its effects on the lipid metabolism, PPARα also acts on pro-inflammatory pathways and negatively affects other signaling pathways, such as NFκB. As a result of its effects on the lipid metabolism and inflammation, PPARα can modulate physiopathological mechanisms implicated in NAFLD and atherosclerosis [65]. After assessing the expression of PPARα in the hepatic tissue, no significant alterations were found in the groups treated with LPSF/GQ-02, suggesting that this thiazolidine derivative does not affect the expression of PPARα. The reduction of lipid content, as well as the reduction of inflammation after treatment with LPSF/GQ-02, could be explained by a possible PPARα agonist action. However, this was not confirmed in the results obtained in the present study.

PPARγ is expressed in elevated levels in adipose tissue and plays an important role in the increased sensitivity to insulin, as well as promoting the capture of fatty acids for adipocytes and their differentiation. The consequent effect of these processes results in increased storage of triglycerides in the adipocytes, reducing the delivery of fatty acids to the liver [66]. On the other hand, it is widely known that the increased expression of PPARγ in the hepatic tissue in a murine model plays an important deleterious role, increasing hepatic steatosis [67]. According to Costa-Leite et al. [25], the molecular structure of LPSF/GQ-02 indicates a possible role as an agonist of

PPARγ. For this reason, the expression of PPARγ was analyzed after treatment with pioglitazone and LPSF/GQ-02. Treatment with pioglitazone increased the expression of PPARγ, although LPSF/GQ-02 was not capable of inducing significant alterations. These results confirm the agonist action of pioglitazone in relation to PPARγ, as previously described [27, 34].

The κB transcription factor (NFκB) is located (although inactive) in the cellular cytoplasm associated with regulatory proteins called κB inhibitors (IκB), such as IκBα [68]. The phosphorylation of IκBα is an important step in the activation of NFκB and, thus, these proteins become targets for specific therapy, given that the activation of NFκB is associated with the transcription of pro-inflammatory genes [69]. In the present study, prolonged treatment with LPSF/GQ-02 for 30 days induced a significant increase in the expression of IκBα. In addition, there was a significant increase in the total expression of cytoplasmic NFκB-65 in the LPSF/GQ-02 group, when compared with the HFD group, indicating an anti-inflammatory action through the inhibition of NFκB-65. By activating NFκB, IκBα liberates NFκB, thereby enabling the NFκB to be translocated to the nucleus [70]. Therefore, it is possible to infer indirectly that the greater the expression of IκBα and NFκB-65 in the cytoplasm, the lower the translocation to the nucleus will be, with a consequent decrease in inflammatory gene transcription. These results confirm the reduction of hepatic inflammation in the group treated with LPSF/GQ-02, as evidenced by the decrease in the tissue levels of IL-6, iNOS, COX-2 and F4/80.

Endothelial sinusoidal dysfunction, with the decrease in the production of intra-hepatic nitric oxide, has been considered for many years as a relevant pathogenic factor in the progression of hepatic deleterious events, such as cirrhosis [71]. Adequately functioning endothelial sinusoidal cells produce NO, inhibiting the activation of hepatic

stellate cells (HSC) and thus, representing a powerful natural antifibrotic [72]. Moreover, NO produced by the enzyme endothelial nitric oxide synthase (eNOS), in nanomolar concentrations, exhibits anti-inflammatory effects through the signaling pathways of the GMPc and can inhibit the activity of NFκB, among other things [73,74]. After the administration of LPSF/GQ-02, a significant increase of eNOS was observed in comparison to the control group, indicating that LPSF/GQ-02 could develop its anti-inflammatory and antifibrotic activities partly due to the activation of this enzyme in the hepatic tissue. It is interesting to note that the treatment with LPSF/GQ-02 was effective in decreasing the quantity of hepatic collagen and thereby reducing tissue fibrosis, as evidenced by the results obtained with eNOS.

Hepatic resistance to insulin is associated with NAFLD and is an important factor in the pathogenesis of type 2 diabetes and metabolic syndrome [75]. Although there is a general consensus that insulin resistance is caused by defects in insulin signaling, many causes have been proposed to explain how these insulin signaling defects appear in NAFLD. Inflammation, oxidative stress of the endoplasmic reticulum and the accumulation of lipids in the liver have been indicated as causes for the development of insulin resistance in animal models of NAFLD [76]. Furthermore, the production of cytokines is known to activate intracellular kinases capable of inhibiting key elements of the insulin signaling route, including proteins of the insulin receptor substrate (IRS) and phosphatidylinositol 3-kinase (PI3K) [77]. For this reason, the expression of two important molecules of the insulin signaling route was analyzed after the administration of LPSF/GQ-02: insulin receptor (CD-220) and IRS-1. Although there were no alterations in the levels of the insulin receptor, LPSF/GQ-02 significantly increased the levels of IRS-1 in comparison to the HFD group. These results possibly indicates an improving of the insulin signaling, although it is necessary to be confirmed

by analyzing Serine/Threonine or Tyrosine phosphorylation grade of IRS-1, akt/PKB or GSK, since they reflect the real state of the hepatic insulin signaling. In a previous study conducted in our laboratory, SILVA et al. [24] observed that LPSF/GQ-02 was capable of improving the sensitivity to insulin in LDLR-/- mice fed with a high-fat diet. Thus, the accumulation of hepatic fat and inflammation seem to be partly associated with altered insulin signaling in animals fed with a high-fat diet, which is restored after the treatment with LPSF/GQ-02.

ATP-binding cassette transporter AI (ABCA1) is a part of the ABC family of transporters. The fundamental role of ABCA1 in the formation of foam cells and atherosclerosis has already been established: it mediates the active transport of intracellular cholesterol and phospholipids to apolipoprotein AI, which is the main lipoprotein of the HDL. Mutations of the ABCA1 gene cause a decrease in the levels of HDL cholesterol, with a consequent increase in atherosclerosis [78]. Although not yet fully defined, it is known that the expression of apolipoprotein AI or ABCA1 in hepatocytes can reduce hepatic steatosis, decreasing the storage of lipids in the hepatocytes by transporting the lipids and also by reducing the oxidative stress of the endoplasmic reticulum, which further favors the diminishment of steatosis [79]. In the present study, although there was an increase in the protein levels of ABCA1 in the hepatic tissue after treatment with LPSF/GQ-02, this increase was not significant in relation to the HFD group. According to Ozasa et al [36], pioglitazone was effective in the efflux of cholesterol, based on the expression of ABCA1, in a manner that was dependant on PPARγ in atherosclerotic plaque. Contradictorily, in the present study, the pioglitazone group also did not increase the protein levels of ABCA1 after the use of the high-fat diet. These differences may be due to the different cellular types studied.

In summary, the present study demonstrated that the chronic consumption of a high-fat diet developed NAFLD characteristics in LDLR-/- mice while also increasing fat, hepatic fibrosis, inflammation and insulin resistance. The administration of LPSF/GQ-02 inhibited the hepatic injury, decreased inflammatory markers and increased sensitivity to insulin, suggesting an important role in the improvement of NAFLD. Therefore, LPSF/GQ-02 has become a potential candidate for the treatment of chronic hepatic pathologies triggered by the consumption of a high-fat diet. New studies are being conducted in our laboratory to investigate the possible active mechanisms of LPSF/GQ-02 on the hepatic lipid metabolism.

Author Contributions

Conceived and designed the experiments: AKSS FOSG ELR ACO BSS CAP. Performed the experiments: AKSS DOCT FOSG ELR BSS ACO LAMS CAP. Analyzed the data: AKSS FOSG CAP. Contributed reagents/materials/analysis tools: AKSS DOCT FOSG ELR BSS ACO MCAL IRP CAP. Wrote the paper: AKSS CAP.

References

1 - De Alwis NM, Day CP. (2008) Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatology 48: 104–112.

2 - Dowman JK, Armstrong MJ, Tomlinson JW, Newsome PN. (2011) Current therapeutic strategies in non-alcoholic fatty liver disease. Diabetes Obes Metab 13: 692-702.

3 – Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. (2005) Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab 288: 462-468.

4 - Tilg H, Moschen AR. (2010) Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 52: 1836-1846.

5 - Day CP, James OF. (1998) Steatohepatitis: a tale of two “hits”? Gastroenterology 114: 842-845.

6 - Keaney JF, Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, et al. (2003) Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol 23: 434–439.

7 - Shoelson SE, Lee J, Goldfine AB. (2006) Inflammation and insulin resistance. J. Clin. Invest 116: 1793–1801.

8 - Muriel P. (2009). NF-kB in liver diseases: a target for drug therapy. J. Appl. Toxicol 29: 91–100.

9 - Lawrence T. (2009). The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb. Perspect. Biol 1: 1-18.

10 - Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, et al. (2005). Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF kappaB. Nat. Med 11: 183–190.

11 - Karin M., Delhase M. (2000). The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin. Immunol. 12: 85–98.

12 - Hayden MS, Ghosh S. (2004). Signaling to NF-kappaB. Genes Dev 18: 2195–2224.

13 - Baeuerle PA, Baltimore D. (1988). I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science 242: 540–546.

14 - Ghosh S, Baltimore D. (1990). Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. Nature 344: 678–682.

15 - Girard J. Mechanisms of action of thiazolidinediones. (2001) Diabetes Metab 27: 271–8.

16 - Yki-Jarvinen H. (2004) Thiazolidinediones. N Engl J Med 351:1106–18.

17 - Watkins PB. Insight into hepatotoxicity: The troglitazone experience. (2005) Hepatology 41:229–30.

18 - Scheen AJ. Hepatotoxicity with thiazolidinediones: is it a class effect? (2001) Drug Saf 24: 873–88.

19 - Lutchman G, Modi A, Kleiner DE, Promrat K, Heller T, Grany M, et al. (2007) The effects of discontinuing pioglitazone in pateints with nonalcoholic steatohepatitis. Hepatology 46:424–9.

20 - Nissen SE, Wolski K. (2007) Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. N Engl J Med 356: 2457–71.

21 - Ratziu V, Giral P, Jacqueminet S, Charlotte F, Hartemann-Heurtier A, Serfaty L, et al. (2008) Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. Gastroenterology 135:100–10.

22 - Schalm, SW. (1997) The diagnosis of cirrhosis: clinical relevance and methodology. J Hepatol. 27(6):1118-9.

23 - Iredale, JP. (2003) Cirrhosis: new research provides a basis for rational and targeted treatments. BMJ 19:143-7.

24 - Silva, AKS, Torres DOC, Rocha SWS, Gomes FOS, Silva BS, Donato MA, et al. (2013) Effect of new thiazolidine derivatives LPSF/GQ-02 and LPSF/GQ-16 on atherosclerotic lesions in LDL receptor-deficient mice LDLR($^{-/-}$). Cardiovasc Pathol 22(1): 81-90.

25 -Leite LFC, Mourão RHV, Lima MC, Galdino SL, Hernandes MZ, Neves FAR, et al. (2007). Synthesis, biological evaluation and molecular modeling studies of arylidene-thiazolidinediones with potential hypoglycemic and hypolipidemic activities. Eur J Med Chem. 42: 1263-1271.

26 - Mourão RH, Silva TG, Soares AL, Vieira ES, Santos JN, Lima MC, et al. (2005) Synthesis and Biological Activity of Novel Acridinylidene and Benzylidene thiazolidinediones. Eur J Med Chemistry 40: 1129-1133.

27 - Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK. et al. (2000) Peroxisome proliferator-activated receptor γ ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J Clin Investig 106: 523-531.

28 - Game BA, He L, Jarido V, Nareika A, Jaffa AA, Lopes-Virella MF, et al. (2007) Pioglitazone inhibits connective tissue growth factor expression in advanced

atherosclerotic plaques in low-density lipoprotein receptor-deficient mice. Atherosclerosis 192: 85-91.

29 – Gupte, AA, Liu JZ, Ren Y, Minze LJ, Wiles JR, Collins AR, et al. (2010) Rosiglitazone attenuates age- and diet-associated nonalcoholic steatohepatitis in male low-density lipoprotein receptor knockout mice. Hepatology. 52(6): 2001-11.

30 – Bradford, MA. (1970) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72: 240–54.

31 – Montes GS, Junqueira LCU. (1988) Histochemical localization of collagen and of proteoglycans in tissues. CRC press. 41-72.

32 - Sehgal PB, Grieninger G, Tosato G (eds) (1989) Regulation of the acute phase and immune responses: interleukin-6. Ann New York Acad Sci 557:1-583.

33- Wilkins T, Tadkod A, Hepburn I, Schade RR. (2013) Nonalcoholic fatty liver disease: diagnosis and management. Am Fam Physician 88(1):35-42.

34 - Collins AR. Meehan WP, Kintscher U, Jackson S, Wakino S, Noh G, et al. (2001). Troglitazone inhibits formation of early atherosclerotic lesions in diabetic and nondiabetic low density lipoprotein receptor-deficient mice. Arterioscler Throm Vasc Biol. 21: 365-371.

35 - Napoli C, Nigris F, Welch JS, Calara FB, Stuart RO, Glass CK, et al. (2002) Maternal hypercholesterolemia during pregnancy promotes early atherogenesis in LDL receptor-deficient mice and alters aortic gene expression determined by microarray. Circulation 105: 1360–7.

36 - Ozasa, H. Ayaori M, Iizuka M, Terao Y, Uto-Kondo H, Yakushiji E, et al. (2011) Pioglitazone enhances cholesterol efflux from macrophages by increasing ABCA1/ABCG1 expressions via PPAR_/LXR_ pathway: Findings from in vitro and ex vivo studies. Atherosclerosis. 219: 141–150.

37 - Varas-Lorenzo C, Margulis AV, Pladevall M, Riera-Guardia N, Calingaert B, Hazell L, et al. (2014) The risk of heart failure associated with the use of noninsulin blood glucose-lowering drugs: systematic review and meta-analysis of published observational studies. BMC Cardiovasc Disord 14(1): 1-17.

38- Camp HS. (2003) Thiazolidinediones in diabetes: current status and future outlook. Curr Opin Investig Drugs 4:406–11.

39 -Yang LY, Kuksis A, Myher JJ, Steiner G. (1995) Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies. Journal of Lipid Research 36: 125–136.

40 - Yamaguchi L, Yang L, McCall S, Huang J, Yu XX, Pandey SK, et al. (2007). Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage

and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatology 45 (6): 1366-1374.

41 - Chao L, Marcus-Samuels B, Mason MM, Moitra J, Vinson C, Arioglu E, et al. (2000) Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. J. Clin. Invest. 106: 1221–1228.

42 - Memon RA, Tecott LH, Nonogaki K, Beigneux A, Moser AH, Grunfeld C, et al. (2000). Up-regulation of peroxisome proliferatoractivated receptors (PPAR-alpha) and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity: troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice. Endocrinology 141: 4021–4031.

43 - Bedoucha M, Atzpodien E, Boelsterli UA. (2001) Diabetic KKAy mice exhibit increased hepatic PPARgamma1 gene expression and develop hepatic steatosis upon chronic treatment with antidiabetic thiazolidinediones. J. Hepatol 35:17–23.

44 - Morán-salvador E, López-Parra M, García-Alonso V, Titos E, Martínez-Clemente M, González-Périz A, et al. (2011) Role for PPARγ in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. FASEB J 25: 2538–2550.

45 - Zhang W, Xu YZ, Liu B, Wu R, Yang YY, Xiao XQ, et al. (2014) Pioglitazone Upregulates Angiotensin Converting Enzyme 2 Expression in Insulin-Sensitive Tissues

in Rats with High-Fat Diet-Induced Nonalcoholic Steatohepatitis. Hindawi Publishing Corporation. the Scientific World Journal. Article ID 603409.

46 – Wang HN, Wang YR, Liu GQ, Liu Z, Wu PX, Wei XL, et al. (2008) Inhibition of hepatic interleukin-18 production by rosiglitazone in a rat model of nonalcoholic fatty liver disease. World J Gastroenterol 14(47): 7240-7246.

47 - Ciupińska-Kajor M, Hartleb M, Kajor M, Kukla M, Wyleżoł M, Lange D, et al. **(**2013) Hepatic angiogenesis and fibrosis are common features in morbidly obese patients. Hepatol Int. v 7 (1): 233-240.

48 - Nasir GA, Mohsin S, Khan M, Shams S, Ali G, Khan SN, et al. (2013) Mesenchymal stem cells and Interleukin-6 attenuate liver fibrosis in mice. Journal of Translational Medicine 11: 1-9.

49 - Yudkin, JS. Kumari M, Humphries SE, Mohamed-Ali V. (2000) Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 148: 209-214.

50 - Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, et al. (2005) Local and systemic insulin resitance resulting from hepatic activation of IKK beta and NF-KappaB. Nat Med 11: 183-190.

51 - Kugelmas, M. Hill DB, Vivian B, Marsano L, McClain CJ. (2003) Cytokines and NASH: a pilot study of the effects of lifestyle modifications and vitamin E. Hepatology 38: 413-419.

52 - Haukeland, JW. Damås JK, Konopski Z, Løberg EM, Haaland T, Goverud I, et al. (2006) Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. J Hepatol 44: 1167-1174.

53 - Mas, E. Danjoux M, Garcia V, Carpentier S, Ségui B, Levade T, et al. (2009) IL-6 deficiency attenuates murine diet-induced non-alcoholic steatohepatitis. PLoS One 4: 7929.

54 - Esterson YB. Zhang K, Koppaka S, Kehlenbrink S, Kishore P, Raghavan P, et al. (2013) Insulin Sensitizing and Anti-Inflammatory Effects of Thiazolidinediones Are Heightened in Obese Patients. Journal of Investigative Medicine. 61(8): 1152-1160.

55 –Mohapatra, J. Sharma M, Singh S, Chatterjee A, Swain P, Balaraman R, et al. (2009) Subtherapeutic dose of pioglitazone reduces expression of inflammatory adipokines in db/db mice. Pharmacology 84(4):203-10.

56 - Yoneda M. Hotta K, Nozaki Y, Endo H, Tomeno W, Watanabe S, et al. (2009) Influence of inducible nitric oxide synthase polymorphisms in Japanese patients with non-alcoholic fatty liver disease. Hepatol Res 39: 963-971.

57 -Salamone F, Galvano F, Cappello F, Mangiameli A, Barbagallo I, Li Volti G, et al. (2012) Silibinin modulates lipid homeostasis and inhibits nuclear factor kappa B activation in experimental nonalcoholic steatohepatitis. Res transl. 159 (6): 477-86.

58 - Tian F. Zhang YJ, Li Y, Xie Y. (2014) Celecoxib ameliorates non-alcoholic steatohepatitis in type 2 diabetic rats via suppression of the non-canonical wnt signaling pathway expression. Plos one 9(1): e83819.

59 - Yu, J. Ip E, Dela Peña A, Hou JY, Sesha J, Pera N, et al. (2006) COX-2 induction in mice with experimental nutritional steatohepatitis: Role as pro-inflammatory mediator. Hepatology 43: 826–36.

60 - Stienstra, R. Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, et al. (2010) Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxime proliferator activated receptor aplha activity. Hepatology. 51(2): 511-22.

61- Su, GL. (2002). Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 283: G256–G265.

62 – Rivera CA. Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M, et al. (2007) Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol. 47: 571–579.

63 - Larter CZ, Yeh MM, Van Rooyen D, Brooling J, Ghatora K, Farrell GC, et al. (2012). Peroxisome proliferator-activated receptor-a agonist, Wy 14 643, improves metabolic indices, steatosis and ballooning in diabetic mice with non-alcoholic steatohepatitis. Journal of Gastroenterology and Hepatology 27 341–350

64 - Gonzalez FJ, Shah YM. (2008). PPARalpha: mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. Toxicology 246: 2–8.

65 -Lalloyer F, Wouters K, Baron M, Caron S, Vallez E, Vanhoutte J, et al. (2011) Peroxisome proliferator-activated receptor-alpha gene level differently affects lipid metabolism and inflammation in apolipoprotein E2 knock-in mice. Arterioscler Thromb Vasc Biol. 31(7): 1573–1579.

66 - Kallwitz ER., MClachlan A, Cotler SJ. (2008). Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease. World J Gastroenterol 14(1): 22-28.

67- Morán-salvador E, López-Parra M, García-Alonso V, Titos E, Martínez-Clemente M, González-Périz A, et al. (2011). Role for PPARγ in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. FASEB J, 25: 2538–2550.

68 –Li Z, Nabel GJ. (1997). A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription. Mol Cell Biol. 17(10): 6184-90.

69 – Tak PP, Firestein GS. (2001) NF-kappaB: um papel chave em doenças inflamatórias. J Clin Invest. 107 (1): 7-11.

70- Thomas D Gilmore. (1999) The Rel/NF-kB signal transduction pathway: introduction. Oncogene 18: 6842 - 6844.

71 - Groszmann RJ, Wiest R. (2002) The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. Hepatology 35: 478–491.

72- Langer DA, Das A, Semela D, Kang-Decker N, Hendrickson H, Bronk SF. et al. (2008) Nitric oxide promotes caspase-independent hepatic stellate cell apoptosis through the generation of reactive oxygen species. Hepatology 47: 1983–1993.

73 - Matthews JR, Botting CH, Panico M, Morris HR, Hay RT, (1996) .Inhibition of NF-kappaB DNA binding by nitric oxide. Nucleic Acids Res. 24: 2236–2242.

74- Grumbach IM, Chen W, Mertens SA, Harrison DG. (2005) A negative feedback mechanism involving nitric oxide and nuclear factor kappa-B modulates endothelial nitric oxide synthase transcription. J Mol Cell Cardiol. 39 : 595–603.

75 - Cheung O, Sanyal AJ. (2010) .Recent advances in nonalcoholic fatty liver disease. Curr Opin Gastroenterol. 26: 202–208.

76- Savage DB, Semple RK. (2010) Recent insights into fatty liver, metabolic dyslipidaemia and their links to insulin resistance. Curr Opin Lipidol 21: 329–336.

77- Hotamisligil, G.S. (2006) Inflammation and metabolic disorders. Nature. v 444: p 860–867.

78 -Bochem AE, van Wijk DF, Holleboom AG, Duivenvoorden R, Motazacker MM, Dallinga-Thie GM, et al. (2013) ABCA1 mutation carriers with low-density lipoprotein cholesterol are characterized by a greater atherosclerotic burden. Eur Heart J. 34 (4): 286 91.

79 -Liu W, Qin L, Yu H, Lv F, Wang Y. (2014) ApoA-1 and ABCA1 expression alleviates lipid accumulation in hepatocytes. J Gastroenterol Hepatol. 29(3): 614-22.

FIGURE LEGENDS

Figure 1- **Effect of treatment with Pioglitazone and LPSF/GQ-02 for 15 and 30 days on biochemical blood parameters in LDLR−/− mice.** TC – total cholesterol, LDL - low-density lipoprotein cholesterol, TG – triglycerides, HDL - high-density lipoprotein cholesterol, AST - aspartate aminotransferase. Values were determined in plasma samples from nonfasting animals. Values expressed as mean ±S.D. Biochemical determinations performed with eight animals per group. Different letters denote significant differences between treatments, $P< 0.05$.

Figure 2 – **Effects of Pioglitazone and LPSF/GQ-02 for 15 days- hepatic histopathological analyses of LDLR-/- mice. Representative liver sections stained with H&E (A–D), oil red O (E–H) and Sirius Red (I-L):** (A) Control group. (B) HFD group exhibiting macro and microvesicular steatosis and inflammatory infiltrates (arrow). (C) Pioglitazone group showing steatosis and inflammatory infiltrates (arrow). (D) LPSF/GQ-02 group showing normal hepatic architecture tissue organization, with few lipid droplets. (E) Control group. (F) HFD group with increase in hepatic lipids. (G) Pioglitazone group showing increase in lipids content (H) LPSF/GQ-02 presenting few lipid inclusions. (I) Control group. (J) HFD group showing a great quantity of collagen in the perivenular and sinusoidal regions. (K) Pioglitazone group exhibiting a great quantity of collagen. (L) LPSF/GQ-02 group exhibiting collagen only in the perivenular region. Bars 20µm. (M,N) Quantification of labelling for Oil Red O and collagen by Sirius Red (N=5), respectively. (O) Quantification of liver triglyceride (N=5). Different letters denote significant differences between treatments, $P< 0.001$ and $P< 0.0001$, respectively.

Figure 3 – **Effects of Pioglitazone and LPSF/GQ-02 for 30 days- hepatic histopathological analyses of LDLR-/- mice. Representative liver sections stained with H&E (A–D), oil red O (E–H) and Sirius Red (I-L):** (A) Control group. (B) HFD group exhibiting macro and microvesicular steatosis and inflammatory infiltrates (arrow). (C) Pioglitazone group showing steatosis and inflammatory infiltrates (arrow). (D) LPSF/GQ-02 group showing normal hepatic architecture tissue organization, with few lipid droplets. (E) Control group. (F) HFD group with increase in hepatic lipids. (G) Pioglitazone group showing increase in lipids content (H) LPSF/GQ-02 presenting few lipid inclusions. (I) Control group. (J) HFD group showing a great quantity of collagen in the perivenular and sinusoidal regions. (K)

Pioglitazone group exhibiting a few quantity of collagen. (L) LPSF/GQ-02 group exhibiting collagen only in the perivenular region. Bars 20µm. (M,N) Quantification of labelling for Oil Red O and collagen by Sirius Red (N=5), respectively. (O) Quantification of liver triglyceride (N=5). Different letters denote significant differences between treatments, P< 0.001 and P< 0.0001, respectively.

Figura 4 - **Immunohistochemical analysis for interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and F4/80 in LDLR-/- mice treated with Pioglitazone and LPSF/GQ-02 for 15 days.** (A, E, I, M) Control group exhibited low reactivity to IL-6, iNOS, COX-2 and F4/80. HFD group showed high reactivity (B, F, J, N). Similarly, Pioglitazone group exhibited strong reaction for IL-6, iNOS, COX-2 and F4/80 (C, G, K, O). (D, H, L, P). Conversely, LPSF/GQ-02 groups exhibited reduced reactivity for IL-6, iNOS, COX-2 and F4/80. Bars 50µm. (Q,R,S,T) Quantification of labeling for IL-6, iNOS, COX-2 and F4/80 (N=5). Different letters denote significant differences between treatments, P< 0.0001, P< 0.005, P< 0.005, P< 0.0001 respectively.

Figura 5 - **Immunohistochemical analysis for interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) and F4/80 in LDLR-/- mice treated with Pioglitazone and LPSF/GQ-02 for 30 days.** (A, E, I, M) Control group exhibited low reactivity to IL-6, iNOS, COX-2 and F4/80. HFD group showed high reactivity (B, F, J, N). Similarly, Pioglitazone group exhibited strong reaction for IL-6, iNOS, COX-2 and F4/80 (C, G, K, O). (D, H, L, P). Conversely, LPSF/GQ-02 groups exhibited reduced reactivity for IL-6, iNOS, COX-2 and F4/80. Bars 50µm. (Q,R,S,T) Quantification of labeling for IL-6, iNOS, COX-2 and F4/80 (N=5). Different letters denote significant differences between treatments, P< 0.0001, P< 0.005, P< 0.005, P< 0.0001 respectively.

Figure 6 - **Western blotting analysis showing the effects of Pioglitazone and LPSF/GQ-02 on the expression of PPARα and PPARγ in the liver of LDLR-/- mice**. Treatment for 15 days did not alter the expression of PPARα (A, B). However, in the 30-day experiment, the HFD and LPSF/GQ-02 groups exhibited a decrease in the expression of PPARα when compared to the control groups (D, E). Treatment for 15 days only increased the expression of PPARγ in the pioglitazone group in relation to all other groups. (A, C) In the 30-day experiment, the HFD and LPSF/GQ-02 groups exhibited a decrease in the

expression of PPARγ when compared to the control and pioglitazone groups. (D, F) The data were analyzed using the Student's t-test. The columns represent the mean ± S.D. of the protein investigated. The results were confirmed in three different experiments (n = 5). Different letters denote significant differences between treatments, P< 0.05.

Figure 7 - **Western blotting analysis showing the effects of Pioglitazone and LPSF/GQ-02 on the expression of IkBα and NFkB p65 in the liver of LDLR-/- mice.** Treatment for 15 days did not alter the expression of the protein IkBα (A, B). However, the prolonged treatment with LPSF/GQ-02 significantly increased the expression of IkBα when compared to the control group (D, E). Treatment for 15 days did not alter the expression of the protein NFkB p65 (A, C) However, the prolonged treatment with pioglitazone and LPSF/GQ-02 significantly increased the expression of NFkB p65 when compared to the HFD group (D, F). The data was analyzed using the Student's t-test. The columns represent the mean ± S.D. of the protein investigated. The results were confirmed in three different experiments (n = 5). Different letters denote significant differences between treatments, P< 0.05.

Figure 8 - **Western blotting analysis showing the effects of Pioglitazone and LPSF/GQ-02 on the expression of eNOS, ABCA1, CD-220 and IRS-1 in the liver of LDLR-/- mice.** Treatment for 15 days reduced the concentration of eNOS in the pioglitazone and LPSF/GQ-02 groups in comparison with the control and HFD groups (A,B) However, after 30 days, an increase was observed in the expression of eNOS in the pioglitazone and LPSF/GQ-02 groups in comparison to the HFD group (F,G). The 15-day treatment did not alter the levels of ABCA1 in relation to all of the groups. (A,C) After 30 days, significant differences of ABCA1 were only found between the pioglitazone and LPSF/GQ-02 groups. (F,H). Treatment for 15 or 30 days did not alter the expression of the insulin receptor CD-220 in all of the groups studied (A,D,F,I). The 15-day treatment with pioglitazone and LPSF/GQ-02 increased the protein levels of IRS-1 when compared with the HFD group. (A,E) LPSF/GQ-02 increased the levels of IRS-1 after thirty days in comparison with the HFD group. (F,J). The data was analyzed using the Student's t-test. The columns represent the mean ± S.D. of the protein investigated. The results were confirmed in three different experiments (n = 5). Different letters denote significant differences between treatments, P< 0.05.

FIGURE 1

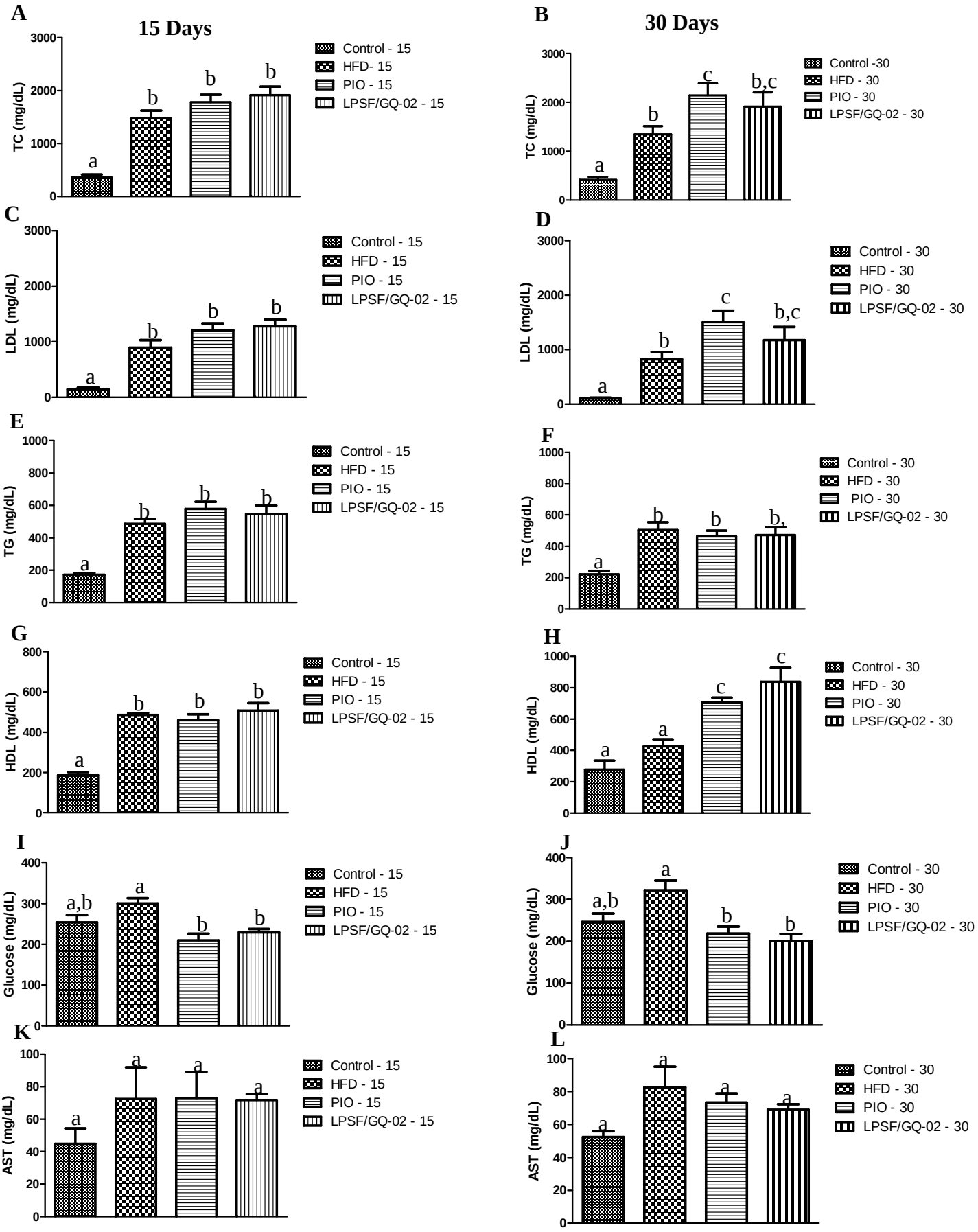
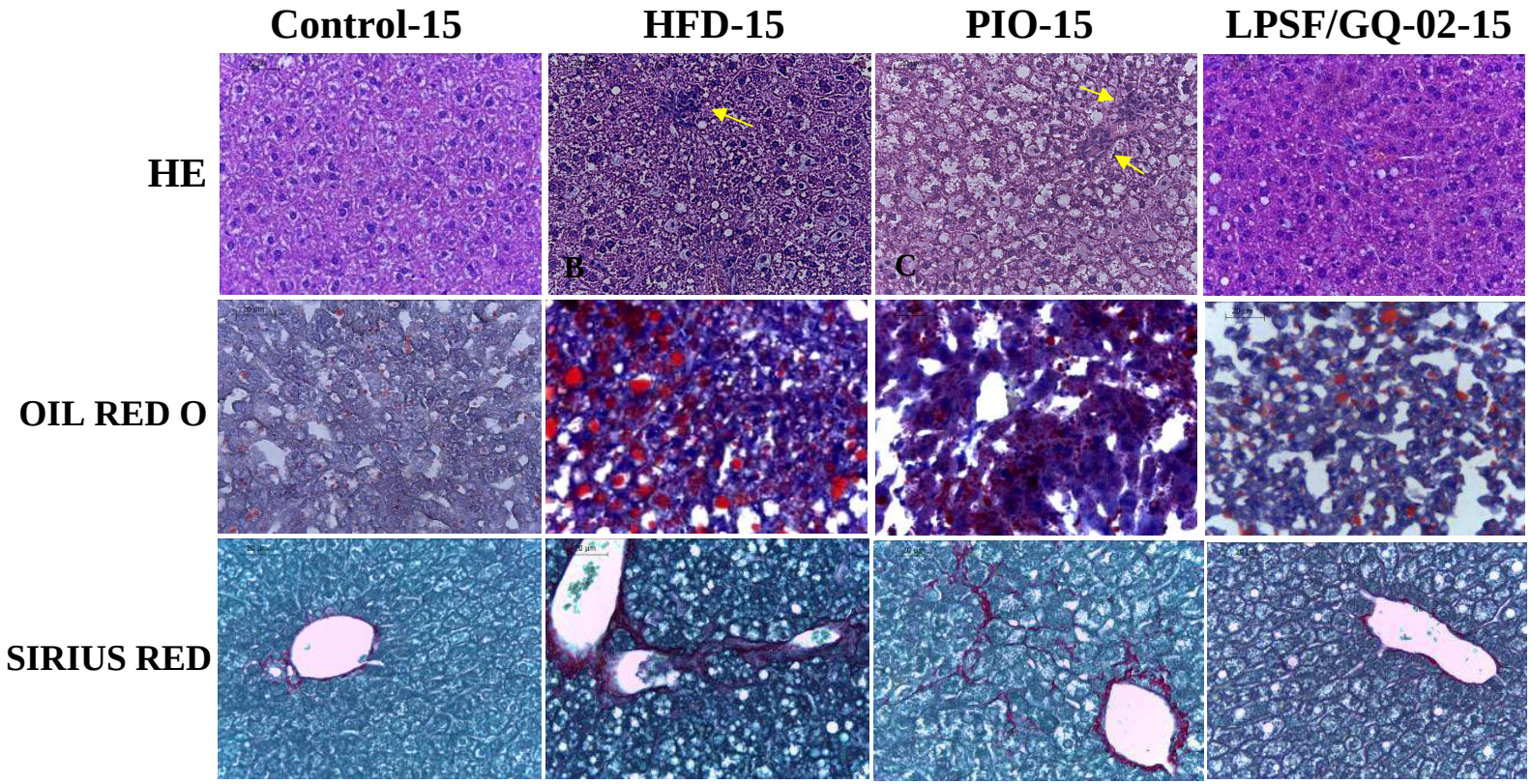
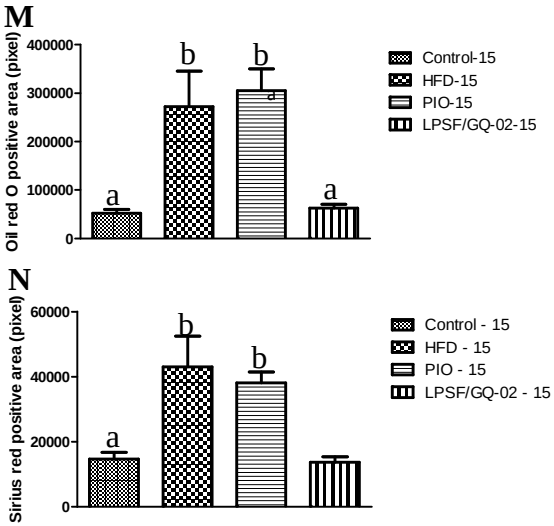

A
15 Days
TC (mg/dL)
Control - 15
HFD- 15
PIO - 15
LPSF/GQ-02 - 15
B
30 Days
TC (mg/dL)
Control -30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
C
LDL (mg/dL)
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
D
LDL (mg/dL)
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
E
TG (mg/dL)
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
F
TG (mg/dL)
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
G
HDL (mg/dL)
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
H
HDL (mg/dL)
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
I
Glucose (mg/dL)
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
J
Glucose (mg/dL)
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
K
AST (mg/dL)
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
L
AST (mg/dL)
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30

FIGURE 2


Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
HE
OIL RED O
SIRIUS RED
B
C


M
Oil red O positive area (pixel)
400000
300000
200000
100000
0
a
b
b
a
Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
N
Sirius red positive area (pixel)
60000
40000
20000
0
a
b
b
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15

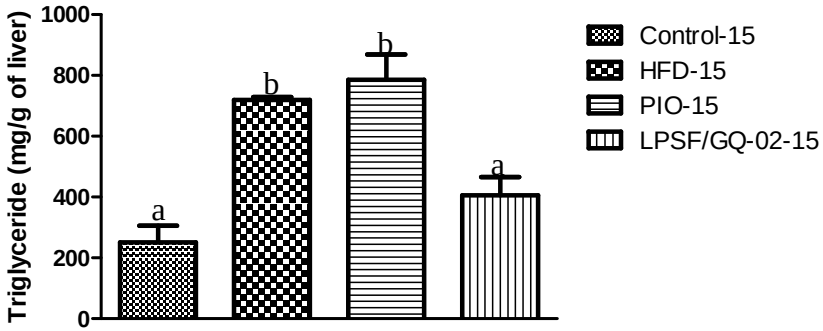
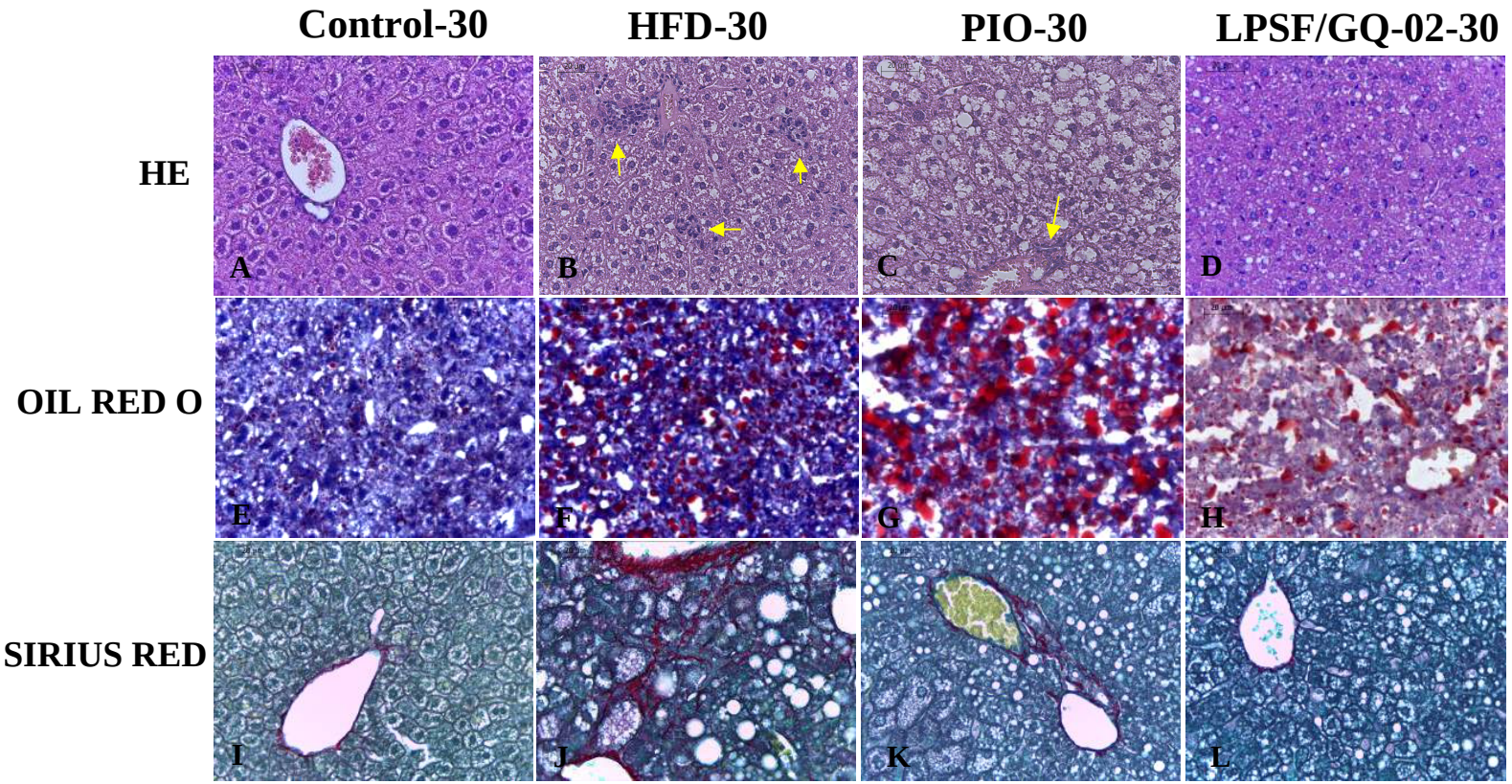
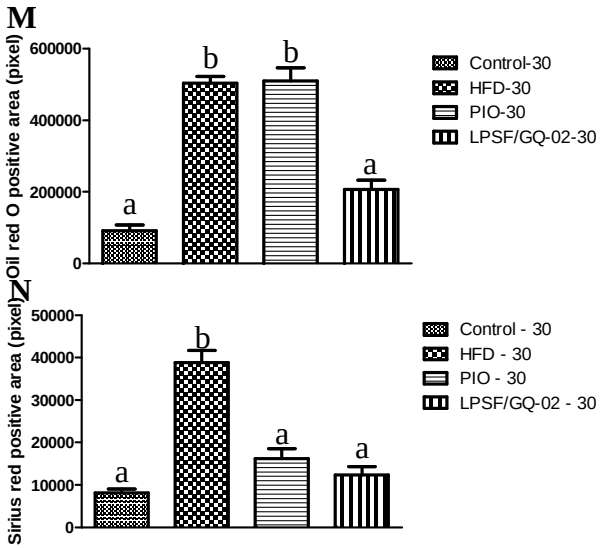

Triglyceride (mg/g of liver)
1000
800
600
400
200
0
a
b
b
a
Control-15
HFD-15
PIO-15
LPSF/GQ-02-15

FIGURE 3


Control-30
HFD-30
PIO-30
LPSF/GQ-02-30
HE
OIL RED O
SIRIUS RED
A
B
C
D
E
F
G
H
I
J
K
L


M
Oil red O positive area (pixel)
600000
400000
200000
0
a
b
b
a
Control-30
HFD-30
PIO-30
LPSF/GQ-02-30
N
Sirius red positive area (pixel)
50000
40000
30000
20000
10000
0
a
b
a
a
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30

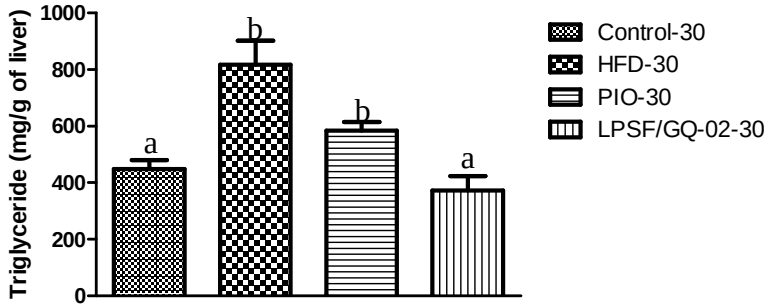

Triglyceride (mg/g of liver)
1000
800
600
400
200
0
a
b
b
a
Control-30
HFD-30
PIO-30
LPSF/GQ-02-30

FIGURE 4

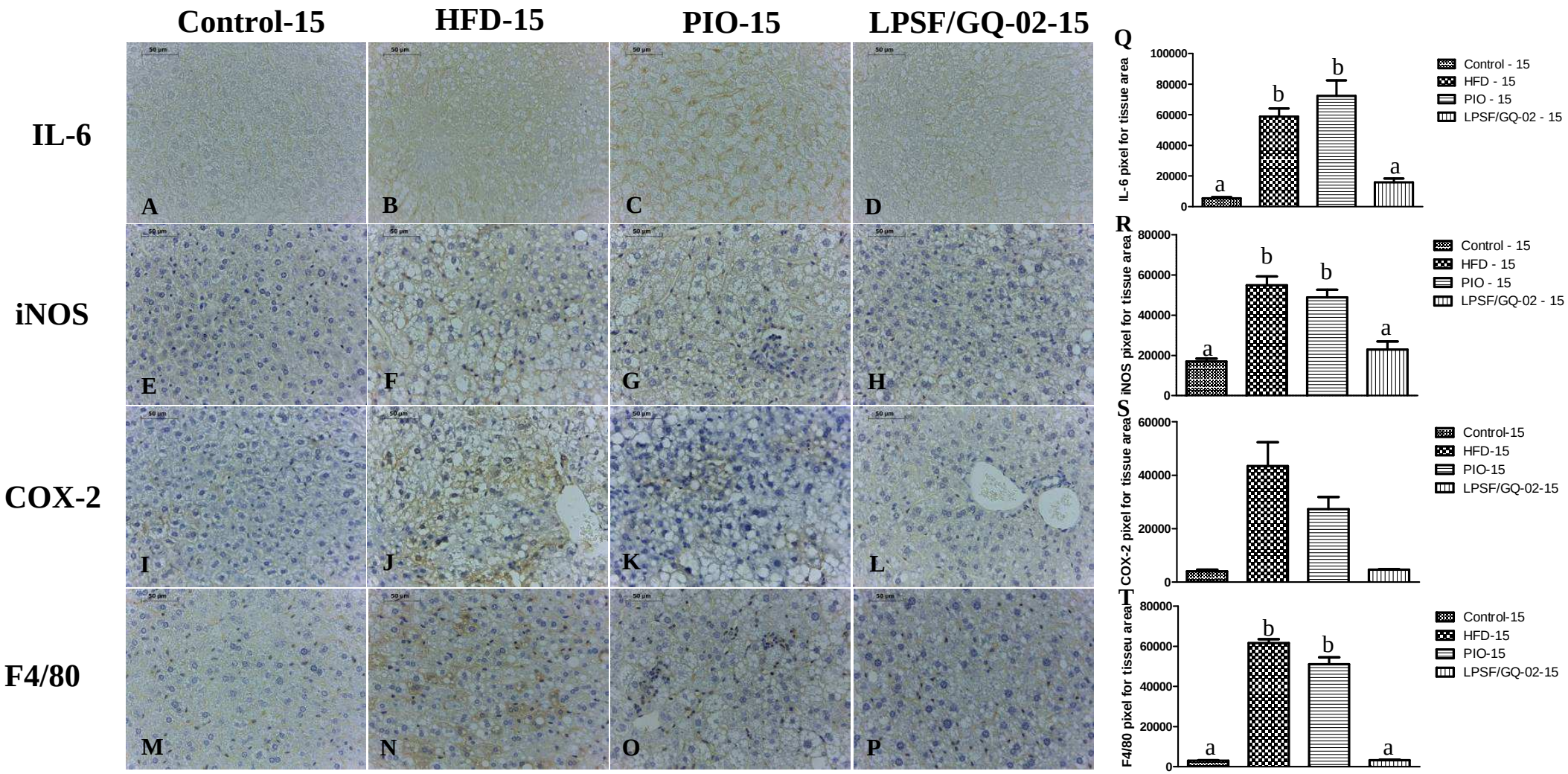

Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
IL-6
iNOS
COX-2
F4/80
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
IL-6 pixel for tissue area
R
iNOS pixel for tissue area
S
COX-2 pixel for tissue area
T
F4/80 pixel for tisseu area
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
a
b

FIGURE 5

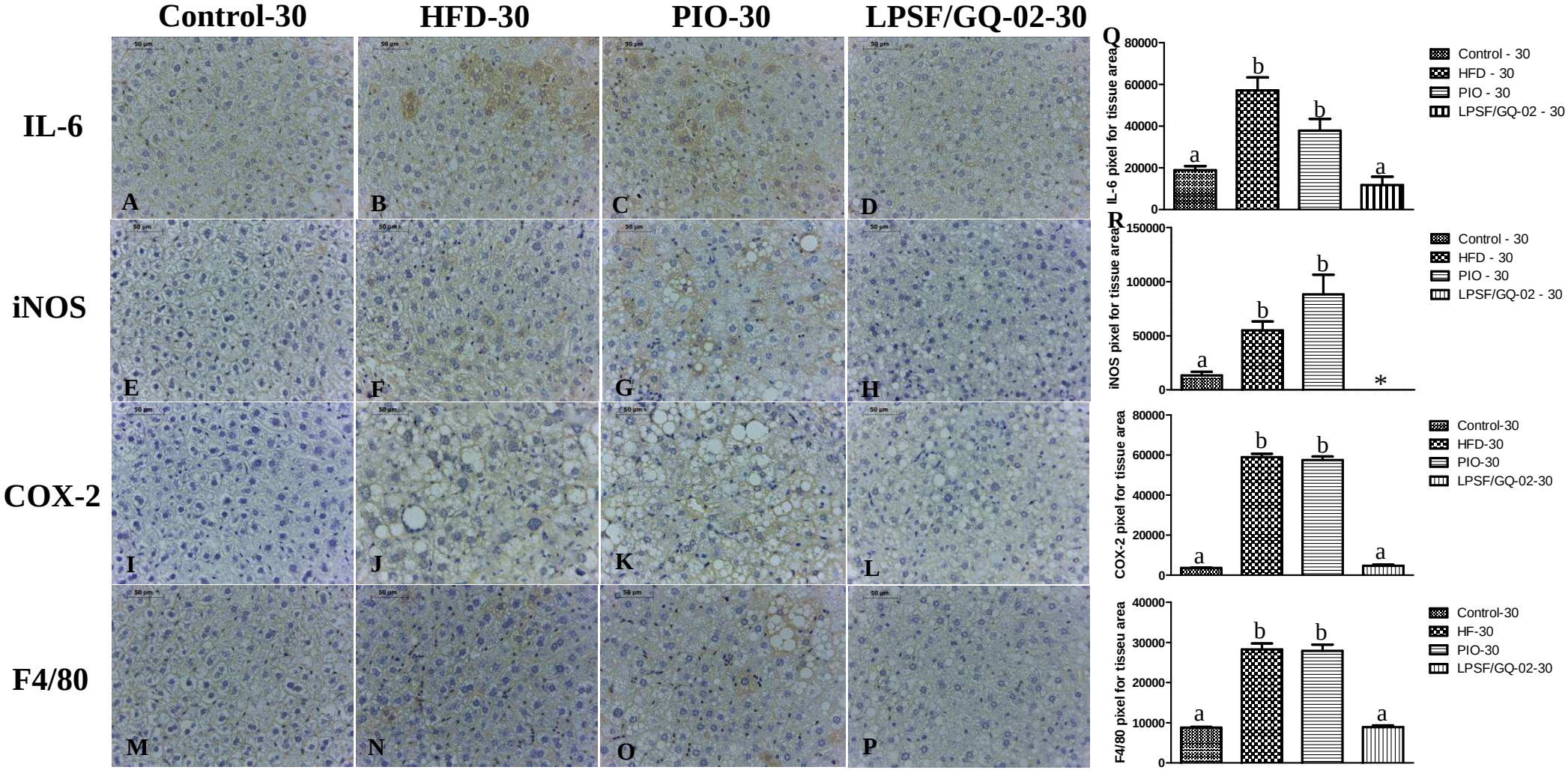

Control-30
HFD-30
PIO-30
LPSF/GQ-02-30
IL-6
iNOS
COX-2
F4/80
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
IL-6 pixel for tissue area
iNOS pixel for tissue area
COX-2 pixel for tissue area
F4/80 pixel for tisseu area
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
Control-30
HFD-30
PIO-30
LPSF/GQ-02-30
HF-30
a
b
*

FIGURE 6

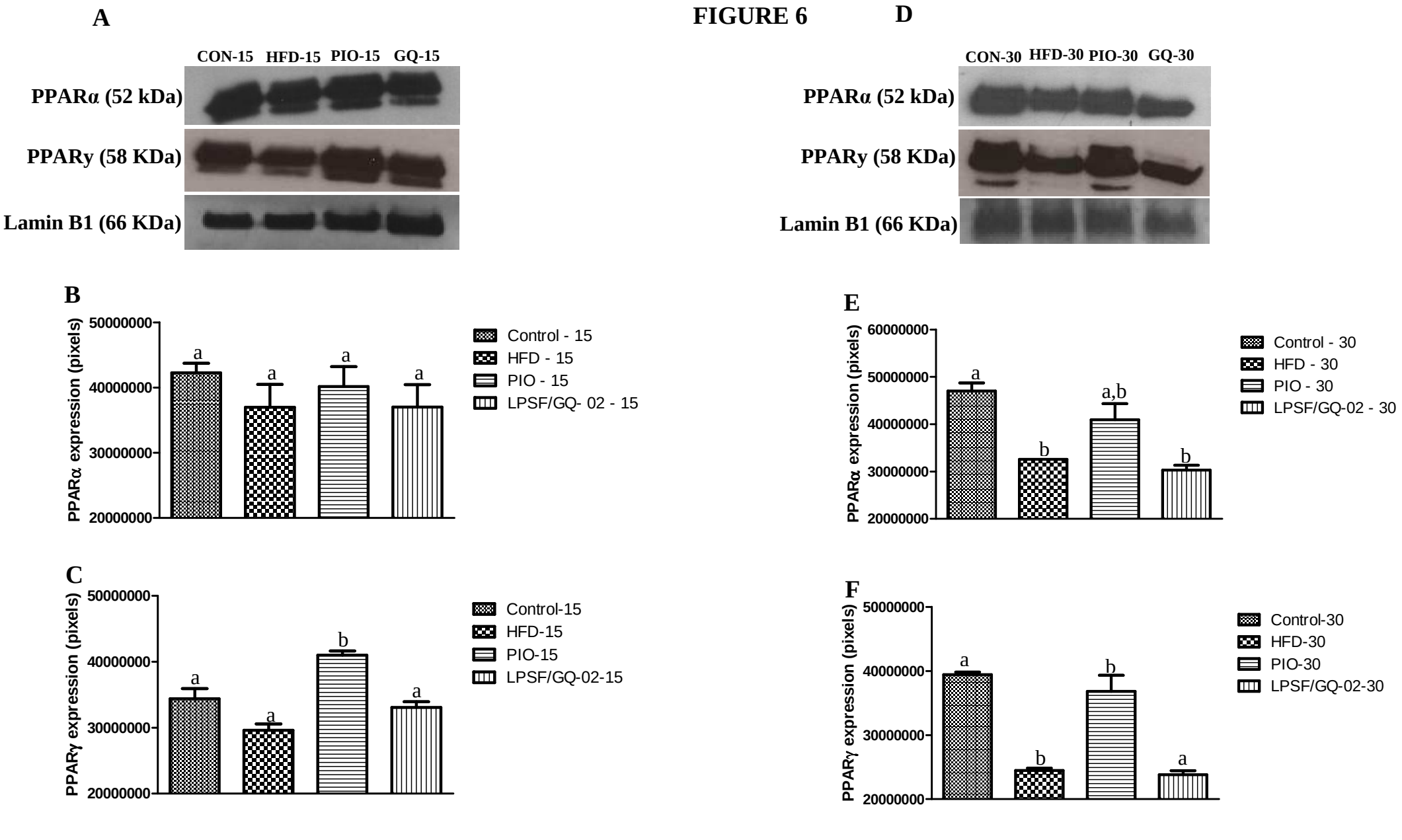
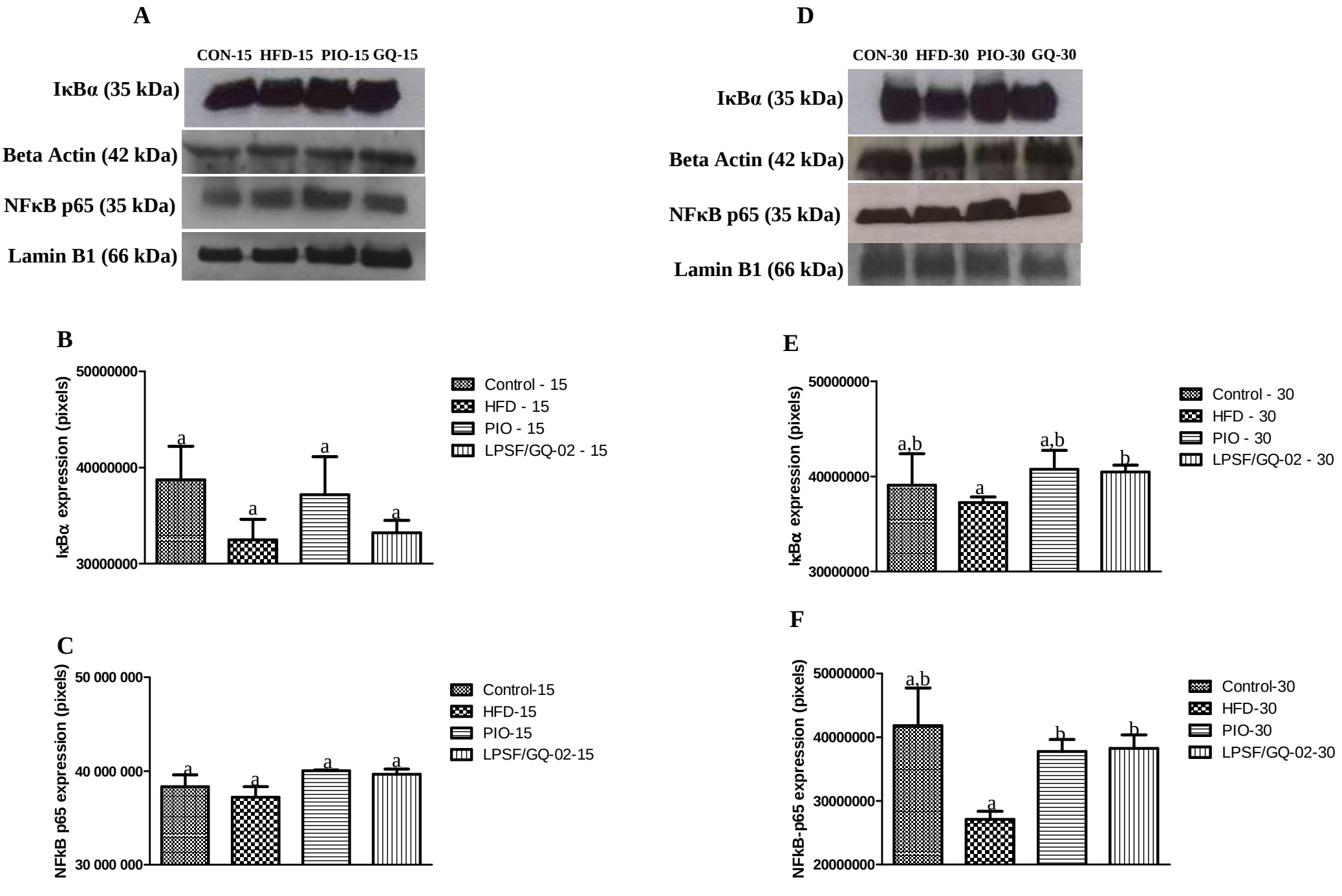
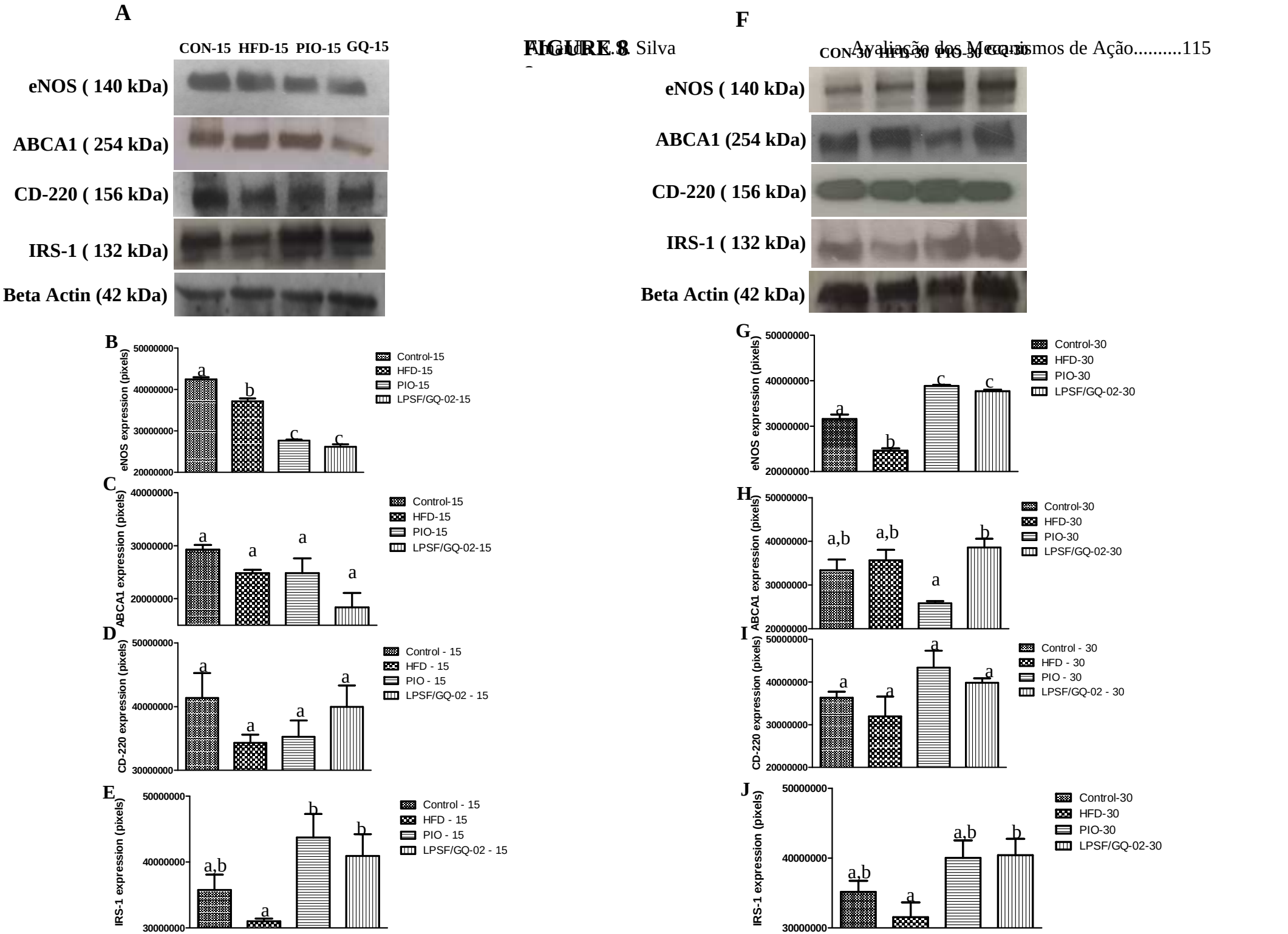
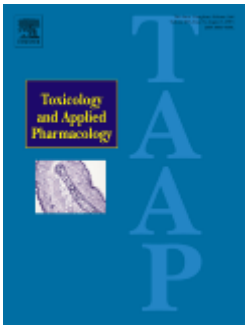

A
CON-15 HFD-15 PIO-15 GQ-15
PPARα (52 kDa)
PPARy (58 KDa)
Lamin B1 (66 KDa)
D
CON-30 HFD-30 PIO-30 GQ-30
PPARα (52 kDa)
PPARy (58 KDa)
Lamin B1 (66 KDa)
B
PPARα expression (pixels)
50000000
40000000
30000000
20000000
a a a a
Control - 15
HFD - 15
PIO - 15
LPSF/GQ- 02 - 15
E
PPARα expression (pixels)
60000000
50000000
40000000
30000000
20000000
a b a,b b
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
C
PPARγ expression (pixels)
50000000
40000000
30000000
20000000
a a b a
Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
F
PPARγ expression (pixels)
50000000
40000000
30000000
20000000
a b b a
Control-30
HFD-30
PIO-30
LPSF/GQ-02-30

FIGURE 7


A
CON-15 HFD-15 PIO-15 GQ-15
IκBα (35 kDa)
Beta Actin (42 kDa)
NFκB p65 (35 kDa)
Lamin B1 (66 kDa)
D
CON-30 HFD-30 PIO-30 GQ-30
IκBα (35 kDa)
Beta Actin (42 kDa)
NFκB p65 (35 kDa)
Lamin B1 (66 kDa)
B
IκBα expression (pixels)
50000000
40000000
30000000
a
a
a
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
E
IκBα expression (pixels)
50000000
40000000
30000000
a,b
a
a,b
b
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
C
NFkB p65 expression (pixels)
50 000 000
40 000 000
30 000 000
a
a
a
a
Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
F
NFkB-p65 expression (pixels)
50000000
40000000
30000000
20000000
a,b
a
b
b
Control-30
HFD-30
PIO-30
LPSF/GQ-02-30


A
CON-15 HFD-15 PIO-15 GQ-15
eNOS (140 kDa)
ABCA1 (254 kDa)
CD-220 (156 kDa)
IRS-1 (132 kDa)
Beta Actin (42 kDa)
F
CON-30 HFD-30 PIO-30 GQ-30
eNOS (140 kDa)
ABCA1 (254 kDa)
CD-220 (156 kDa)
IRS-1 (132 kDa)
Beta Actin (42 kDa)
B
eNOS expression (pixels)
Control-15
HFD-15
PIO-15
LPSF/GQ-02-15
G
eNOS expression (pixels)
Control-30
HFD-30
PIO-30
LPSF/GQ-02-30
C
ABCA1 expression (pixels)
H
ABCA1 expression (pixels)
D
CD-220 expression (pixels)
Control - 15
HFD - 15
PIO - 15
LPSF/GQ-02 - 15
I
CD-220 expression (pixels)
Control - 30
HFD - 30
PIO - 30
LPSF/GQ-02 - 30
E
IRS-1 expression (pixels)
J
IRS-1 expression (pixels)

Capítulo III



Artigo II (Submetido)

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Chronic LPSF/GQ-02 treatment attenuates inflammation and atherosclerosis development in LDLr-/- mice

Amanda Karolina Soares e Silva[a,b*], Fabiana Oliveira dos Santos Gomes[a,b], Bruna dos Santos Silva[a,b], Edlene Lima Ribeiro[a,b], Amanda Costa Oliveira[a,b], Shyrlene Meyre da Rocha Araújo[a,b], Ingrid Tavares de Lima[a,b], Anne Gabrielle Vasconcelos Oliveira[c], Martina Rudnicki[d], Dulcineia S P Abdalla[d], Ivan da Rocha Pitta[e], Christina Alves Peixoto[a]

[a]*Laboratório de Ultraestrutura, Centro de Pesquisa Aggeu Magalhães (FIOCRUZ), Recife, Pernambuco,Brasil*

[b]*Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.*

[c]*Centro de Tecnologias Estratégicas do Nordeste (CETENE), Recife, Pernambuco,Brasil*

[d]*Departmento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêutica, Universidade de São Paulo, São Paulo, SP, Brasil*

[e]*Laboratório de Planejamento e Síntese de Fármacos, Universidade Federal de Pernambuco, Recife, Brasil*

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[a]*Laboratório de Ultraestrutura, Centro de Pesquisa Aggeu Magalhães (FIOCRUZ), Recife, Pernambuco,Brasil*

[b]*Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.*

[c]*Centro de Tecnologias Estratégicas do Nordeste (CETENE), Recife, Pernambuco,Brasil*

[d]*Departmento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêutica, Universidade de São Paulo, São Paulo, SP, Brasil*

[e]*Laboratório de Planejamento e Síntese de Fármacos, Universidade Federal de Pernambuco, Recife, Brasil*

[*]Corresponding address: MSc Amanda Karolina Soares e Silva, Laboratório de Ultraestrutura, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ, Av. Moraes Rego s/n, Recife, Brasil. CEP 50670-420.

Fax number: (55) 81 21012557. Email: kls.amanda@gmail.com

ABSTRACT

Background: Atherosclerosis is a complex disorder with a multifactorial pathogenesis. We previously indicated that the new TZD LPSF/GQ-02 inhibits hepatic steatosis and inflammation, which are reported as risk factors for atherosclerosis development. Here, we explored the effects of LPSF/GQ-02 on atherosclerosis in $LDLr^{-/-}$ mice comparing two treatment periods.

Methods and results: $LDLr^{-/-}$ mice were fed a high-fat diet for 10 and 12 weeks and received oral treatment with LPSF/GQ-02 (30mg/kg/day) or pioglitazone (20mg/kg/day) for 15 and 30 days, respectively. Both treatment protocols with LPSF/GQ-02 resulted in lower collagen density in the atherosclerotic lesions. In addition, the treatment for 15 days also decreased mRNA levels of CD40, MCP-1, ABCG1 and upregulated PPARα, whereas the 30-days treatment reduced the protein levels of LOX-1, p-IκBα and p-NFκB.

Conclusion: This study provides evidence that LPSF/GQ-02 affects the composition and growth of atherosclerotic lesions in $LDLr^{-/-}$ mice. Moreover, our data also support previous findings showing anti-inflammatory properties of LPSF/GQ-02 and reinforce the therapeutic potential of this TZD for treating atherosclerosis and inflammation-related disorders.

Keywords: Atherosclerosis, LPSF/GQ-02, anti-inflammatory, Thiazolidinediones.

INTRODUCTION

According to the World Health Organization, cardiovascular diseases (CVD) are responsible for the highest number of death compared to any other pathology. In this context, ischemic heart diseases and stroke are the leading causes of heart disease. Atherosclerosis, the underlying cause of most CVD is a chronic inflammatory disease characterized by the accumulation of modified lipids, cholesterol and inflammatory cells in the walls of medium and large arteries (Mclaren et al., 2011). In addition, atherosclerosis presents a multifactorial pathogenesis with numerous risk factors, including diabetes, dyslipidemia, smoking, hypertension and non-alcoholic fatty liver disease (NAFLD) (Fruchart et al., 2004).

NAFLD refers to a wide spectrum of liver diseases caused by fat deposit in the liver cells in the absence of excess alcohol consumption. Compelling evidence has demonstrated that NAFLD is not only an independent risk factor for CVD, but also might be a predictor of CVD (Targher et al., 2008, Ahmed, et al. 2012). In fact, previous studies have revealed that the severity of liver histology in NAFLD patients closely correlates with markers of early atherosclerosis (Alkhouri et al., 2010). Moreover, atherosclerosis and NAFLD might share common metabolic and molecular mediators. As a result, NAFLD plays a relevant role in the pathogenesis of atherosclerosis (Than and Newsome, 2015).

Since atherosclerosis and NAFLD might share common mechanisms, similar therapeutic strategies could be employed in the treatment of both disorders. The improvement in insulin resistance, thereby modulating cardiometabolic risk factors comprises an effective treatment of NAFLD that might prevent the development of atherosclerosis (Brea and Puzo, 2013; Than and Newsome, 2015). Thiazolidinediones (TZDs), which served as high-affinity the peroxisome proliferator-activated receptor

gamma (PPARγ) agonists, are widely used as insulin-sensitizers in the treatment of type 2 diabetes. In addition to their antidiabetic properties, recent evidence suggests that TZDs also displayed potent antiatherosclerotic effects and might improve steatosis and hepatocyte ballooning described in NAFLD conditions (Rakoski et al., 2010).

LPSF/GQ-02, compound 5-(4-Chloro-benzylidene)-3-(4-methylbenzyl)-thiazolidine-2,4-dione is a new TZD derivate. Previous results demonstrated that the treatment with LPSF/GQ-02 for 15 days effectively decreased the risk factors associated with the development of atherosclerosis, such as insulin resistance and inflammation, consequently reducing atherosclerotic lesions in LDL receptor deficient ($LDLr^{-/-}$) mice (Silva, et al., 2013). Interestingly, the treatment with LPSF/GQ-02 for 15 days also presented beneficial effects in a mouse model of NAFLD, improving the hepatic architecture, decreasing fat and collagen accumulation as well as inflammatory marker levels (Silva et al, 2015). It is also noteworthy that prolonged treatment with LPSF/GQ-02 leads to greater beneficial effects on mice model of NAFLD. In fact, besides similar results observed with the short treatment, the treatment with LPSF/GQ-02 for 30 days also improved the lipid profile of mice and modulate NF-κB signaling pathway. Since only the effects of short treatment with LPSF/GQ-02 were explored on atherogenesis, the purpose of this study was to establish the effects of prolonged LPSF/GQ-02 treatment on atherosclerosis in $LDLr^{-/-}$ mice and compare to those findings from short LPSF/GQ-02 treatment.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the ethical principles in animal experimentation adopted from the Colégio Brasileiro de Experimentação Animal (COBEA). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Fundação Oswaldo Cruz – FIOCRUZ (Permit Number: L-010/09).

Synthesis of Thiazolidine Derivative LPSF/GQ-02

LPSF/GQ-02, 5-(4-chloro-benzylidene)-3-(4-methyl benzyl)-thiazolidine- 2,4-dione was synthesized at the Department of Antibiotics of the Universidade Federal de Pernambuco (Brazil) following the methodology described by Mourão et al., 2005.

Study design

Homozygous LDLr$^{-/-}$ male mice (C57BL/6 background) were obtained from Jackson Laboratories (USA) and bred in the vivarium of the *Centro de Pesquisas Aggeu Magalhães*. Prior to the protocol, the state of the health of mice was determined. After weaning, sixty mice were fed a standard diet for eight days of adaptation. After this period, mice received an atherogenic diet (*PragSoluções Biociências*) for 10 weeks (Li et al., 2000), were separated into three groups (n=10) and submitted to daily treatment by gavage with pioglitazone and LPSF/GQ-02 for 15 days, as follows:

HFD15 – This group received the atherogenic diet (HFD) consisting of 21% milk fat and 1.25% cholesterol (Li et al., 2000).

PIO15 – This group received HFD and was treated with 20mg/kg/day of pioglitazone (Actos®) for 15 days (Game et al., 2007)

LPSF/GQ-02–15 - This group received HFD and was treated with 30mg/kg/day of glitazone LPSF/GQ-02 for 15 days (Mourão et al., 2005).

A second experiment assessed the glitazones for a longer period of time. The animals were submitted to the above mentioned criteria, although they received the atherogenic diet for 12 weeks (Gupte et al., 2010) and the drugs were administered during the last four weeks of the experimental diet, totaling 30 days of treatment with glitazones (*HFD30/ PIO30/ LPSF/GQ-02–30*). Mice were maintained in plastic cages at 22ºC (±1°C) on a 12 h light-dark cycle and received water and chow *ad libitum* during all the experiment. At the end of the treatment, mice were anesthetized (Ketamine/Xylasine). Blood was collected by cardiac puncture and it was transferred into dry tubes. The serum was separated and stored at -20°C for biochemical measurements. Aortas were dissected, fixed for posterior processing (morphological analysis) and frozen at - 80°C for posterior Western blotting and real-time PCR analysis.

Masson's Trichrome Stain

The cuts were stained with Masson's trichrome to assess the amount of collagen in the aorta. The slides were pre-treated with xylene to remove paraffin and hydrated with a decreasing amount of ethanol. Subsequently, the cuts were stained with working hematoxylin solution for 10 minutes. Next, the slides were immersed in Biebrich scarlet for 5 minutes and Rinse in distilled water. Next, slides were treated with Phosphotungstic/phosphomolybdic acid solution for another 10 minutes and immediately transferred directly into Aniline blue for 5 minutes, washed in running water for 2 minutes and lastly treated with 1% acetic acid solution for 1 minute. After this process, the slides were dehydrated in ethanol at 100%, cleared in xylene and

mounted. Five images of the same magnification were quantitatively analyzed using Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

Cytosolic and Nuclear protein extraction

Cytosolic and nuclear proteins from aortas were isolated using Cayman's Nuclear Extraction kit (Cayman chemical company, Ann Arbor, Michigan, USA). Aorta fragments were homogenized in a hypotonic buffer supplemented with DTT and Nonidet P-40 per gram of tissue. Aortas were then centrifuged and re-suspended by adding specified assay reagents, following the manufacturer's instructions. The cytosolic and nuclear fractions were stored in pre-chilled vials at -80˚C until further analysis. Aorta cytosol proteins were used to determine the quantity LOX-1 and p-IkBα in the immunoblotting, whereas nuclear fractions were used for p-NFkB-p65.

Measurement of protein levels

The total, cytosolic and nuclear extraction protein levels were determined using the Bradford method, with bovine serum albumin as standard (Bradford, 1970). The optical density of samples was measured at 660 nm on a spectrophotometer. All samples were run in duplicate and the mean of the two absorbance levels was used to determine protein quantification. The protein concentration per sample amount was determined using the equation from a calibration curve. The curve was generated using the same method as the samples, with the substitution of bovine serum albumin at five concentration levels.

Western blot analysis

After protein quantification, 40 μg of total protein was loaded on 12% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. (Bio Rad, CA, USA, Ref. 162-0115). The membranes were blocked overnight at 4ºC with 5% non-fat milk in TBS-T (Tris-buffered saline 0.1% plus 0.05% Tween 20, pH 7.4) followed by incubation with antibodies against the following: LOX-1 (1:500, Santa Cruz Biotechnology, CA), p-IkBα (1:1000, Abcam Cambridge, UK) and p-NFkB-p65 (1:1000, Abcam Cambridge, UK) diluted in TBS-T buffer solution containing 3% non-fat milk for 3h at room temperature. After washing (six times, 10 min each) in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:80000, Sigma, USA), diluted in TBS-T with 1% nonfat milk, for 1h 30min at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used to visualize the labeled protein bands and the blots were developed on X-ray film (Fuji Medical, Kodak, Ref. Z358487-50EA). For quantification, the density of pixels of each band was determined by Image J 1.38 software (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD). The results were confirmed in three sets of experiments for each protein investigated. Immunoblotting for β-actin was performed as a control for the above protein blots. After protein blot visualization with enhanced chemiluminescence, the protein antibodies were stripped from the membranes, which were reprobed with monoclonal anti-β-actin antibody (1:2000, Sigma, USA), and protein densitometry was performed.

Quantitative real-time PCR analysis

Total RNA was isolated from cells of atherosclerotic lesion areas using trizol reagent (Life Technologies), and 2 μg of RNA were reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit, according to the manufacturer's instructions (Life Technologies). Quantitative real-time PCR was performed in an ABI 7500 Fast System (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Life Technologies) and specific primer pairs for ABCA-1, ABCG-1, MCP-1, CD-36, CD-40, LXRα, PPARα, PPARγ and GAPDH (table 1). The primers were designed using Primer Express version 2.0 (Applied Biosystems). The cycling program was 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and single-cycle dissociation stage. Each sample was run in triplicate. The expression of each target gene was normalized GAPDH RNA relative expression as an internal efficiency control. The mRNA fold change relative to the control was calculated using the 2(−Delta Delta c(t)) method (Livak and Schmittgen, 2001).

Table 1. Forward and reverse primers used for real-time PCR

Gene	Forward primer	Reverse primer
ABCA-1	5´-GGTTTGGAGATGGTTATACAATAGTTGT-3'	5´-TTCCCGGAAACGCAAGTC-3'
ABCG-1	5´-CCTTCCTCAGCATCATGCG-3'	5´-CCGATCCCAATGTGCGA-3'
CD-36	5´- TTTCCTCTGACATTTGCAGGTCTA -3'	5´- AAAGGCATTGGCTGGAAGAA -3'
CD-40	5’ – CTGGACAAGCTGTGAGGATAAG – 3’	5’ – TAGAGAAACACCCCGAAAATG – 3’
GAPDH	5’- TGCACCACCAACTGCTTAGC -3’	5’-GCCCACGGCCATCA -3’
LXRα	5´-CAGAGAGGAAGCCAGGATGCC–3´	5´-GAGCGCCGGTTACACTGTTGC–3´
MCP-1	5’-TGAGTAGGCTGGAGAGCTACAA-3’	5’-ATGTCTGGACCCATTCCTTC-3’
PPARα	5´- TCAGGGTACCACTACGGAGT–3´	5´-CTTGGCATTCTTCCAAAGCG–3´
PPARγ	5’-CACAGAGATGCCATTCTGGC-3’	5’-GGCCTGTTGTAGAGCTGGGT-3’

Statistical analysis

GraphPad Prism software (version 5) was used for the statistical analysis. Data were expressed as mean ± standard deviation (S.D.). Differences between the control

and treated groups were analyzed using analysis of variance (ANOVA), prior to the performance Tukey's post hoc test or the Student's t-test. Probability values less than 0.05 were considered significant.

RESULTS

Effects of LPSF/GQ-02 on the content collagen in aorta

Collagen and proteins that constitute the extracellular matrix are suggested to play important roles in the progression of atherosclerotic lesion. Therefore, we evaluated the content of collagen in the aorta of LDLr -/- mice after LPSF/GQ-02 treatment. As illustrated in Fig 1, collagen accumulation was decreased by treatment with LPSF/GQ-02. Although the reduction in the collagen accumulation induced by LPSF/GQ-02 treatment for 15 days was significant when compared to HFD and PIO groups (Fig. 1A-C), the treatment with LPSF/GQ-02 for 30 days reduced the deposition of collagen significantly only when compared to PIO group (Fig. 1D-F). Quantitative analysis of the collagen content was performed in all groups using ANOVA and Tukey's post hoc tests (Fig. 1G and 1H).

Influence of LPSF/GQ-02 on gene expression of protein involved in reverse cholesterol transport

Both ABCA-1 and ABCG-1 play a pivotal role in the removal of excess intracellular cholesterol and phospholipids to lipid-poor apolipoprotein acceptors in the initial step of reverse cholesterol transport. As shown in Fig. 2, only the treatment with PIO for 30 days increased the mRNA levels of ABCA-1 and ABCG1. On the other

hand, expression of ABCA-1 mRNA was unchanged in aorta of LPSF/GQ-02-treated mice (Fig. 2A and B). In addition, the treatment with LPSF/GQ-02 for 15 days suppressed ABCG1 mRNA whereas no effect was observed in the aorta of mice treated with LPSF/GQ-02 for 30 days (Fig. 2C and D).

Action of LPSF/GQ-02 on expression of scavenger receptors LOX-1 and CD-36

LOX-1 and CD-36 are receptors involved in the binding and internalization of oxidized low-density lipoprotein (ox-LDL) cholesterol particles, thereby playing an essential role in pro-atherogenic mechanisms including endothelial dysfunction and foam cell formation. In order to establish if LPSF/GQ-02 treatment could modulate levels of these receptors, we evaluated CD-36 mRNA and LOX-1 protein levels. As can be seen in Fig. 3A, no significant difference was detected on LOX-1 expression after 15 days of treatment with the TZDs (Fig. 3A). Conversely, after 30 days of treatment, the LSF/GQ-02 downregulated the expression of LOX-1 comparing with the HFD and PIO groups, indicating a possible beneficial effect in reducing atherosclerotic plaques. On the other hand, treatment with PIO for 15 and 30 days upregulated the mRNA levels of CD-36 when compared to HFD and LPSF/GQ-02 groups (Fig. 3C-D). Unlike PIO, treatment with LPSF/GQ-02 did not affect the mRNA level of CD-36.

LPSF/GQ-02 treatment induces anti-inflammatory effects in mice with atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by lipid and cholesterol accumulation within the walls of large and medium arteries. Thus, we investigated the anti-inflammatory properties of LPSF/GQ-02 on mice after development of atherosclerosis. The p-IkBα is the active form of the inhibitory protein of NFκB. The cytoplasmic p-IkBα expression was evaluated after 15 and 30 days of

treatment with TZDs. As showed in the Fig 4, no effect was observed after 15 days of treatment with LPSF/GQ-02 (Fig. 4A). By contrast, LPSF/GQ-02 treatment for 30 days decreased the p-IkBα levels (Fig. 4B). Consistently, the expression of nuclear p-NFκB was unchanged by the treatment with LPSF/GQ-02 for 15 days whereas the treatment for 30 days suppressed p-NFκB expression.

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a potent chemoattractant for mononuclear cell fundamental in the development of inflammatory diseases (Inoshima et al., 2004). In atherosclerosis, MCP-1 might affect monocyte recruitment, thereby contributing to the progression of atherosclerotic lesions (Gu et al., 1998). Also, CD40, a trimeric transmembrane glycoprotein structurally related to TNF-α is described as an relevant marker of atherogenesis (Henn et al., 1998; Aukrust et al., 1999). Therefore, to verify the impact of LPSF/GQ-02 treatment on important markers of atherosclerosis, the mRNA levels of MCP-1 and CD-40 were evaluated. As depicted in Figure 4, MCP-1 and CD-40 mRNA levels were found to be decreased after 15 days of treatment with LPSF/GQ-02 (Figs. 4E and 4D). On contrary, no effect was observed after the treatment with LPSF/GQ-02 for 30 days (Fig. 4F e 4H). Interestingly, after 30 days of treatment with PIO, aortic tissue showed elevated expression of MCP-1 e CD-40 (Fig. 4F e 4H).

Effect of LPSF/GQ-02 on genic expression of PPARα, PPARγ e LXRα

To determine whether LPSF/GQ-02 treatment could modulate also the gene expression of its potential targets, the mRNA levels three transcriptional factors (PPARα, PPARγ e LXRα.), were analysed.

After 15 days of treatment, the TZDs treatment upregulated the expression of PPARα when compared to HFD group (Fig. 5A). On the other hand, after 30 days of

treatment, only PIO treatment increased the mRNA levels of PPARα (Fig. 5B). Interestingly, treatment with PIO induced PPARγ gene expression after 15 and 30 days of treatment when compared with the HFD group whereas no effect wes observed with LPSF/GQ-02 treatment (Fig. 5C-D).

The liver X receptor (LXR) α is a member of the nuclear receptor family of transcription factors. These transcription factors form heterodimers with retinoic acid receptor (RXR) and regulate the expression of genes involved in lipid metabolism, glucose and reverse cholesterol transport (Im and Osborne, 2011), while increasing high density lipoprotein (HDL) levels (Miao et al, 2004). Thus, LXRs decrease the risk of development of cardiovascular disorders including atherosclerosis. After 15 days of treatment, PIO enhances LXRα expression when compared to HFD group (Fig. 5E). Unexpectedly, treatment with LPSF/GQ-02 for 30 days decreased LXRα mRNA levels when compared with the other groups (Fig. 5F).

DISCUSSION

This study provides evidence that the new TZD LPSF/GQ-02 affects atherosclerotic lesion composition in LDLr -/- mice. The treatment with LPSF/GQ-02 for 15 days decreases collagen accumulation and downregulated the mRNA levels of CD40, MCP-1 and ABCG1 in aorta. Importantly, the treatment with LPSF/GQ-02 for 30 days not only reduced total collagen content on the plaque, but also decreased the protein levels of LOX-1, p-IκBα and p-NFκB. However, no effect was detected on the mRNA levels of ABC transporters, CD-40 and MCP-1 with the prolonged treatment. Overall, although both treatment protocols with LPSF/GQ-02 leads to beneficial effects preventing atherosclerosis progression, the duration of treatment is also correlates to particular findings.

An important observation derived from our data is that the treatment with LPSF/GQ-02 for 30 days modulates the expression of LOX-1. There is increasing evidence that LOX-1, a versatile scavenger receptor that is ubiquitously expressed in vascular cells (ECs, SMCs and macrophages), may target diverse cellular events, such as endothelial dysfunction, foam-cell formation (Xu et al., 2013) as well as modulate collagen accumulation in atherosclerotic plaques (Hu et al., 2008). As a result, LOX-1 is critical for the pathogenesis of atherosclerosis (Inoue et al., 2005; Mehta et al., 2007; White et al., 2011).

It is interesting to note that LPSF/GQ-02 treatment for 15 and 30 days reduced the collagen accumulation in the atherosclerotic lesions. Collagen consists in one of the components of the extracellular matrix (ECM), which is critical for the plaques stability. However, it is worth to point out that total collagen content in atherosclerotic lesions results from the balance between its synthesis by SMCs and its degradation by macrophage-derived matrix metalloproteinase (MMPs). Consistently, in a previous

report, LPSF/GQ-02 suppressed the expression of MMP-9, which degrades collagen by-products after initial cleavage by collagenases (Silva et al, 2013). Remarkably, MMP-9 deficiency has been associated not only with lower collagen content on plaques, but also with smaller lesions in a mouse model of atherosclerosis (Luttun et al., 2004). Thus, it may be reasonable to suppose that the modulation of LOX-1 and MMP-9 induced by LPSF/GQ-02 may contribute to the decreased collagen accumulation, thereby altering composition and growth of atherosclerotic lesions.

Inflammation has been implicated in every stage of atherosclerosis, ranging from onset of plaque to its rupture (Wong et al., 2012). It has been proposed that the interplay between insulin resistance and inflammation can affect atherogenesis. On this basis, the clinical use of insulin sensitizers, such as thiazolidinediones (TZDs), has been proposed as a therapeutic alternative to improve aspects related to atherosclerosis. In this context, experimental studies have shown that beyond the antidiabetic effect of TZDs, these compounds can attenuate inflammation by decreasing inflammatory markers such as C-reactive protein (CRP) and MMP-9 and inhibit proliferation of macrophages (Loke et al., 2011; He et al., 2006). A previous work has demonstrated that LPSF/GQ-02 could decrease inflammation by reducing IL-6, iNOS, COX-2 and F4/80, and increasing the protein expression of IκBα and cytoplasmic NFκB-65 in a mouse model of NAFLD (Silva et al, 2015). NFκB activation is associated with atherosclerosis and involves a complex series of signaling events that begins with the activation of the inhibitor κB (IκB) kinase complex, which, in turn, phosphorylates IκB (Karin and Delhase, 2000; Hayden and Ghosh, 2004). As a result, NFκB regulates the expression of several genes related to the atherogenesis. Corroborating previous findings in NAFLD, our data have shown that the treatment with LPSF/GQ-02 for 15 days suppressed mRNA of inflammatory markers, CD-40 and MCP-1 whereas 30-days treatment reduced p-IκBα

and p-NFκB protein levels. These results reinforce the anti-inflammatory properties of LPSF/GQ-02.

Previously, we have also shown that LPSF/GQ-02 treatment for 30 days increases HDL levels in high-fat fed LDLr $^{-/-}$ mice (Silva et al, 2015). Although reverse cholesterol transport (RCT) from macrophage-derived foam cells present in the atherosclerotic lesions only provides a small amount of the cholesterol content to the HDL particles, this process is relevant during atherogenesis (Rosenson et al., 2012). In this regard, RCT from macrophage-derived foam cells contributes not only to the total content of HDL particles, but also decreases cholesterol concentration in plaques. Important pathways for RCT macrophage-derived foam cells involve ABCA1 and ABCG1 transporters. ABCA1 mediates cholesterol efflux to lipid-free apolipoproteins such as apoA-I and apoE whereas ABCG1 mediates cholesterol efflux from macrophages through interactions to HDL particles. (Kennedy et al., 2005; Wang et al., 2004). Thus, ABCA1 and ABCG1 have complementary roles in mediating cholesterol efflux to HDL (Gelissen et al., 2006). Surprisingly, we show here only the short period treatment with LPSF/GQ-02 downregulated ABCG1 suggesting that RCT from macrophage-derived foam cells might be not essential to HDL increase induced by treatment with LPSF/GQ-02 for 30 days. In contrast, in line with a previous work, the treatment with pioglitazone for 30 days increased gene expression of ABCA1 and ABCG1 (Ozasa et al., 2011).

There is evidence suggesting that the molecular structure of LPSF/GQ-02 indicates a possible role as an agonist of PPARγ (Leite et al., 2007). Indeed, TZDs are described as synthetic PPARγ ligands. However, some TZDs, including pioglitazone might also demonstrate PPARα-dependent effects (Orasanu et al., 2008). PPARα is expressed in tissues such as the heart, kidneys, intestinal mucous, skeletal mucous and

liver, regulating genes involved in the lipid metabolism, gluconeogenesis and amino acids (Gonzalez et al., 2008). In addition, PPARα also acts on pro-inflammatory pathways and negatively affects other signaling pathways, such as NFκB (Lalloyer et al., 2011). As a result of its effects on the lipid metabolism and inflammation, PPARα plays a fundamental role on the progression of atherosclerosis in LDLr $^{-/-}$ mice (Li et al., 2004). In this study, we report that similar to pioglitazone, the treatment with LPSF/GQ-02 increased the mRNA levels of PPARα. In contrast, LPSF/GQ-02 treatment has no effect on the mRNA expression of PPARγ whereas pioglitazone also upregulated the mRNA levels of PPARγ in aorta. It is tempting to speculate that the anti-inflammatory effects of LPSF/GQ-02 described here and in our previous work (Silva et al, 2015) might be resulting of PPARα activation. However, further studies are necessary to address whether LPSF/GQ-02-induced effects are likely to be mediated by PPARα and/or PPARγ dependent-mechanisms.

Taken together, our results demonstrate that treatment with LPSF/GQ-02 affects the composition and growth of atherosclerotic lesions in LDLr $^{-/-}$ mice. Moreover, we demonstrate that LPSF/GQ-02 presents anti-inflammatory effects that seen to be probable consequent to PPARα activation. These observations, together with recent findings that LPSF/GQ-02 inhibits the development of hepatic steatosis and inflammation suggest this new TZD as a remarkable drug candidate.

REFERENCES

Ahmed,M.H., Barakat,S., and Almobarak,A.O. (2012). Nonalcoholic fatty liver disease and cardiovascular disease: has the time come for cardiologists to be hepatologists? J. Obes. 2012, 483135.

Alkhouri,N., Tamimi,T.A., Yerian,L., Lopez,R., Zein,N.N., and Feldstein,A.E. (2010). The inflamed liver and atherosclerosis: a link between histologic severity of nonalcoholic fatty liver disease and increased cardiovascular risk. Dig. Dis. Sci. 55, 2644-2650.

Aukrust P, Muller F, Ueland Thor BS, Berget T, Aaser E, Brunsvig BS, et al. (1999). Enhanced levels of soluble and membrane bound CD40 Ligand in platelets with unstable angina. Circulation. 100, 614-20.

Bradford, MA. (1970) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem. 72, 240–54.

Brea,A. and Puzo,J. (2013). Non-alcoholic fatty liver disease and cardiovascular risk. Int. J. Cardiol. 167, 1109-1117.

Fruchart,J.C., Nierman,M.C., Stroes,E.S., Kastelein,J.J., and Duriez,P. (2004). New risk factors for atherosclerosis and patient risk assessment. Circulation 109, III15-III19.

Game, BA. et al. (2007) Pioglitazone inhibits connective tissue growth factor expression in advanced atherosclerotic plaques in low-density lipoprotein receptor-deficient mice. Atherosclerosis 192: 85-91.

Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, Cartland S, Packianathan M, Kritharides L, Jessup W (2006). ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. Arterioscler Thromb Vasc Biol. 26, 534–540.

Gonzalez FJ, Shah YM. (2008). PPARalpha: mechanismof species differences and hepatocarcinogenesis of peroxisome proliferators. Toxicology 246: 2–8.

Gu, L.; Okada, Y.; Clinton, S. K.; Gerard, C.; Sukhova, G. K.; Libby, P.; Rollins, B. J (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor deficient mice. Mol. Cell. 2(2), 275-281.

Gupte AA, Liu JZ, Ren Y, Minze LJ,Wiles JR, Collins AR, et al. (2010) Rosiglitazone attenuates age and diet-associated nonalcoholic steatohepatitis in male low-density lipoprotein receptor knockout mice. Hepatology. 52(6), 2001–11.

Hayden MS, Ghosh S. (2004). Signaling to NF-kappaB. Genes Dev 18, 2195–2224.

He L, Game BA, Nareika A, Garvey WT, Huang Y (2006) Administration of pioglitazone in low-density lipoprotein receptor-deficient mice inhibits lesion progression and matrix metalloproteinase expression in advanced atherosclerotic plaques. J Cardiovasc Pharmacol 48, 212-222.

Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA (1998). CD40 Ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature. 391, 591-4.

Hu,C., Dandapat,A., Sun,L., Chen,J., Marwali,M.R., Romeo,F., Sawamura,T., and Mehta,J.L. (2008). LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet. Cardiovasc. Res. 79, 287-293.

Im, S.S.; Osborne, T.F (2011). Liver X receptors in atherosclerosis and inflammation. Circ Res. 108, 996–1001.

Inoshima, I.; Kuwano, K.; Hamada, N.; Hagimoto, N.; Yoshimi, M.; Maeyama, T.; Takeshita, A.; Kitamoto, S.; Egashira, K.; Hara, N (2004). Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary fibrosis in mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 286(5), L1038-1044.

Inoue K, Arai Y, Kurihara H, Kita T, Sawamura T (2005). Overexpression of lectin-like oxidized low-density lipoprotein receptor-1 induces intramyocardial vasculopathy in apolipoprotein E-null mice. Circ Res 97, 176–184.

Karin M., Delhase M. (2000). The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin. Immunol. 12, 85–98.

Kennedy MA, Barrera GC, Nakamura K, Baldán A, Tarr P, Fishbein MC, Frank J, Francone OL, Edwards PA (2005). ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab. 1, 121–131.

Lalloyer F,Wouters K, Baron M, Caron S, Vallez E, Vanhoutte J, et al. (2011) Peroxisome proliferator activated receptor-alpha gene level differently affects lipid metabolism and inflammation in apolipoprotein E2 knock-in mice. Arterioscler Thromb Vasc Biol. 31(7), 1573–1579.

Leite LFC, Mourão RHV, Lima MC, Galdino SL, Hernandes MZ, Neves FAR, et al. (2007). Synthesis, biological evaluation and molecular modeling studies of arylidene-thiazolidinediones with potential hypoglycemic and hypolipidemic activities. Eur J Med Chem. 42, 1263–1271.

Li AC, Binder CJ, Gutierrez A, Brown KK, Plotkin CR, Pattison JW, Valledor AF, Davis RA, Willson TM, Witztum JL, Palinski W, Glass CK (2004) Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARalpha, beta/delta, and gamma. J Clin Invest. 114, 1564–1576

Li, AC. et al. (2000) Peroxisome proliferator-activated receptor γ ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J Clin Investig 106, 523-531.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods Dec. 25(4), 402 8.

Loke YK, Kwok CS, Singh S (2011) Comparative cardiovascular effects of thiazolidinediones: systematic review and meta-analysis of observational studies. BMJ 342, d1309.

Luttun,A., Lutgens,E., Manderveld,A., Maris,K., Collen,D., Carmeliet,P., and Moons,L. (2004). Loss of matrix metalloproteinase-9 or matrix metalloproteinase-12 protects apolipoprotein E-deficient mice against atherosclerotic media destruction but differentially affects plaque growth. Circulation 109, 1408-1414.

Mehta JL, Sanada N, Hu CP, Chen J, Dandapat A, Sugawara F et al. (2007). Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. Circ Res 100, 1634–1642.

Miao, B. et al. HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator. J Lipid Res. 45(8), 1410-7. 2004.

Mourão, RH. et al. (2005) Synthesis and Biological Activity of Novel Acridinylidene and Benzylidene thiazolidinediones. Eur J Med Chemistry 40, 1129-1133.

Orasanu,G., Ziouzenkova,O., Devchand,P.R., Nehra,V., Hamdy,O., Horton,E.S., and Plutzky,J. (2008). The peroxisome proliferator-activated receptor-gamma agonist pioglitazone represses inflammation in a peroxisome proliferator-activated receptor alpha-dependent manner in vitro and in vivo in mice. J. Am. Coll. Cardiol. 52, 869-881.

Ozasa H, Ayaori M, Iizuka M, Terao Y, Uto-Kondo H et al (2011). Pioglitazone enhances cholesterol efflux from macrophages by increasing ABCA1/ABCG1 expressions via PPARγ/LXRα pathway: findings from in vitro and ex vivo studies. Atherosclerosis. 219(1), 141-50.

Rakoski,M.O., Singal,A.G., Rogers,M.A., and Conjeevaram,H. (2010). Meta-analysis: insulin sensitizers for the treatment of non-alcoholic steatohepatitis. Aliment. Pharmacol. Ther. 32, 1211-1221.

Rosenson,R.S., Brewer,H.B., Jr., Davidson,W.S., Fayad,Z.A., Fuster,V., Goldstein,J., Hellerstein,M., Jiang,X.C., Phillips,M.C., Rader,D.J., Remaley,A.T., Rothblat,G.H.,

Tall,A.R., and Yvan-Charvet,L. (2012). Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125, 1905-1919.

Silva AKS, de Oliveira Cipriano Torres D, dos Santos Gomes FO, dos Santos Silva B, Lima Ribeiro E, Costa Oliveira A, et al. (2015) LPSF/GQ-02 Inhibits the Development of Hepatic Steatosis and Inflammation in a Mouse Model of Non-Alcoholic Fatty Liver Disease (NAFLD). PLoS ONE 10(4), e0123787.

Silva, AKS. et al. (2013) Effect of new thiazolidine derivatives LPSF/GQ-02 and LPSF/GQ-16 on atherosclerotic lesions in LDL receptor-deficient mice LDLR($^{-/-}$). Cardiovasc Pathol 22(1), 81-90.

Targher,G., Marra,F., and Marchesini,G. (2008). Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? Diabetologia 51, 1947-1953.

Than,N.N. and Newsome,P.N. (2015). A concise review of non-alcoholic fatty liver disease. Atherosclerosis 239, 192-202.

Wang N, Lan D, Chen W, Matsuura F, Tall AR (2004). ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. Proc Natl Acad Sci U S A. 01, 9774–9779.

White SJ, Sala-Newby GB, Newby AC (2011). Overexpression of scavenger receptor LOX-1 in endothelial cells promotes atherogenesis in the ApoE(−/−) mouse model. Cardiovasc Pathol 20, 369–373.

Wong, B.W., A. Meredith, D. Lin, B.M. McManus (2012). The biological role of inflammation in atherosclerosis, Can. J. Cardiol. 28, 631-641

Xu,S., Ogura,S., Chen,J., Little,P.J., Moss,J., and Liu,P. (2013). LOX-1 in atherosclerosis: biological functions and pharmacological modifiers. Cell Mol. Life Sci. 70, 2859-2872.

FIGURE LEGENDS

Figure 2- Action of LPSF/GQ-02 on the deposition of collagen in the aorta of LDLr-/- mice. (A,D) HFD groups showing a great quantity of collagen in adventitial and medium layers. (B,E) Pioglitazone groups did not reverse the conditions caused by the high-fat diet, exhibiting a great quantity of collagen in adventitial and medium layers. (C,F) LPSF/GQ-02 groups exhibiting reduced collagen deposition in the aorta (G,H) Quantification of labelling for collagen by Masson's Trichrome (N=5). Bars 20µm. a= adventitia, m= media, i= intima. Results are expressed as mean ± SD. $p < 0.05$, ANOVA with Tukey post hoc test. a=HFD group, b= PIO group, c= LPSF/GQ-02 group.

Figure 3– Effects of LPSF/GQ-02 on gene expression of the cholesterol reverse transport, ABCA-1 and ABCG-1. Male mice were treated as described, and the expression levels of genes that might be involved in the atherosclerotic process were evaluated by real-time PCR. Results are expressed as mean ± SD. $p < 0.05$, ANOVA with Tukey post hoc test. a=HFD group, b= PIO group, c= LPSF/GQ-02 group.

Figure 4 – Effects of LPSF/GQ-02 on protein expression of LOX-1 and gene expression of CD-36 in the aorta of LDLr-/- mice after induced atherosclerotic lesion. (A) LOX-1 expression after 15 days of treatment with LPSF/GQ-02. (B) LOX-1 expression after 30 days of treatment with LPSF/GQ-02. (C) CD-36 gene expression after 15 of treatment with LPSF/GQ-02. (D) CD-36 gene expression after 15 of treatment with LPSF/GQ-02. Results are expressed as mean ± SD. $p < 0.05$, ANOVA with Tukey post hoc test. a=HFD group, b= PIO group, c= LPSF/GQ-02 group.

Figure 5 – Anti-inflammatory effects of LPSF/GQ-02 in LDLr-/- mice. (A, C) western blot of p-IκBα and p-NFκB after 15 days of treatment with LPSF/GQ-02. (B, D) western blot of p-IκBα and p-NFκB after 30 days of treatment with LPSF/GQ-02. (E, G) Real-time PCR mRNA expression of MCP-1 and CD-40 after 15 days of treatment with LPSF/GQ-02. (F, H) Real-time PCR mRNA expression of MCP-1 and CD-40 after 30 days of treatment with LPSF/GQ-02. Results are expressed as mean ± SD. $p < 0.05$, ANOVA with Tukey post hoc test. a=HFD group, b= PIO group, c= LPSF/GQ-02 group.

Figure 6 – action of LPSF/GQ-02 on gene expression of PPARα, PPARγ and LXRα in LDLr-/- mice. (A, C, E) Real-time PCR mRNA expression of PPARα, PPARγ and LXRα, respectively after 15 days of treatment with LPSF/GQ-02. (B, D, F). Real-time PCR mRNA expression of PPARα, PPARγ and LXRα, respectively after 30 days of treatment with LPSF/GQ-02. Results are expressed as mean ± SD. $p < 0.05$, ANOVA with Tukey post hoc test. a=HFD group, b= PIO group, c= LPSF/GQ-02 group.

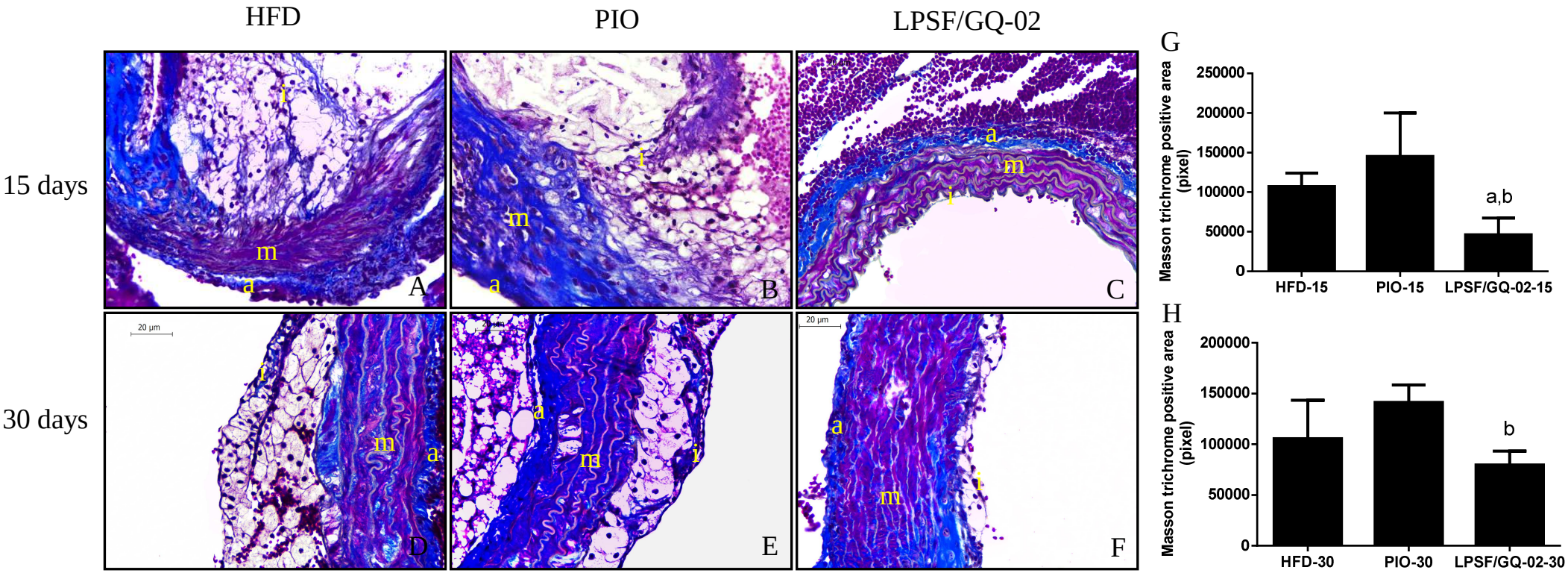
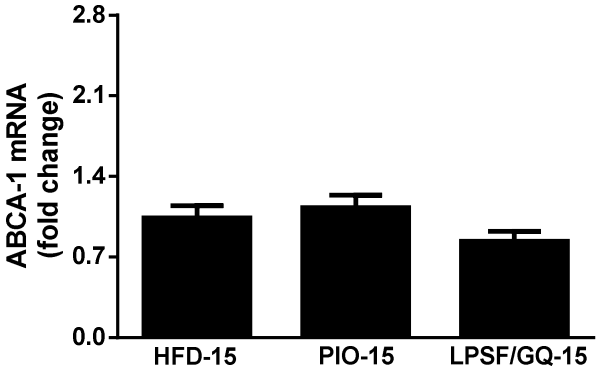
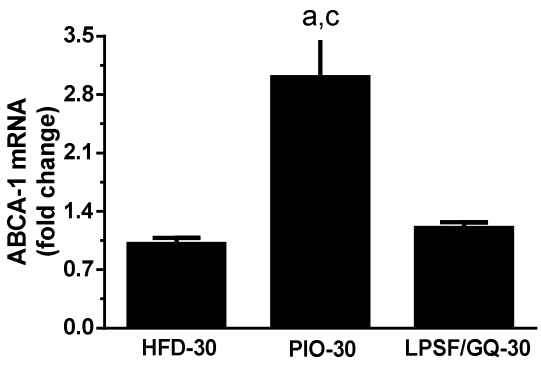
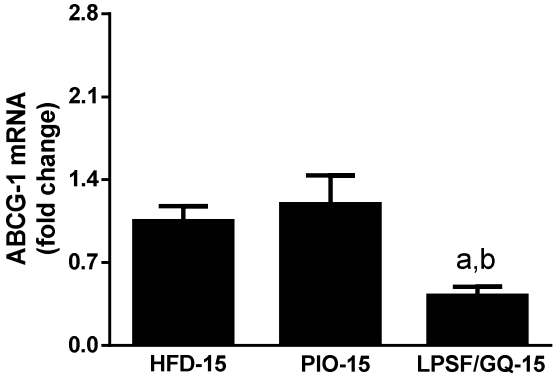

HFD
PIO
LPSF/GQ-02
15 days
30 days
A
B
C
D
E
F
G
H
Masson trichrome positive area (pixel)
250000
200000
150000
100000
50000
0
a,b
HFD-15
PIO-15
LPSF/GQ-02-15
b
HFD-30
PIO-30
LPSF/GQ-02-30

Figure 1

Figure 2


ABCA-1 mRNA
(fold change)
2.8
2.1
1.4
0.7
0.0
HFD-15
PIO-15
LPSF/GQ-15


a,c
ABCA-1 mRNA
(fold change)
3.5
2.8
2.1
1.4
0.7
0.0
HFD-30
PIO-30
LPSF/GQ-30


ABCG-1 mRNA
(fold change)
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2.1
1.4
0.7
0.0
a,b
HFD-15
PIO-15
LPSF/GQ-15

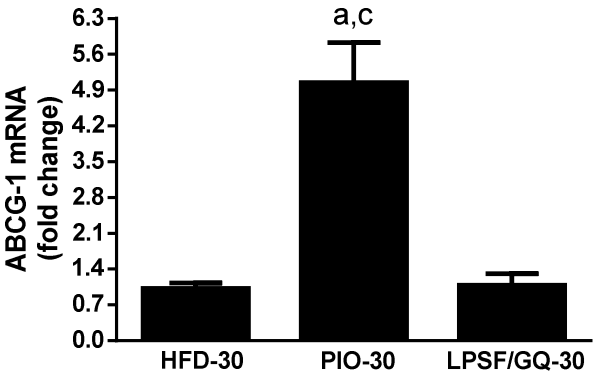

a,c
ABCG-1 mRNA
(fold change)
6.3
5.6
4.9
4.2
3.5
2.8
2.1
1.4
0.7
0.0
HFD-30
PIO-30
LPSF/GQ-30

Figure 3

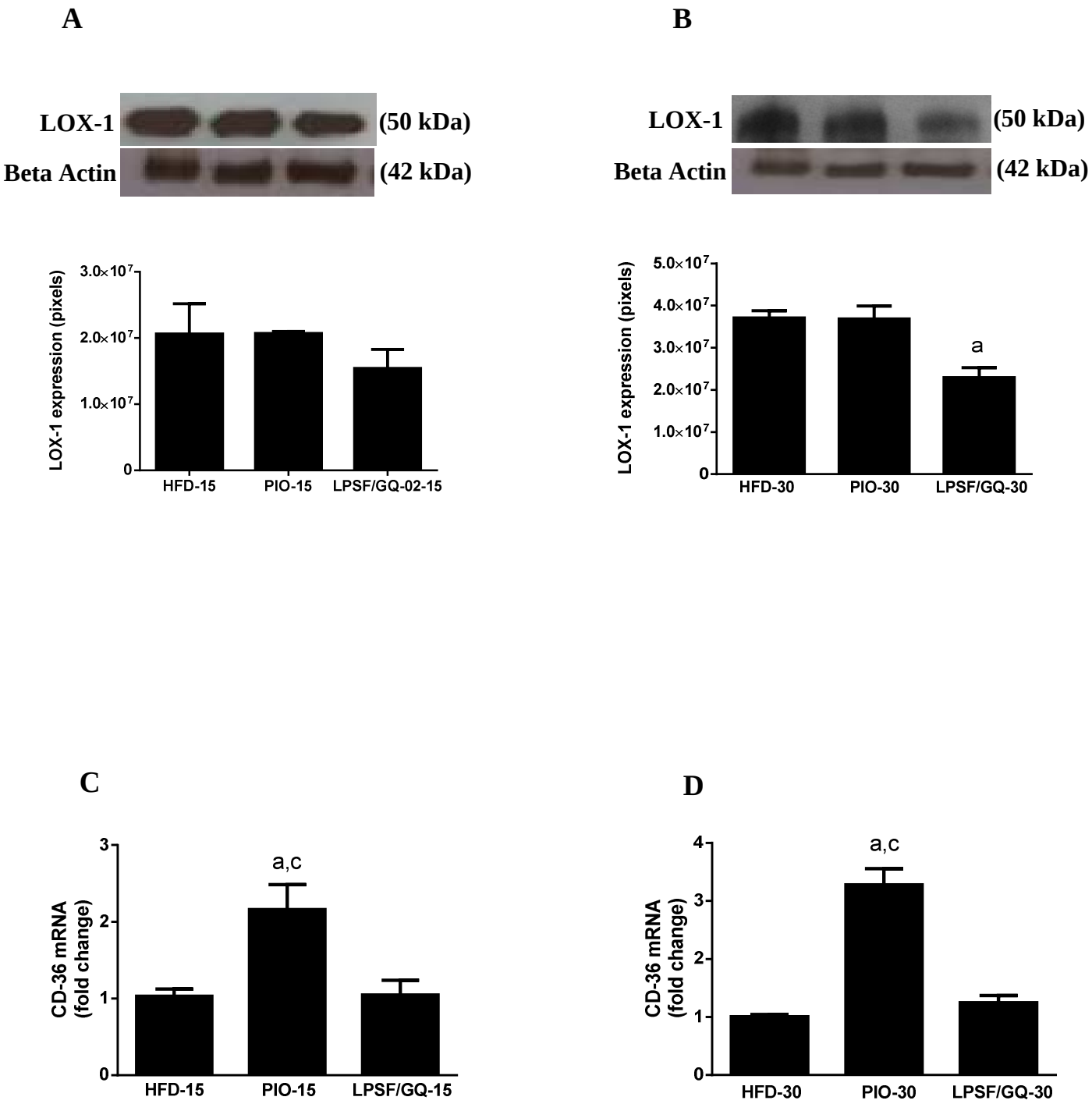
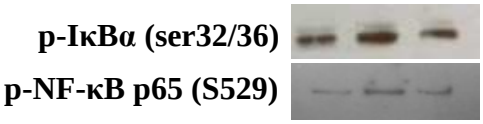
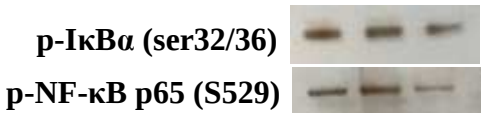
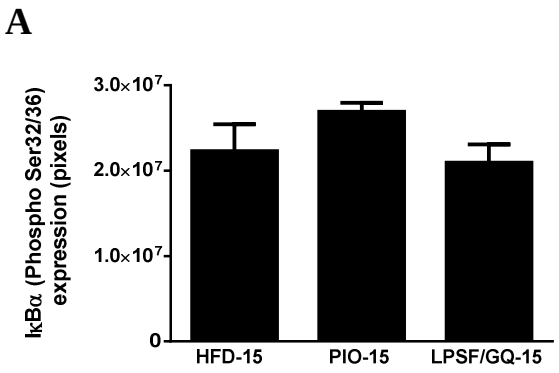
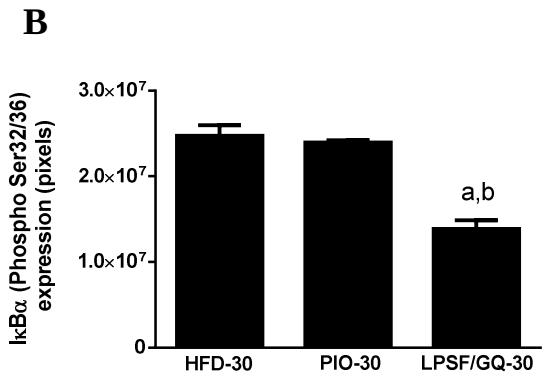

A
LOX-1
(50 kDa)
Beta Actin
(42 kDa)
LOX-1 expression (pixels)
3.0×10^7
2.0×10^7
1.0×10^7
0
HFD-15
PIO-15
LPSF/GQ-02-15
B
LOX-1
(50 kDa)
Beta Actin
(42 kDa)
LOX-1 expression (pixels)
5.0×10^7
4.0×10^7
3.0×10^7
2.0×10^7
1.0×10^7
0
a
HFD-30
PIO-30
LPSF/GQ-30
C
CD-36 mRNA (fold change)
3
2
1
0
a,c
HFD-15
PIO-15
LPSF/GQ-15
D
CD-36 mRNA (fold change)
4
3
2
1
0
a,c
HFD-30
PIO-30
LPSF/GQ-30

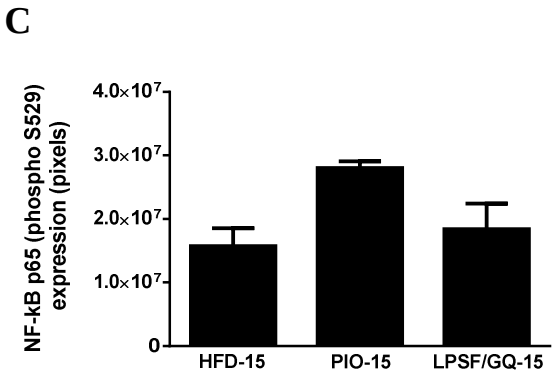
Figure 4

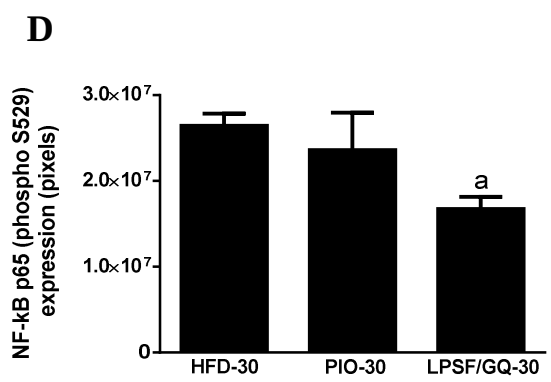

p-IκBα (ser32/36)
p-NF-κB p65 (S529)

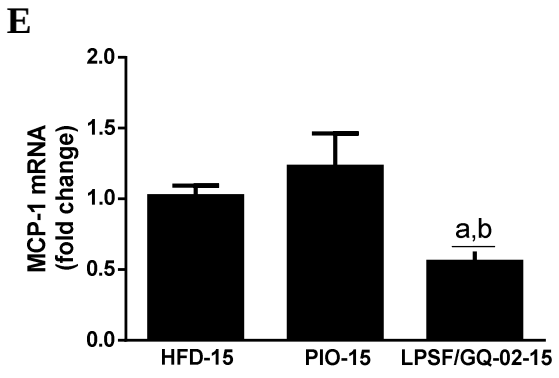

p-IκBα (ser32/36)
p-NF-κB p65 (S529)

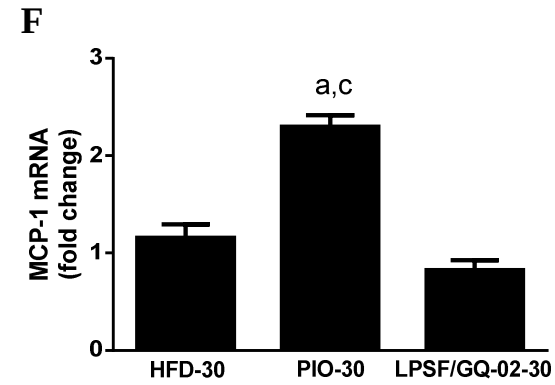

A
IκBα (Phospho Ser32/36) expression (pixels)
3.0×10^7
2.0×10^7
1.0×10^7
0
HFD-15
PIO-15
LPSF/GQ-15

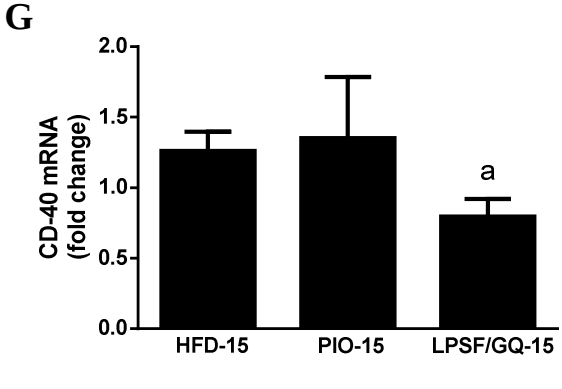

B
IκBα (Phospho Ser32/36) expression (pixels)
3.0×10^7
2.0×10^7
1.0×10^7
0
a,b
HFD-30
PIO-30
LPSF/GQ-30


C
NF-kB p65 (phospho S529) expression (pixels)
4.0×10^7
3.0×10^7
2.0×10^7
1.0×10^7
0
HFD-15
PIO-15
LPSF/GQ-15


D
NF-kB p65 (phospho S529) expression (pixels)
3.0×10^7
2.0×10^7
1.0×10^7
0
a
HFD-30
PIO-30
LPSF/GQ-30


E
MCP-1 mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
a,b
HFD-15
PIO-15
LPSF/GQ-02-15


F
MCP-1 mRNA (fold change)
3
2
1
0
a,c
HFD-30
PIO-30
LPSF/GQ-02-30


G
CD-40 mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
a
HFD-15
PIO-15
LPSF/GQ-15

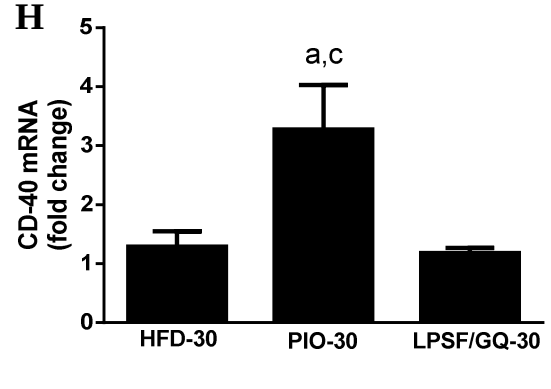
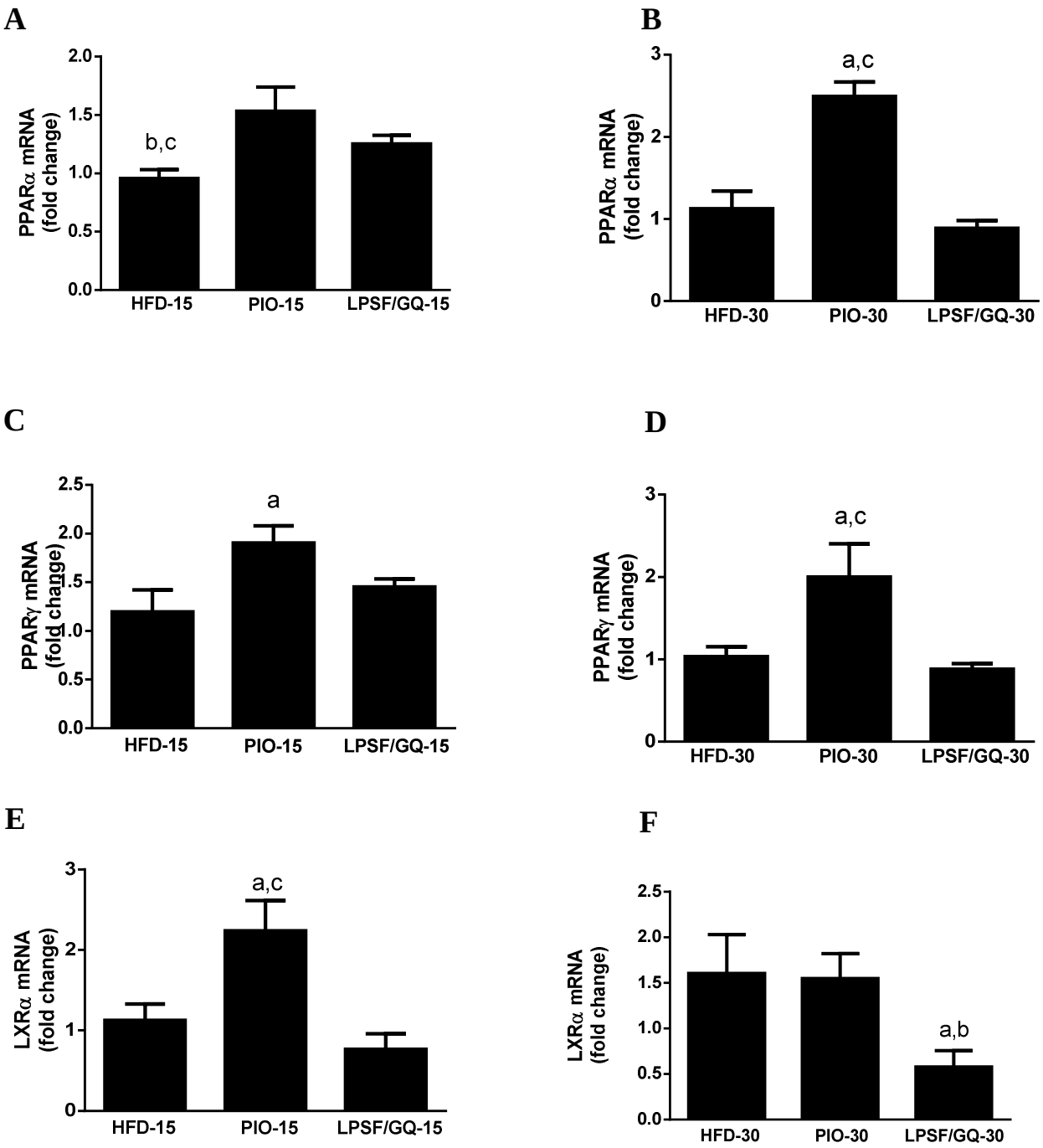

H
CD-40 mRNA (fold change)
5
4
3
2
1
0
a,c
HFD-30
PIO-30
LPSF/GQ-30

Figure 5


A
PPARα mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
b,c
HFD-15
PIO-15
LPSF/GQ-15
B
PPARα mRNA (fold change)
3
2
1
0
a,c
HFD-30
PIO-30
LPSF/GQ-30
C
PPARγ mRNA (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
a
HFD-15
PIO-15
LPSF/GQ-15
D
PPARγ mRNA (fold change)
3
2
1
0
a,c
HFD-30
PIO-30
LPSF/GQ-30
E
LXRα mRNA (fold change)
3
2
1
0
a,c
HFD-15
PIO-15
LPSF/GQ-15
F
LXRα mRNA (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
a,b
HFD-30
PIO-30
LPSF/GQ-30

6. CONCLUSÃO

Os camundongos LDLr-/- apresentaram acúmulo de gordura e inflamação no fígado e aorta após ingestão crônica de uma dieta rica em gordura. A LPSF/GQ-02 foi capaz de atuar sobre os fatores de afetam a inflamação, resistência à insulina e acúmulo de gordura no fígado. Na aorta, a LPSF/GQ-02 atuou sobre a composição, crescimento e inflamação nas lesões ateroscleróticas dos camundongos LDLr-/-. Esses dados sugerem que a LPSF/GQ-02 exerce efeitos modulatórios sobre o metabolismo lipidico, inflamação e resistência à insulina, sugerindo que a LPSF/GQ-02 pode ser considerada como uma molécula promissora para o tratamento de desordens como a aterosclerose e NAFLD.

Anexos

ANEXO A - Parecer do comitê de Ética

MINISTÉRIO DA SAÚDE / FUNDAÇÃO OSWALDO CRUZ
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Comissão de Ética no Uso de Animais
CEUA-FIOCRUZ

CERTIFICADO

Certificamos que o protocolo intitulado :

" Estudo comparativo de tratamentos com glitazonas sobre o processo aterosclerótico em camundongos C57BL/6J. "

número P-510/08, proposto por Christina Alves Peixoto, foi licenciado pelo Nº L-010/09.

Sua licença de Nº L-010/09 autoriza o uso anual de :

- 120 Mus musculus

Esse protocolo está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi APROVADO pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA - FIOCRUZ). Na presente formatação, este projeto está licenciado e tem validade até 21 de janeiro de 2013.

Rio de Janeiro, 21/01/2009

Dra. Norma Vollmer Labarthe
Coordenadora da CEUA
FIOCRUZ

ANEXO B - Normas da Revista do Artigo 1

Modified January 2015

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Abstract

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Introduction

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Materials and Methods

Lorem ipsum dolor sit amet, consectetur adipiscing elit. Vestibulum adipiscing urna ut lectus gravida, vitae (Fig. 1) interdum. Donec tincidunt porta sem nec hendrerit. Vestibulum nec pharetra quam, vitae convallis nunc. Mauris in mattis sapien. Fusce sodales vulputate auctor. Nam sit amet nulla lacus a, Figs. 1 and 2 ultrices tellus. Integer rutrum aliquet sapien, eu fermentum magna pellentesque vitae.

Fig. 1. This is the Fig. 1 Title. This is the Fig. 1 legend.

Fig. 2. This is the Fig. 2 Title. This is the Fig. 2 legend.

Level 1 Heading

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- Do not use ALL CAPS.

NOTE: Do not cite figures, tables, supporting information, or references in the Abstract.

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- Cite multiple figures as “Figs. 1 and 2”, “Figs. 1-3”, etc.

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- Use bold type for the figure titles.

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- Figures should be uploaded separately as individual files.

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Lorem ipsum dolor sit amet, consectetur adipiscing elit. Vestibulum adipiscing urna ut lectus gravida, vitae blandit tortor interdum. Donec p^2 et q^2 tincidunt porta sem nec hendrerit.

$$p^2 + 2pq + q^2 = 1 \quad (1)$$

Display/Numbered Equation

- Format display equations in Mathtype or Equation Tools.
- Do not use Graphic Objects.

Vestibulum nec pharetra quam, vitae convallis nunc. Mauris in mattis sapien. Fusce sodales vulputate auctor. Nam lacus felis, fermentum sit amet nulla ac, tristique ultrices tellus. Integer rutrum aliquet sapien, eu fermentum magna pellentesque vitae. Integer semper viverra mauris vel pulvinar dolor sit amet en $(p+q)^2 = 1$.

Inline Equation

- Format in regular text or as an inline equation in Mathtype or Equation Tools
- Do not use Symbol Font.
- Do not use Graphic Objects.

Genotyping

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Whole genome RFLP analysis

Level 3 heading

- Use Level 3 headings for sub-sections within Level 2 headings.
- Bold type, 14pt font.
- Only use italics and text formatting where needed.
- Do not use ALL CAPS.

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Results and Discussion

Lorem ipsum dolor sit amet, consectetur adipiscing elit. Vestibulum adipiscing urna ut lectus gravida, et bland Table 1 Donec tincidunt porta sem nec hendrerit. Vestibulum nec pharetra quam, vitae convalli. Fido nemo.

Table 1. This is the Table 1 Title.

	Chemical W	Chemical X	Chemical Y	Chemical Z
Chemical 1	Reaction 1W	Reaction 1X	Reaction 1Y	Reaction 1Z
Chemical 2	Reaction 2W	Reaction 2X	Reaction 2Y	Reaction 2Z
Chemical 3	Reaction 3W[a]	Reaction 3X	Reaction 3Y[b]	Reaction 3Z
Chemical 4	Reaction 4W	Reaction 4X	Reaction 4Y	Reaction 4Z
Chemical 5	Reaction 5W	Reaction 5X	Reaction 5Y	Reaction 5Z

This is the Table 1 legend.
[a]Table footnotes belong here.
[b]Footnotes should have corresponding symbols in the table.

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Conclusions

Lorem ipsum dolor sit amet, consectetur adipiscing [1-5]. Vestibulum adipiscing urna ut lectus gravida, vitae blandit tortor interdum. Donec tincidunt porta sem nec hendrerit. Vestibulum nec pharetra quam, vitae convallis nunc. Mauris in mattis sapien. Fusce sodales vulputate auctor S1 Fig. Dolor sit amet S1 and S2 Tables.

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Acknowledgments

- Do not include funding or competing interests information in Acknowledgments.

References

1. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr, McBio GHT, et al. This is the article title. PLoS One 2014 Dec 18; 9(12).
2. Doe J, Data A, van Stats J, Testperson M, Ribosome D Jr, McBio, GHT et al. Bunny dynamics in cartoon landscapes. PLOS One. Forthcoming 2015.

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Supporting Information

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S2 Fig. This is the S2 Fig. Title. This is the S2 Fig. legend.

S1 Table. This is the S1 Table Title. This is the S1 Table legend.

S2 Table. This is the S2 Table Title. This is the S2 Table legend.

S1 File. This is the S1 File Title. This is the S1 File legend.

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#b	Pound/number sign	Second Current address
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[4] Department of Restoration, National Contemporary Bunny Museum, Lagomorph, Connecticut, United States of America

[5] Department of Archaeology, Bunny University, Lagomorph, Connecticut, United States of America

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ANEXO C: Normas da Revista do artigo 2

TOXICOLOGY AND APPLIED PHARMACOLOGY

AUTHOR INFORMATION PACK

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Xianglin Shi, University of Kentucky College of Medicine, Lexington, Kentucky, USA
Anna A. Shvedova, National Institute for Occupational Safety and Health (NIOSH), Morgantown, West Virginia, USA
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Reference to a book:
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Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

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ANEXO D: Artigos Publicados no Período de 2011-2015

Santos Rocha, Sura Wanessa ; Santos Silva, Bruna ; Santos Gomes, Fabiana Oliveira dos ; Soares e Silva, Amanda Karolina; Raposo, Catarina; Sousa Barbosa, Karla Patrícia ; Cipriano Torres, Dilênia de Oliveira ; Oliveira dos Santos, Ana Célia ; Alves Peixoto, Christina . Effect of diethylcarbamazine on chronic hepatic inflammation induced by alcohol in C57BL/6 mice. European Journal of Pharmacology, v. 689, p. 194-203, 2012.

Dilênia de Oliveira Cipriano Torres; Ana Célia Oliveira Santos; Silva, A. K. S.; Moura, P.M.M.F; Eduardo Isidoro Carneiro Beltrão ; Peixoto, C.A . Influence of fatty acids in maternal diet on atherogenesis in offspring of LDL receptor-deficient mice. International Journal of Clinical and Experimental Medicine, v. 5, p. 56-63, 2012.

Soares e Silva, Amanda Karolina; De Oliveira Cipriano Torres, Dilênia; Santos Rocha, Sura Wanessa ; dos Santos Gomes, Fabiana Oliveira ; dos Santos Silva, Bruna ; Donato, Mariana Aragão Matos ; Raposo, Catarina ; Santos, Ana Célia oliveira ; de Lima, Maria do Carmo Alves ; Galdino, Suely Lins ; da Rocha Pitta, Ivan ; De Souza, José Roberto Botelho ; Peixoto, Christina Alves . Effect of new thiazolidine derivatives LPSF/GQ-02 and LPSF/GQ-16 on atherosclerotic lesions in LDL receptor-deficient mice (LDLR−/−). Cardiovascular Pathology, v. 22, p. 81-90, 2013.

Dos Santos, Laise Aline Martins ; Ribeiro, Edlene Lima ; Barbosa, Karla Patrícia Sousa ;Fragoso, Ingrid Tavares ; Gomes, Fabiana Oliveira Dos Santos ; Donato, Mariana Aragão Matos ; Da Silva, Bruna Santos ; Silva, Amanda Karolina Soares ; Rocha, Sura Wanessa Santos ; De França, Maria Eduarda Rocha ; Rodrigues, Gabriel Barros ; Silva, Teresinha Gonçalves ; Peixoto, Christina Alves . Diethylcarbamazine inhibits nuclear factor-κb activation in acute lung injury induced by carrageenan in mice. International Immunopharmacology (Print), v. 23, p. 153-162, 2014.

Dos Santos Gomes, Fabiana Oliveira; Carvalho, Maria Da Conceição ; Saraiva, Karina L.A. ; Ribeiro, Edlene Lima ; Soares E Silva, Amanda Karolina ; Donato, Mariana Matos Aragão ; Rocha, Sura Wanessa Santos ; Silva, Bruna Santos E ; Peixoto, Christina Alves . Effect of chronic sildenafil treatment on the prostate of C57Bl/6 mice. Tissue & Cell, v. 46, p. 439-449, 2014.

Lemos, Ana Janaina Jeanine M.; Peixoto, Christina A.; Teixeira, Álvaro Aguiar C.; Luna, Rayana Leal A.; Rocha, Sura Wanessa S.; Santos, Hilda Michelly P.; Silva, Amanda Karolina S.; Nunes, Ana Karolina S.; Wanderley-Teixeira, Valéria . Effect of the combination of metformin hydrochloride and melatonin on oxidative stress before and during pregnancy, and biochemical and histopathological analysis of the livers of rats after treatment for polycystic ovary syndrome. Toxicology and Applied Pharmacology, v. 2014, p. 00, 2014.

Ribeiro, Edlene Lima; Barbosa, Karla Patricia De Souza ; Fragoso, Ingrid Tavares; Donato, Mariana Aragão Matos ; Oliveira Dos Santos Gomes, Fabiana ; Silva, Bruna Santos Da ; Silva, Amanda Karolina Soares E ; Rocha, Sura Wanessa Santos ; Amaro Da Silva Junior, Valdemiro ; Peixoto, Christina Alves . Diethylcarbamazine Attenuates

the Development of Carrageenan-Induced Lung Injury in Mice. Mediators of Inflammation (Print), v. 2014, p. 1-12, 2014.

Da Silva, Bruna Santos; Rodrigues, Gabriel Barros ; Rocha, Sura Wanessa Santos; Ribeiro, Edlene Lima; Gomes, Fabiana Oliveira Dos Santos ; E Silva, Amanda Karolina Soares; Peixoto, Christina Alves . Inhibition of NF-κB activation by diethylcarbamazine prevents alcohol-induced liver injury in C57BL/6 mice. Tissue & Cell, v. 00, p. 00, 2014.

Silva, A.K.S; Cipriano Torres, Dilênia De Oliveira; Gomes, F. O. S. ; Silva, B. S. ; Ribeiro, E. L. ; Oliveira, A. C. ; Dos Santos, Laise Aline Martins ; Lima, M. C. A. ; Pitta, Ivan R. ; Peixoto, Christina Alves . LPSF/GQ-02 Inhibits the Development of Hepatic Steatosis and Inflammation in a Mouse Model of Non-Alcoholic Fatty Liver Disease (NAFLD). Plos One, v. 10, p. 1-23, 2015.