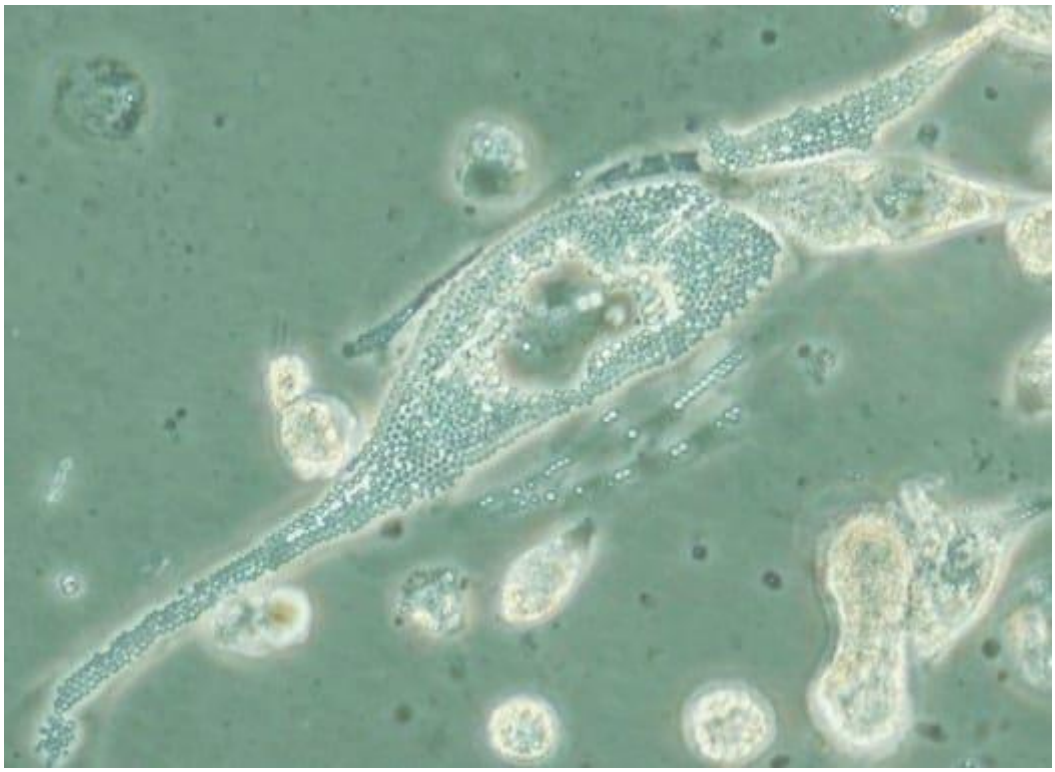
IMPACT OF TRIGLYCERIDE-RICH LIPOPROTEINS ON THE INFLAMMATORY FUNCTION OF MONOCYTES

Roser Franquesa Cortés

TREBALL FINAL DE GRAU BIOTECNOLOGIA



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In cooperation with Pere Virgili Health Research Institute

Date: June 2021

Jo, **Roser Franquesa Cortés**, amb DNI **47127124-D**, sóc coneixedor de la guia de prevenció del plagi a la URV *Prevenció, detecció i tractament del plagi en la docència*: guia per a estudiants (aprovada el juliol 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) i afirmo que aquest TFG no constitueix cap de les conductes considerades com a plagi per la URV.

Tarragona, 07 de juny de 2021

ACKNOWLEDGEMENTS

I would like to begin to express my gratitude to Juan Moreno Vedia, URLA's PhD student and my friend, for his extraordinary patience and kind guidance, for his mentorship and teamwork that he allowed me to grow in my learning and become a better future researcher. In addition, I would like to thank my professional tutor, Dra. Josefa Girona, for providing me with the chance to work in her research unit and lab, for her patience and for her admirable help and advice that she offered throughout these months.

The experiments described in this project would not have been possible without URLA's research unit. Under the unique expertise and competency of all PhD students like Juan Moreno and Jose Adria and the technicians created an efficient environment to learn.

I would like to thank my academic tutor, Dra. Neus Martínez, for his collaboration and contribution that made this project possible.

Finally, a special thanks to my family and friends, who always supported and encouraged me through this entire process.

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ABBREVIATIONS

ASCVD	atherosclerosis cardiovascular disease
ApoB	apolipoprotein B
CAD	coronary artery disease
CHOL	cholesterol
CD206	mannose receptor (cluster of differentiation 206)
CVD	cardiovascular disease
DM	diabetes mellitus
ECs	endothelial cells
FBS	fetal bovine serum
FH	familial hyperchylomicronemia
HDL	high-density lipoprotein
^{1}H-NMR	proton nuclear magnetic resonance
ICAM-1	intercellular adhesion molecule 1
IDL	intermediate-density lipoprotein
IFN-γ	interferon gamma
IL-1β	interleukin 1 beta
IL-4	interleukin 4
IL-6	interleukin 6
IL-8	interleukin 8
IL-12	interleukin 12
IL-13	interleukin 13
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LP	lipoprotein
Lp (a)	lipoprotein (a)

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MMPs	matrix metalloproteinase
NaBr	sodium bromide
NO	nitric oxide
oxLDL	oxidized low-density lipoprotein
PBS	phosphate-buffered saline
PCSK9	protein convertase subtilisin/kexin type 9
Pen/Strep	penicillin-streptomycin
PL	phospholipid
PMA	phorbol 12-myristate 13-acetate
PROT	protein
PRR	pattern recognition receptors
Q1 to Q4	quartile 1 to quartile 4
q-PCR	quantitative polymerase chain reaction
RPMI1640	Roswell Park Memorial Institute 1640
RT-PCR	reverse transcription polymerase chain reaction
SRs	scavengers
TG	triglycerides
TMB	tetramethylbenzidine
TLRs	toll-like receptors
TNF-α	tumor necrosis factor alpha
TRL	triglyceride-rich lipoprotein
VCAM-1	vascular adhesion molecule 1
VLDL	very low-density lipoprotein
VSMCs	vascular smooth muscle cells

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Pere Virgili Institute (IISPV) promotes, develops, manages and makes research dissemination in scientific and technologic fields of life health. The IISPV manages biomedical research activity of the following institutions: ICS Camp de Tarragona (Hospital Universitari de Tarragona Joan XXIII), ICS Terres de l'Ebre (Hospital de Tortosa verge de la Cinta), Grup SAGESSA, Grup Pere Mata and Universitat Rovira i Virgili.

IISPV (founded in 2005) is a national and international center of reference in biomedical research with the objective to provide and improve the health and well-being of population. In order to meet the requirements, Pere Virgili is activate in different strategic areas like Nutrition and Metabolism, Neurosciences and Mental health, Health and Environment, and Oncology and Hematology.

Within the area of Nutrition and Metabolism, we can find different research units as Lipids and Atherosclerosis Research Unit (URLA). The principal common objectives consists in the study of alterations in lipid metabolism and metabolism disorders (dyslipidemia, obesity, and diabetes mellitus) as well as its relationship with cardiovascular disease.

URLA employs 18 people involved in different research lines. One of the main lines of research is Lipoprotein metabolism, Atherosclerosis, and Diabetes, which is responsible for Lluís Masana and Josefa Girona.

In order to write this project, I cursed my internship in the research line of Lluís Masana and Josefa Girona, in URLA's group.

ABSTRACT

Lipoproteins are one of the main determinants in the pathogenesis of atherosclerosis changing the phenotype of macrophages, being involved in lipid deposition and plaque formation among others. Although it is well known the proatherogenic effect of LDL, recent works highlight the importance of TGs and the particles that transport them. High concentrations of triglyceride-rich lipoproteins (TRL) are related to metabolic disorders. TRL can be internalized to the arterial wall and activate the inflammatory response of macrophages present in the vascular wall. Therefore, TRL might be considered as a cardiovascular risk factor.

The main objective of this study is to analyze the effects of TRL derived from a cohort of patients with metabolic disorders on the inflammatory status of macrophages, incubating these cells with isolated TRL according to growing concentrations of the triglycerides transported in those particles.

Plasma samples of 72 patients from the METBANC cohort were used to isolate TRL fraction by ultracentrifugation. Pools of four growing quartiles of triglycerides concentration in TRL fraction were incubated with THP-1 macrophages at different times to study the mRNA expression, protein secretion of IL-1β (pro-inflammatory) and CD206 (anti-inflammatory).

TRL induced IL-1β expression at 4 hours of treatment accompanied by a progressive increase of its secretion through time. IL-1β expression was reduced at 24 hours while CD206 mRNA expression and protein secretion increased. The growing TG-TRL quartiles induced a regulated M1 polarization of macrophages, suggesting that TRL have a pro-inflammatory effect early in time. The reduction of IL-1β expression level after 4 hours of TRL treatment and the later CD206 secretion led us to propose a transcriptional regulatory effect as a possible consequence of a M2 polarization. Overall, our results support the role of TRL derived from cardiometabolic patients on the inflammatory response of macrophages.

KEY WORDS

Triglyceride-rich lipoprotein, inflammation, IL-1β, CD206, THP-1 macrophages atherosclerosis.

INTRODUCTION

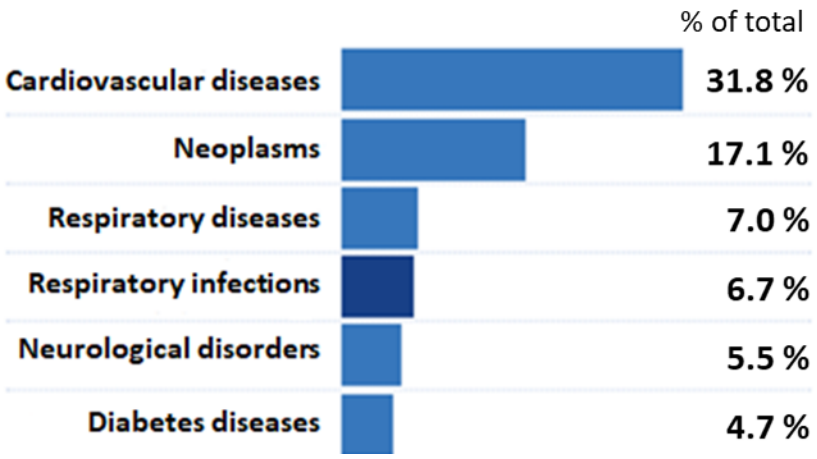
Cardiovascular diseases and epidemiology

Cardiovascular disease is a general term for a group of different disorders related to the heart and blood vessels. These chronic diseases are gradually in development throughout life being asymptomatic during years and, usually, the advanced stages of the diseases cause the first symptoms (1).

The principal advanced diseases include coronary artery disease (the arteries of the heart are blocked resulting in a decrease in oxygen-rich blood flow to the heart), cerebrovascular disease (which affects the blood flow through the brain), and peripheral arterial disease (which affects the blood flow through the arms and legs), among others.

Cardiovascular diseases (CVDs) are the leading cause of death globally. An estimated 17.9 million people die from CVDs each year, representing 31% of all global deaths (Figure 1). According to the World health organization (WHO), 4 out of 5 cardiovascular disease deaths are due to heart attacks and strokes.

Figure 1: Global causes of death in 2017.



Top global causes of death in 2017, the remaining 27.2% correspond to other causes such as digestive diseases and infectious diseases. Source: IHME (Institute for Health Metrics and Evaluation).

Different risk factors can modify the incidence of cardiovascular disease worldwide. Those risk factor can be classified as invariable or modifiable. Invariable factors include gender, age and heritable genetic disorders (2). Between heritable genetic disorders, we could find familial hyperchylomicronemia (FH), which is caused by mutations in one of the genes that control the way cholesterol is cleared by the body, principally the genes that codify for the LDLR, ApoB and PCSK9, mutation that results in the accumulation of low-density lipoprotein cholesterol (LDL) in the bloodstream (3). Modifiable factors

include hypertension, tobacco use, physical inactivity, and a high-fat diet (rich in cholesterol and triglycerides), among others. Modern lifestyle, with high alcohol consumption, high-fat diets, and smoking habits is worsening the incidence of CVD around the world and, as a matter of fact, obesity is closely related to suffering a cardiovascular disorder. Moreover, numerous studies have related obesity with an increase in the development of different diseases, including Type 2 Diabetes Mellitus and dyslipidemias like hypercholesterolemia or hypertriglyceridemia (1).

Pathogenesis of atherosclerosis

Among CVD, atherosclerosis contributes to major mortality of cardiovascular diseases including coronary artery disease (CAD) (2,4). The clinical manifestations are the different consequences of thrombotic complications on ruptured lesions, being "atherothrombosis" (atherosclerosis plus thrombosis) the major cause of mortality in Western countries (5,6).

Atherosclerosis is a complex and multifactorial disease, characterized by lipid deposition and other blood-borne material within the vessel wall of medium or large-sized elastic or muscular arteries. This process is the product of a slow lipid deposition, which results in the formation of atheroma or fibroinflammatory lipid plaque which may restrict blood flow or, under certain circumstances, may proceed to an acute cardiovascular clinical event as a result of plaque rupture that leads to thrombosis (5–8).

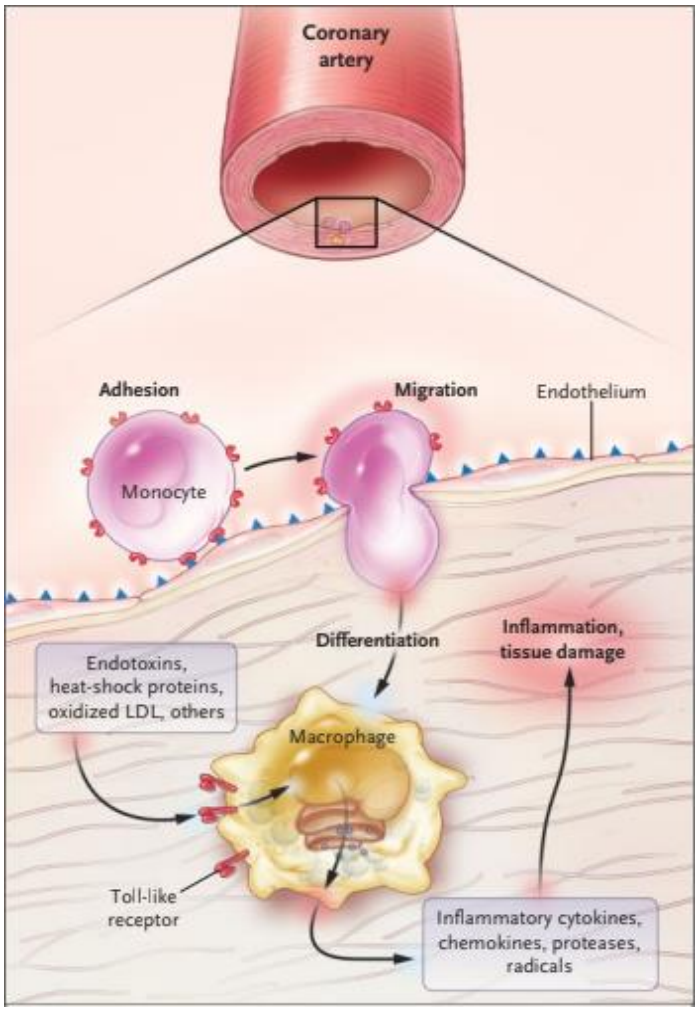
Atherosclerosis is a chronic inflammatory vascular disease. Lipid deposition within the innermost layer of the artery (intima) promotes an inflammatory response that plays an important role in the atheroma plaque formation (8).

The principal early stages in atherosclerosis disease are endothelial injury, due to the dysfunction in the pattern of secretion and synthesis of different endothelium substances as well as abnormal lipid metabolism (5,8). Endothelial cells (ECs) are activated causing two consequences at the systemic and vascular level (5,7) (Figure 2). Firstly, at systemic level, ECs cells express several types of leukocyte adhesion molecules like interleukin-8 (IL-8), intercellular and vascular adhesion molecule-1 (ICAM-1 and VCAM-1), among others. These inflammatory factors cause blood cells to roll along the intima layer of the artery attracting monocytes at the site of ECs activation (5–8). Secondly, at vascular level, the up-regulation of adhesion molecules facilitates the internalization of monocytes. Monocytes bind to the innermost layer and migrate through the arterial wall and the inflammation process begins, perpetuating the pathological process (5–8). The

activation of ECs promotes the infiltration of circulating lipids as cholesterol into the arterial wall as well.

Cytokines, inflammatory factors, and growth factors secreted in the inflamed arteria, induce the differentiation of the infiltrated monocytes into macrophages. As an innate immune response to the secreted molecules in the injured arterial wall, macrophages up-regulate pattern recognition receptors (PRR), including scavengers (SRs) and toll-like receptors (TLRs) (6,7) (Figure 2). The expression of TLRs induces macrophage activation and SRs internalize a broad range of apoptotic cell fragments, bacterial endotoxins, and oxidized low-density lipoprotein (oxLDL) (7,9).

Figure 2: Role of macrophages at inflammation vascular disease.



Role of macrophages at early stages of atheroma formation. Source: Inflammation, atherosclerosis, and coronary artery disease (7).

TLRs are typical pattern recognition receptors in the innate response, and can induce cell activation, producing pro-inflammatory cytokines as IL-1β, TNF-α, IL-6, IFN-γ, proteases, cytotoxic oxygen, and nitrogen radical molecules (7,8).

SRs internalize molecules and particles with pathogen molecular patterns, including oxLDL. The endothelium injury produces an enhancement of circulating lipids into the intimal layer, mainly LDL particles enriched in cholesterol and other lipoproteins like TRL enriched in triglycerides as recent studies suggest (7,9–12).

LDL, especially small dense LDL particles are strongly related to atherosclerosis cardiovascular disease because of its size. The abundance of small dense LDL facilitates its entry into the arterial wall enhancing the presence of lipids in atheroma plaque (10).

Moreover, LDL particles can bind to the proteoglycans present in the matrix of the subendothelial space and be oxidized by the action of different enzymes, becoming oxLDL. High levels of LDL modified to oxidized LDL accumulate in the subendothelial level as cytosolic droplets. Oxidized cholesterol is highly toxic, producing pro-inflammatory cascade reactions (secretion of IL-1β, TNF-α, etc) and perpetuating the recruitment of immune cells (6–9). (Figure 2)

Infiltrated macrophages engulf oxLDL deposits due to their size and oxidative modification. The differentiation into macrophages may have a protective role at a vascular level by providing phagocytic capacity to remove oxLDL particles. However, the progressive accumulation of lipids leads to the transformation of macrophages into foam cells, a lipid-rich macrophage. Foam cells are the prototypical cells in atherosclerosis (7–10) (Figure 3).

Accumulation of foam cells, lipids, and other immune cells into the endothelium promote a fatty streak. Fatty streak precedes the formation of atheroma plaque being the initial lesion of the arteria. This fatty streak can be detected clinically. In addition, the early stages, as well as visual lesions, can progress without symptoms, and this progression could be stopped or even reversed until disappears (5,7).

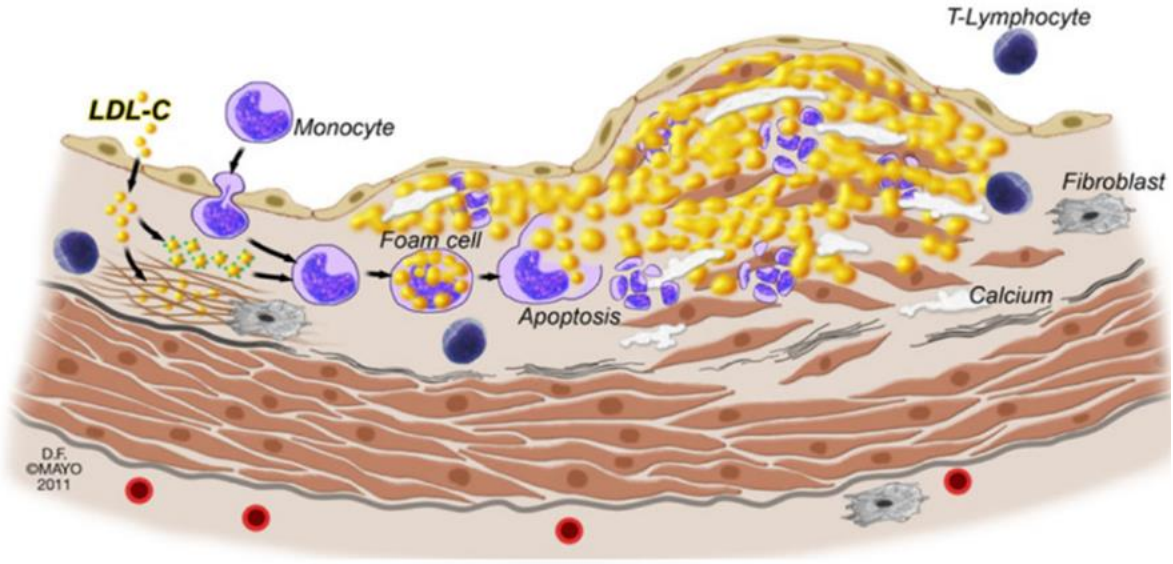
Over time, foam cells cannot remove lipids from the vessel wall and this promotes its apoptotic death. As a consequence, cholesterol and other lipids, as well as secreted inflammatory substances and apoptotic cells are accumulated in the vessel wall developing a more complex lesion (5,8). (Figure 3)

Following foam cell formation and the presence of fatty streak, other cell types play a key role in the atherosclerosis process. Vascular smooth muscle cells (VSMCs) are activated due to pro-inflammatory cytokines and migrate from the media to the intima layer. VSMCs proliferate and participate in the remodeling of the intima, releasing a variety of pro-inflammatory cytokines and adhesion molecules that enhance inflammation and fibroatheroma plaque formation (8).

Moreover, T-cells and antigen-presenting dendritic cells play an important role too. Antigens presented by recruited macrophages (acting as antigen-presenting cells), trigger the activation of T cells. T cells produce a Th1 response, producing more cytokines, enzymes, and other molecules, which in turn, activate other cells such as macrophages and vascular cells (7). (Figure 3)

In the late stages of atherosclerosis, infiltrated immune and non-immune cells secrete matrix metalloproteinase (MMPs), which mediates the stable lesion to an unstable phenotype and degrades collagen fibers. Degrading the extracellular matrix of the plaque induces its rupture and thrombus formation (8).

Figure 3: Lipid deposition and plaque formation process.



Progression of atherosclerosis in the intima layer of the artery. Source: Inflammation, atherosclerosis, and coronary artery disease (7).

Lipids and lipoproteins

Lipids can be absorbed from meals or synthesized in the liver, being cholesterol and triglycerides of special importance. Lipids are insoluble in plasma and must be bound to proteins (named lipoproteins) to be transported. A lipoprotein is a biochemical complex with a hydrophobic core containing neutral lipids like triglycerides and cholesterol esters surrounded by an outer hydrophilic membrane compounded by phospholipids, free cholesterol, and apolipoproteins (affording the structural form as well as participating in the metabolism of different lipoproteins) (10).

The principal role of lipoproteins is providing the transport for lipids to peripheral tissues including dietary lipids. Lipoproteins are divided into seven groups based on density, size

and lipid composition determined by ultracentrifugation (Table 1). As we can see in Table 1, chylomicrons are the largest particles made by the intestine. A dietary meal rich in lipids produce chylomicron particles increased in the number of triglycerides. On the contrary, poor fat ingestion produces small particles decreased in triglycerides (10).

The next lipoprotein group depending on density is remnant lipoproteins or triglycerides-rich lipoprotein (TRL). TRL contains high amounts of triglycerides. This group includes chylomicrons remnants, very low-density lipoprotein (VLDL), and intermediate-density lipoprotein (IDL) (10,13). Chylomicron remnant is obtained by the hydrolysis from chylomicrons, resulting in a smaller particle. Remnants are lipolyzed to form VLDL, and in turn, VLDL are lipolyzed to form IDL. VLDL are the most abundant triglyceride-rich lipoprotein, so the size of that particle varies depend on the number of transported triglycerides (10,13).

Moreover, the group derived from VDLD and IDL hydrolysis are LDL. LDL transport cholesterol from the liver to other tissues. LDL in fact, could be modified to obtain the smallest density group named lipoprotein (a) (Lp (a)), that enhances atherosclerotic progression (14).

On the other hand, another lipoprotein group is high-density lipoproteins (HDL). HDL absorbs cholesterol from peripheral tissues to the liver, playing a key role in reverse cholesterol transport.

Table 1: Classes of lipoproteins (10)**.**

Lipoprotein	**Density (g/mL)**	**Size (nm)**	**Major Lipids**	**Major Apoproteins**
Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
Chylomicron Remnants	0.930- 1.006	30-80	Triglycerides Cholesterol	Apo B-48, Apo E
VLDL	0.930- 1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
IDL	1.006- 1.019	25-35	Triglycerides Cholesterol	Apo B-100, Apo E, Apo C
LDL	1.019- 1.063	18- 25	Cholesterol	Apo B-100
HDL	1.063- 1.210	5- 12	Cholesterol Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E
Lp (a)	1.055- 1.085	~30	Cholesterol	Apo B-100, Apo (a)

Lipoproteins and atherosclerotic cardiovascular disease

In the atherosclerosis process, endothelium injury produces an enhancement of circulating lipids into the intimal layer, especially LDL particles enriched with cholesterol. LDL and its oxidative modification oxLDL represent an important initial factor in the development of this disease, which is followed and proceeded by infiltration of immune cells such as monocytes. Monocytes differentiate into activated macrophages and lead to the secretion of pro-inflammatory cytokines as IL-1β as they engulf lipoproteins particles (2,5–9).

Lipoproteins are one of the major risk factors among all ASCVDs. Lipoproteins play a key role in atherogenesis changing the phenotype and different properties of cells like macrophages involved in plaque formation (13).

Novel ^{1}H-NMR technique allows us to determine the lipoprotein size and the differentiation up to nine lipoprotein fraction in which the number of particles, their size and their cholesterol and triglycerides content is determined. Advanced lipoprotein test is necessary to improve cardiovascular risk prediction (15).

Between all lipoproteins (Table 1), LDL has been directly implicated in atherosclerosis. Some studies establish that LDL is not only a biomarker of atherosclerosis but also a causal factor of ASCVD. It is known that cumulative LDL into the arteria is determinant for the initiation and development of atheroma plaque as well as atherosclerotic cardiovascular disease (16).

It is known that small dense LDL particles could penetrate the arterial wall without being limited from its size or other biophysical issues. Moreover, these particles due to non-affinity for its receptor, resulted in a prolonged retention in the blood system, which finally produces an enhancement of its entry. Abundance of small LDL particles into arteria are more susceptible to oxidation and finally, they are taken by macrophages becoming foam cells (10,13,16).

On contrary, HDL has been associated with atheroprotection. These particles play an important role in reverse cholesterol transport, a process believed to contribute to the protection of atherosclerotic diseases. In reverse cholesterol transport, HDL promotes cholesterol efflux from macrophages being a critical and important step (10,17,18). Cholesterol efflux is considered a new biomarker due to its important protective function. It has been demonstrated that reverse cholesterol transport (with cholesterol efflux from macrophages as a critical step), is inversely associated with lesion and atheroma plaque size as well as clinical manifestations (10,17,18).

Another lipoprotein group, mentioned before, is triglyceride-rich lipoprotein (TRL) that includes chylomicrons remnants, VLDL, and IDL. The focus on the role of TRL has increased through the last five years (11–13). Furthermore, some studies, as well as epidemiology, suggest that high concentrations of triglyceride-rich lipoproteins are related to metabolic disorders as obesity, type 2 diabetes and metabolic syndrome. Therefore, elevated triglyceride levels are an ASCVD risk factor and they have been associated with mortality. These lipoproteins enriched in triglycerides are considered a new biomarker of cardiovascular risk because of their pathological role, independent of LDL particles (11,12).

TRLs are a strong predictor of ASCVD on account of its ability to penetrate arterial walls and can lead to plaque formation. Between TRL lipoproteins, chylomicron remnant is the largest particle so they suffer biophysical issues to enter into injured endothelium. On contrary, the other TRL lipoproteins, VLDL and IDL, are smaller particles and can enter into arteria as well as LDL particles do (10,13). Recent studies demonstrated that these medium-size TRL particles go through arteria and on account of their size and they get trapped by different immune (macrophages) and non-immune cells (12).

These particles could also be taken up by macrophages, growing atheroma plaque and contributing to the phenotype change to foam cells. Whereas LDL particles needs oxidation to enter into macrophages, TRLs do not require any modification. In addition, macrophages have a VLDL receptor and apolipoprotein receptor (11–13).

Inflammation role of macrophages

Macrophages are cells of the innate immune system taking part in multiple functions. They are in charge of carrying out a “first-line” defense against foreign organisms by phagocytosis process and/or presenting antigens to lymphocytes cells and initiate inflammation by releasing molecules like cytokines (19). These cytokines are also released in conditions like obesity and cancer (among others), affecting other immune and non-immune cells (20).

Macrophages show high plasticity, being able to adapt their phenotype against different environmental stimuli. The different phenotypic changes are known as macrophage polarization. Two major polarization states give rise to the two major functional phenotypes: classically activated type 1 (M1) and alternatively activated type 2 (M2) (19–21).

M1 response is based on the response to produced TH1 cytokines. M1 activation occurs due to microbial products (LPS), lymphocyte Th1 cytokines (TNF-α), or both of them. An M1 macrophage is characterized by enhanced production of pro-inflammatory cytokines like IL-1β, TNF-α, IL-6, and IL-12, a high antigen presentation and production of nitric oxide (NO) and other nitrogen species that enhance inflammation (19–21).

M2 response is based on the response to TH2 cytokines. M2 phenotype can be induced by IL-4, IL-13, toll-like receptors between others. An M2 macrophage is characterized by the decreased production of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines like IL-10 and other mannose receptors like CD206 (19–21).

IL-1β

Interleukin 1β is an established mediator of inflammation that acts as a regulator of endothelial activation. This M1 marker is part of a family of cytokines that play a relevant role in atherosclerosis. IL-1β propeptide requires activation. Propeptide can be activated by macrophages and its expression can be upstream or downstream depending on the stimuli (19–22).

CD206

Mannose receptor (cluster of differentiation 206) is an established M2 marker. M2 polarization macrophages express high levels of CD206 and produce anti-inflammatory cytokines like IL-10 (19–22).

HYPOTHESIS

Lipoproteins derived from triglyceride-rich particles (VLDL, IDL) increase their concentration in situations of metabolic alteration such as obesity, metabolic syndrome and diabetes. Given that their size allows them to reach the subendothelial area of the vascular wall, they exert a direct atherogenic role in addition to that of LDL, depositing cholesterol and other lipids such as certain classes of fatty acids that activate the inflammatory response of macrophages present in the vascular wall of the arteries. The quantitative determination of these particles can also be carried out by ^{1}H-NMR with great precision. Both the composition and the inflammatory role of these lipoproteins can contribute to a better understanding of the pathogenesis of vascular and metabolic alterations, as well as to detect possible new biomarkers of vascular risk and define new therapeutic targets.

OBJECTIVES

General objective:

To analyze the effect of TRLs derived from patients with metabolic disorders such as obesity, metabolic syndrome and diabetes on the inflammatory status of macrophages.

Specific objectives:

1. Isolate TRL by ultracentrifugation from the plasma of the mentioned patients.

2. Determine by ^{1}H-NMR the number of TRL particles and their size in patients.

3. Analyze the composition of TRL according to quartiles of TG.

4. Incubate TRL, pooled according to TG quartiles with macrophages to determine inflammatory function (IL-1beta and CD206), cytotoxicity and lipid uptake.

MATERIAL AND METHODS

TRL isolation and analysis

Plasma samples from 340 individuals from the METBANC collection was used to isolate TRL by ultracentrifugation. The METBANC collection includes patients with obesity, diabetes and atherogenic dyslipidemia who were recruited during the years 2010-2015 in the UVASMED unit of the Hospital Universitari de Sant Joan de Reus (23). Plasma samples were stored at -80º in the Biobanc of IISPV until use.

With the purpose of studying the inflammatory function of TRL for the present project, it is necessary to obtain and extract lipoprotein fractions from the plasma samples. The method used for the separation of plasma lipoproteins was preparative sequential ultracentrifugation, which is based on using different densities for their separation. The aim of ultracentrifugation method consists in the extraction of the desired lipoprotein fraction concentrated in a layer at the top of ultracentrifuge tube. Moreover, subfractions of each plasma lipoproteins could be isolated due to its particular density.

The separation of plasma lipoproteins was carried out in the to the Optima XPN-100 ultracentrifuge model (Beckman Coulter) with a rotor fixed angle Kontron 45.6. ANNEX 1

First of all, samples were defrosted at room temperature. To separate lipoproteins fractions, 2 mL of each plasma sample is needed and caught in 6.5 mL tubes (Beckman Coulter). Then, salt solutions were prepared at 7.4 pH just to obtain the defined density on each fraction and then added to Beckman Coulter tubes following the explained protocol:

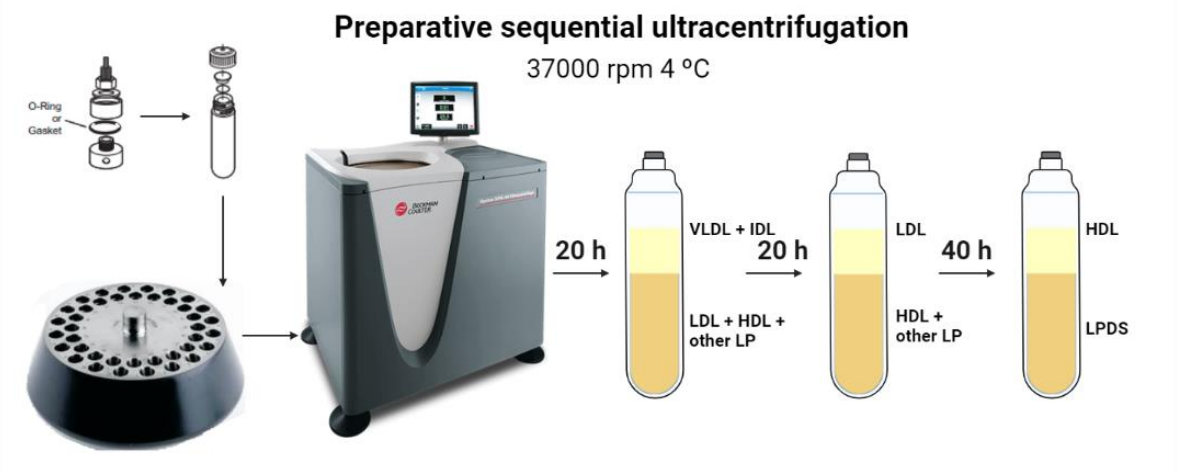
1. **TRL isolation (VLDL + IDL)**: Very Low-Density and Intermediate-Density Lipoproteins were isolated, so they were arranged in a tube, 2 mL of plasma sample plus 2 mL of 1.006 g/mL salt solution (NaBr) plus 1 mL of 1.070 g /mL just to obtain the desired fraction with a density between 1.006 g/mL and 1.019 g/mL. The samples were centrifuged at 37.000 rpm at 4 ºC during 20 hours. After these time, 2 mL were taken from the upper zone of the tub corresponding to the VLDL and IDL fraction.

Once lipoproteins were separated by sequential preparative ultracentrifugation they could be biochemically analyzed. The purpose was to obtain biochemical parameters including cholesterol, triglycerides, and phospholipids using a colorimetric assay (Spinreact) and apolipoprotein B-100 using an immunoturbidimetric assay (Horiba)

which were adapted to the Cobas Mira Plus Autoanalyser (Roche Diagnostics). In addition, total protein parameter was obtained using Lowry method. ANNEX 2

In this project, only TRL fraction was analyzed requiring 150 µl of each VLDL + IDL isolation sample by Cobas Mira Plus Autoanalyser and at the same time analyzing total protein concentration by Lowry method.

Figure 4: Preparative sequential ultracentrifugation process.



Workflow of preparative sequential ultracentrifugation to obtain lipoproteins fractions from plasma samples. VLDL (very-low density lipoprotein), IDL (intermediate-density lipoprotein), LDL (low-density lipoprotein), HDL (high-density lipoprotein), other LP (other lipoproteins) and LPDS (lipoprotein deficient serum). Rpm (revolutions per minute).

TRL particle analysis by ^{1}H-NMR

The determination of lipoprotein particle size and particle number concentration of three subtypes of TRL (including large, medium and small) were NMR analyzed so as to achieve a more detailed lipid profile.

NMR spectroscopy is a technique that enables analysis of structural determination or organics compounds such as lipoprotein particles. A novel method developed with the collaboration of our group based on 2D diffusion-ordered ^{1}H-NMR (proton nuclear magnetic) spectroscopy (DOSY) named Liposcale test was used to obtain a detailed lipid profile from plasma samples. Liposcale test has high sensitivity without handling samples and the obtaining of results is fast (15).

In order to obtain an extensive analysis of TRL fraction, a 250 µl of plasma sample was shipped to Biosfer Teslab, creators of Liposcale Test (Reus, Spain). Before ^{1}H-NMR analysis, 200 µl of TRL samples were diluted with 50 µl of deuterated water and 300 µl

of 50 mM PBS were added. ^{1}H-NMR spectra were recorded on a Bruker Avance III 600 spectrometer operating at a proton frequency of 600.2 MHz, at a 305.95 K.

This test obtains particle concentrations and diffusion coefficients from the methyl moieties of the lipids in lipoproteins after the analysis of NMR pulse. Lipoproteins particles resonate at slightly different frequencies, each LP subtype has hydrodynamic characteristics associated. Diffusion coefficients allow the parameter size arise from Stokes-Einstein equation.

The measurement of the size is used to calculate the number of LP. Each LP subtype is associated with a Lorentzian function (F1 to F9). The area of each Lorentzian function reflected the lipid concentration of each subtype.

Cell culture

Human monocyte THP-1 (ATCC, cell line derived from an acute monocytic leukemia) cells were maintained in RPMI1640 (Roswell Park Memorial Institute 1640, Biowest-Labclinics) supplemented with 10% (v/v) FBS (fetal bovine serum, Biowest-Labclinics) and 1% (v/v) Pen/Strep (penicillin-streptomycin, Biowest-Labclinics) with a density $<1x10^{6}$ cells/mL. THP-1 cell line was incubated at 37 ºC with 5% CO_2.

THP-1 monocytes were differentiated into macrophages by 72 hours incubation with PMA (phorbol 12-myristate 13-acetate, Sigma) at 200 nM in the supplemented RPMI1640 medium (Bio-west). Macrophages cells were wash out with PBS (phosphate-buffered saline, Bio-west) and followed by 24 hours incubation in RPMI supplemented medium (24).

In vitro treatment of macrophages with TRL

THP-1 monocytes were seeded at 700.000 cells/well in 6 well plates. Monocytes are differentiated into macrophages as described. Cells were washed out with PBS and incubated with TRL at different times (4h, 8h, 24h, 30h) and different concentrations.

TRL were separated into quartiles of triglycerides concentration in VLDL + IDL (TRL). Six samples of each quartile were caught with 150 µl for sample and added all together to form 4 different pools named Q1, Q2, Q3 and Q4.

Once the pools were prepared, it must be dialyzed to remove the overconcentration of salt present in all the samples and pools due to the ultracentrifugation process. In order to start the dialysis process, a fragment of semipermeable dialysis membrane

(MW 12-14.000 Da, Medicell), was cut for each quartile pool. The membranes were placed in distilled water and boiled. Then membranes are removed from the boiled water, closed at one end and inserted the pool sample. Membranes were closed and inserted into an Erlenmeyer flask containing autoclaved PBS (pH at 7.4) stirring at 4 ºC. After 1 hour PBS was removed and replaced with new PBS so that the PBS solution was not saturated and after another hour the membranes of the flask were removed and the TRL content was recollected into 1.5 mL tube (Eppendorf).

LPS (10 ng/mL) in RPMI serum free was used as positive control for the expression of pro-inflammatory cytokines. Finally, macrophages were then treated with 10% v/v of dialyzed quartile in RPMI serum free, forming Q1, Q2, Q3 and Q4 different conditions. Moreover, cells were cultured with TRL quartiles at different times (4 h, 24h) at 37 ºC with 5% CO_2.

After the incubation time, the supernatants were collected and stored at -80 ºC until used LDH cytotoxicity and cytokine assays.

mRNA analysis

RNA was extracted from the TRL conditioned macrophages and control wells using a PureLink RNA Mini Kit (Invitrogen) according to the company protocol. Extracted RNA was quantified by Sinergy HT reader (Biotek) and finally stored at -80 ºC until needed for the RT-qPCR. ANNEX 3

RT-qPCR

Extracted RNA was reverse transcribed using PrimeScript RT reagen Kit (Takara Bio) so as to obtain cDNA following the protocol. Q-PCR was performed using Taqman (Life Technologies) in a LightCycler 96 (Roche) and primers of different genes as IL-1β, CD206 and the reference primer gene for normalization GADPH.

Table 2: Primers used in q-PCR.

GENE	COMPANY	REFERENCE
IL-1B	Life Technologies	Hs01555410_m1
CD206	Life Technologies	Hs00267207_m1
GADPH	Life Technologies	Hs99999905_m1

LDH cytotoxicity assay

LDH is released into the cell culture medium upon damage to the plasma membrane. For this reason, LDH assay was used to determine cytotoxicity in supernatant THP-1 cells upon TRL treatment.

Briefly, 100 µl culture medium from each sample was introduced into 96-well plate and 100 µl of reaction mixture (Roche) were added to each well. After 30 minutes, the activity was quantified using a plate reader measured at Δ620/492 nm in Sinergy HT reader (Biotek).

Determination of IL-1β and CD206 secretion by ELISA

The supernatants collected and stored after incubation period time and conditions were defrosted at room temperature. Cytokine secretion in the supernatant medium was assayed using ELISA kits following the procedure recommended.

The cytokines analyzed were IL-1β and CD206 (RayBiotech). These assays were based on a quantitative type enzyme-linked immunosorbent sandwich assay. The kit provides a 96-well plate on which an antibody specific for human IL-1β or CD206 was coated. Previous to analyze the samples, we have tested different dilution to optimize the protocol. Standards and diluted samples (optimal dilution factor was 1:2 for IL-1β and no dilution for CD206) are pipetted into the wells and cytokines present in cell culture supernatants samples were bounded by the immobilized antibody. After the incubation period, wells were washed out and the biotinylated antihuman IL-1β or CD206 was added. After washing antihuman antibody streptavidin was pipetted to the wells. The wells were again washed and TMB (tetramethylbenzidine) substrate solution was added growing a blue color proportion to the amount of cytokine bounded to the well; then the addition of the stop solution changed the color from blue to yellow and was finally measured at 450 nm at Sinergy HT reader (Biotek).

Cell staining

Nile red

Nile red (9-diethylamino-5H-benzo[α]phenoaxazine-5-one) is a fluorescent vital stain used for the detection of intracellular lipid droplets. Nile red binds to neutral lipids as cytoplasmic droplets usually triglycerides or cholesterol esters and polar lipids too.

This stain assay was performed to observe differences between the fatty uptake of differentiated macrophages incubated with and without TRL. Nile red was treated on macrophages after the exposure of 8 hours with TRL. The different conditions of THP-1 macrophages were washed twice with PBS and finally incubated with diluted 500-fold Nile Red dye (Sigma-Aldrich). After 5 minutes of exposure in the dark, fluorescence intensity was captured using an inverted microscope (Olympus IX71).

Fluorescence intensity was detected and quantified after washing with PBS and adding 300 µl of cholic acid 1% (Sigma) in methanol for 1 hour, finally 100 µl were picked for duplicate sample. The intensity was detected at λ_{ex} 485/20 an λ_{em} 590/35 using a fluorescence reader (Sinergy HT reader (Biotek).

Oil red

Oil red is a fat-soluble dye used for the detection of triglycerides and lipoproteins. Oil red is highly soluble in lipids binding to neutral lipids within cultured cells. Lipid droplets appear in a red color.

This stain assay was performed to observe differences between control macrophages and incubated 8 hours TRL macrophages. Cells were fixed using paraformaldehyde 4% and washed with isopropanol. Then oil red (Sigma-Aldrich) was added during 10-20 minutes of exposure to samples and controls, cells were washed with water and finally viewed and captured under an inverted microscope (Olympus IX71).

Statistical analysis

The normality of continuous variables was determined by the Kolmogorov–Smirnov test. Data are presented as the medians and 25th and 75th percentiles (IQR) for continuous variables not normally distributed or the mean and standard deviation (SD) when normally distributed. Categorical variables are expressed as percentages unless otherwise indicated. To evaluate differences between quartiles ANOVA or Kruskal-Wallis tests were used. To evaluate differences between control and quartiles in the in vitro experiments, Mann-Whitney U test was used. Statistical analyses for clinical data were performed using SPSS software (IBM SPSS Statistics, version 27.0.1.0) and GraphPad Prism 5 for analysis of in vitro experiments. Statistical tests $p < 0.05$ were taken as significant.

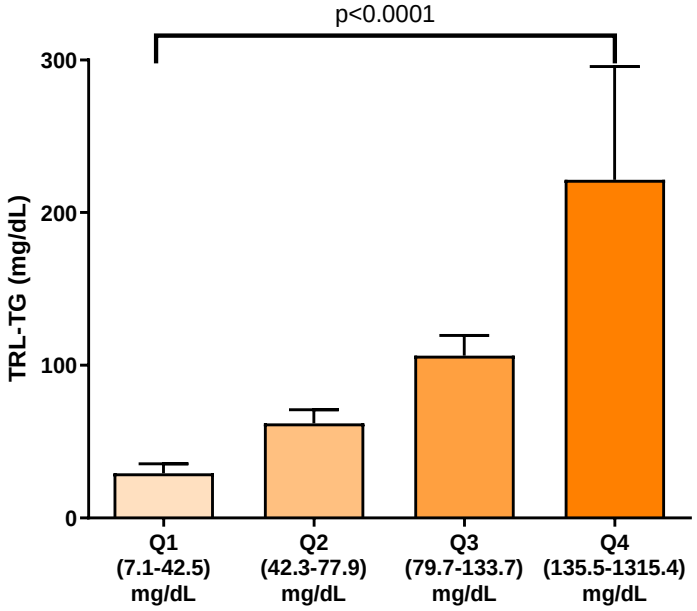
RESULTS

CLINICAL STUDY

Clinical, anthropometric and lipid characteristics of the study subjects

In the study METBANC cohort, obesity was present in 52.5%, diabetes mellitus (DM) in 72.8%, and metabolic syndrome (MS) in 78.6% of the patients (23). The METBANC cohort (n=340) was divided into four growing quartiles of triglycerides concentration in TRL previously isolated. Figure 5 shows the median range of each quartile which were statistically different (p<0.0001).

Figure 5: Patients of the METBANC cohort divided into quartiles of TG-TRL (Q1 n=84; Q2 n=85; Q3 n=86; Q4 n=84).



Bar graphs of TG-TRL divided into four quartiles. Each bar represents the median with interquartile range of TG-TRL. X axis shows the minimum-maximum value of each quartile in mg/dL. p-value from Kruskal-Wallis test.

Table 3 summarizes the clinical, anthropometric, biochemical characteristics and TRL lipidomics of the 72 patients (18 patients/quartile) that were selected for the in vitro study. Patients included had a median age of 61 (51-67) years, of which 45.8% were female. Metabolic syndrome was present in 84.7%.

With respect to metabolic disorders, obesity, atherogenic dyslipidemia and metabolic syndrome increased in each quartile (p<0.05), but diabetes decreased (p=0.024). All biochemical data but glucose deferred statistically (p<0.01), with cholesterol and triglycerides increasing in each quartile. Moreover, TRL lipidomics parameters except size had growing concentration values between quartiles (p<0.001).

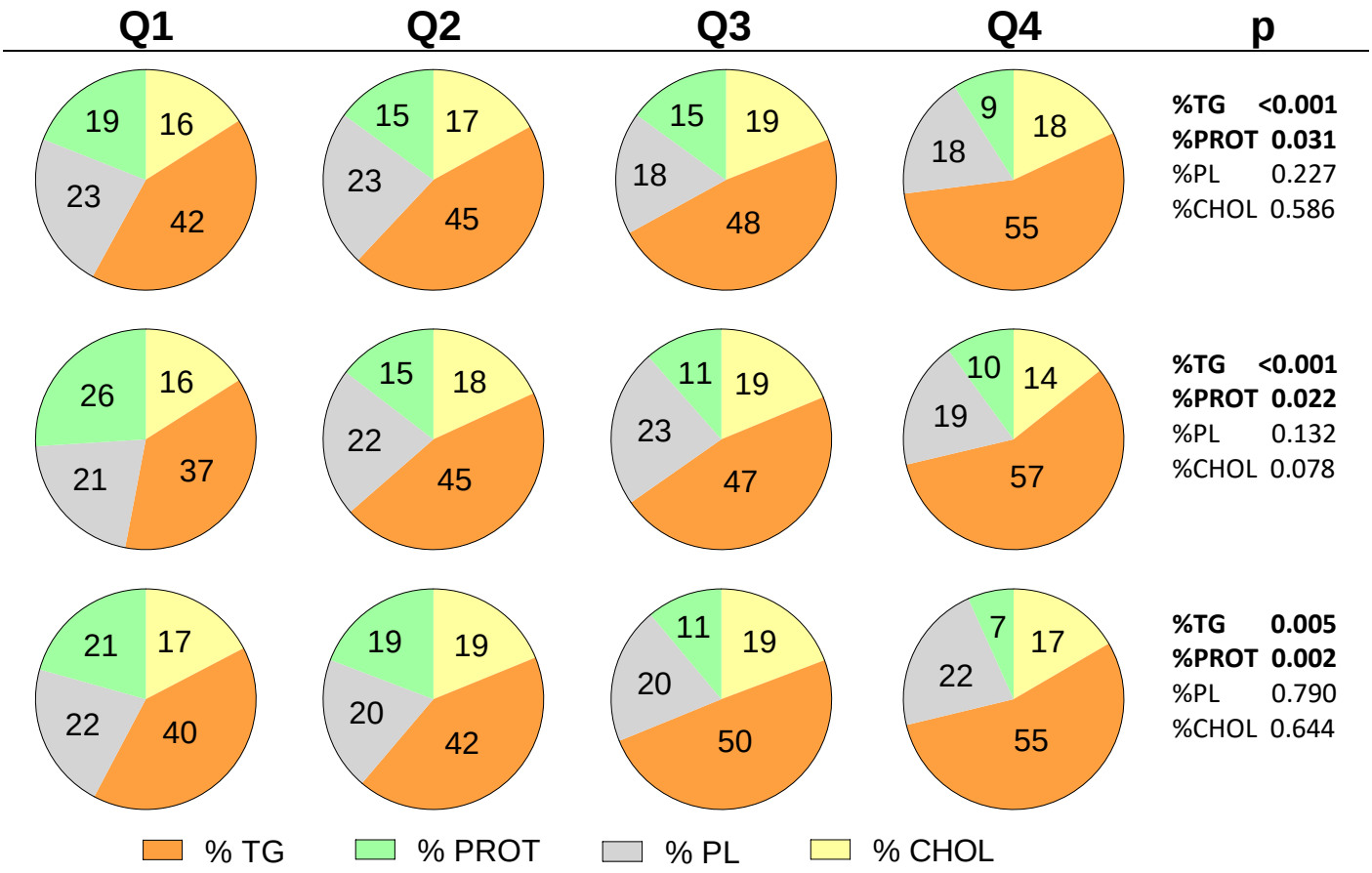
Table 3: Clinical, anthropometric, biochemical characteristics data and TRL lipidomics of the study population grouped by growing quartiles of triglycerides in TRL fraction.

	Q1 (N=18)	Q2 (N=18)	Q3 (N=18)	Q4 (N=18)	p
Clinical data					
Sex, women %	50	55.6	38.9	38.9	0.680
Obesity, %	33.3	66.7	77.8	77.8	**0.016**
Diabetes, %	94.4	94.4	77.8	77.8	**0.024**
Atherogenic dyslipidemia, %	0	16.7	77.8	61.1	**<0.001**
Metabolic Syndrome, %	61.1	88.9	94.4	94.4	**0.014**
Age, years	65 (50-70)	64 (57-69)	62 (48-66)	53 (49-59)	**0.049**
BMI, kg/m^2	28.66 ± 4.35	32.06 ± 6.11	34.03 ± 5.90	32.77 ± 3.67	**0.017**
Waist circumference, cm	98.6 ± 10.7	109.7 ± 12.0	108.1 ± 11.5	108.5 ± 8.5	**0.010**
Biochemical data					
Glucose, mg/dL	135.5 (112-207)	134.5 (120-150)	143.5 (114-164)	138.0 (119-195)	0.879
Cholesterol, mmol/L	5.12 (4.60-5.92)	5.14 (4.69-6.09)	6.93 (5.48-7.44)	7.19 (6.17-7.77)	**<0.001**
Triglycerides, mmol/L	0.80 (0.77-0.96)	1.42 (1.28-1.50)	2.16 (1.98-2.65)	4.40 (4.06-4.84)	**<0.001**
HDL-C, mmol/L	1.45 ± 0.22	1.14 ± 0.23	1.14 ± 0.20	1.16 ± 0.27	**<0.001**
LDL-C, mmol/L	3.39 ± 0.81	3.45 ± 0.70	4.39 ± 1.25	3.75 ± 1.07	**0.012**
Apo A-I, mg/dL	145.61 ± 20.36	125.22 ± 24.93	122.33 ± 14.72	131.56 ± 23.54	**0.008**
Apo B-100, mg/dL	101.56 ± 20.56	111.72 ± 19.87	145.17 ± 31.25	143.56 ± 28.49	**<0.001**
TRL lipidomics					
Total, nmol/L	29.01 (27.35-31.63)	49.28 (45.70-58.83)	79.84 (67.19-86.28)	156.91 (134.68-166.66)	**<0.001**
Large, nmol/L	0.86 (0.76-0.93)	1.22 (1.05-1.43)	1.72 (1.50-1.99)	3.30 (3.03-3.57)	**<0.001**
Medium, nmol/L	3.87 (3.21-4.36)	6.21 (5.73-6.85)	9.75 (8.37-10.73)	21.35 (19.56-24.99)	**<0.001**
Small, nmol/L	24.53 (23.37-25.81)	42.61 (38.40-50.63)	68.65 (57.32-74.63)	130.43 (110.51-141.76)	**<0.001**
Size, nm	42.4 ± 0.13	42.27 ± 0.08	42.24 ± 0.12	42.36 ± 0.17	**0.001**

Data are means ± SD for normally distributed variables, medians with interquartile range for nonparametric data or n (%). BMI, body mass index; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; Apo A-I, apolipoprotein A1; Apo B-100, apolipoprotein B100; TRL, triglyceride-rich lipoprotein. p-values are for group comparisons. Statistical analysis: $\chi 2$ for categorical data; t-test or Kruskal-Wallis tests are for continuous variables.

The isolated TRL fraction from the 72 patients (18 samples/quartile) described were pooled in three different experiments and grouped in four quartiles of TG-TRL as previously described. For each experiment, six different TRL samples were used to generate a pool for quartile. Figure 6 shows the biochemical composition of each quartile used for the three independent experiments: percentage of triglycerides increased in each quartile ($p \leq 0.005$) whereas the percentage of total proteins in the TRL fraction decreased ($p < 0.05$). ANNEX 4

Figure 6: Biochemical composition of the pooled TRL fractions divided into quartiles.



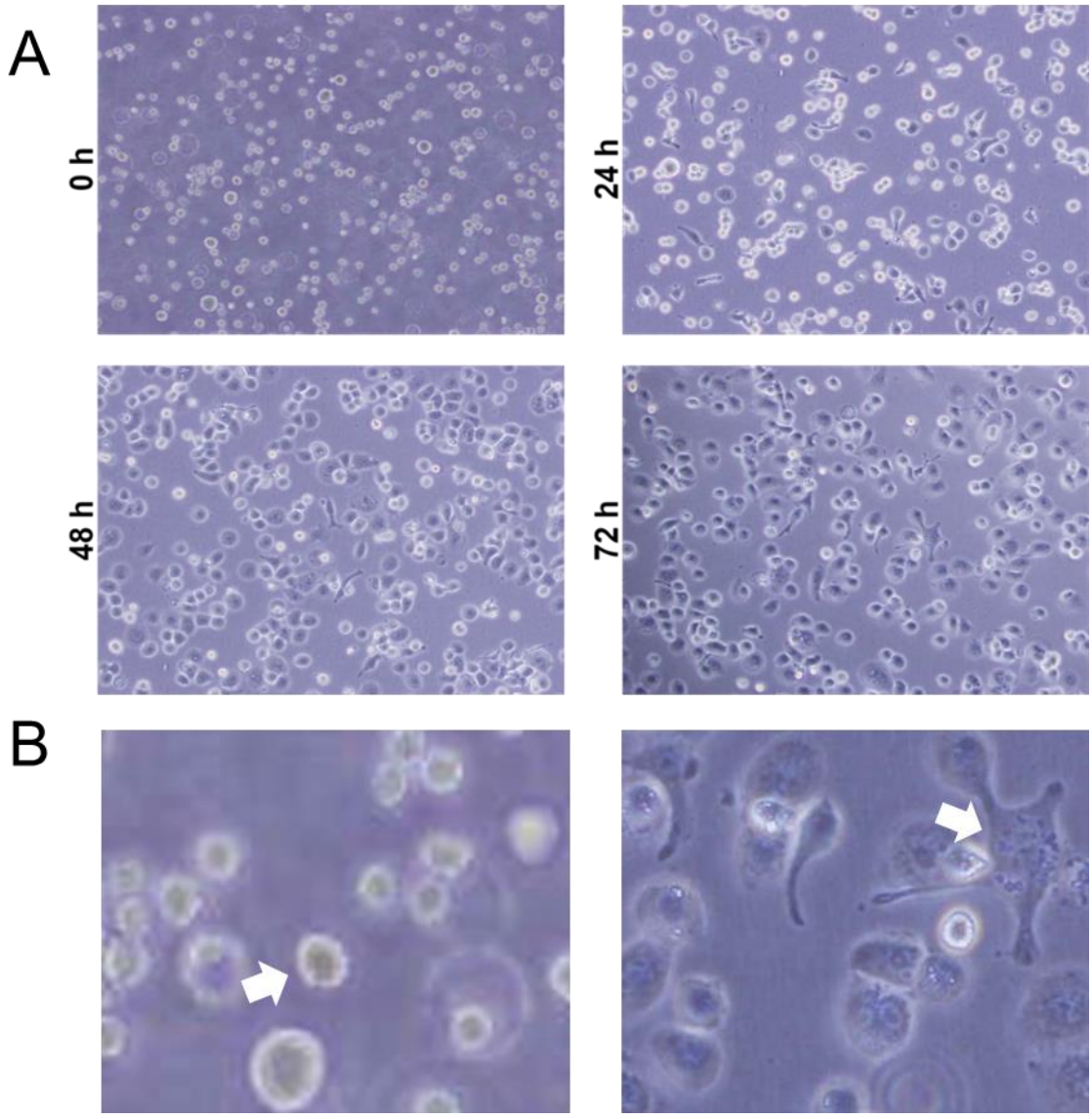
Pie chart of the biochemical composition of TRL pools divided in four quartiles of TG-TRL. The mean percentage of each parameter was obtained by the media of the six samples used for each quartile. CHOL, cholesterol; TG, triglycerides; PL, phospholipid; PROT, protein. p-values from Kruskal-Wallis test.

IN VITRO STUDY

Differentiation of THP-1 monocytes to macrophages with PMA

THP-1 monocytes were cultured in suspension and treated for 72 hours with 200nM PMA. Figure 7A shows the time-course differentiation of monocytes to macrophages at 24h, 48h and 72h. Monocytes changed to an adherent phenotype and their morphology from round to spindle (shown in Figure 7B).

Figure 7: Differentiation of THP-1 monocytes to macrophages with PMA.



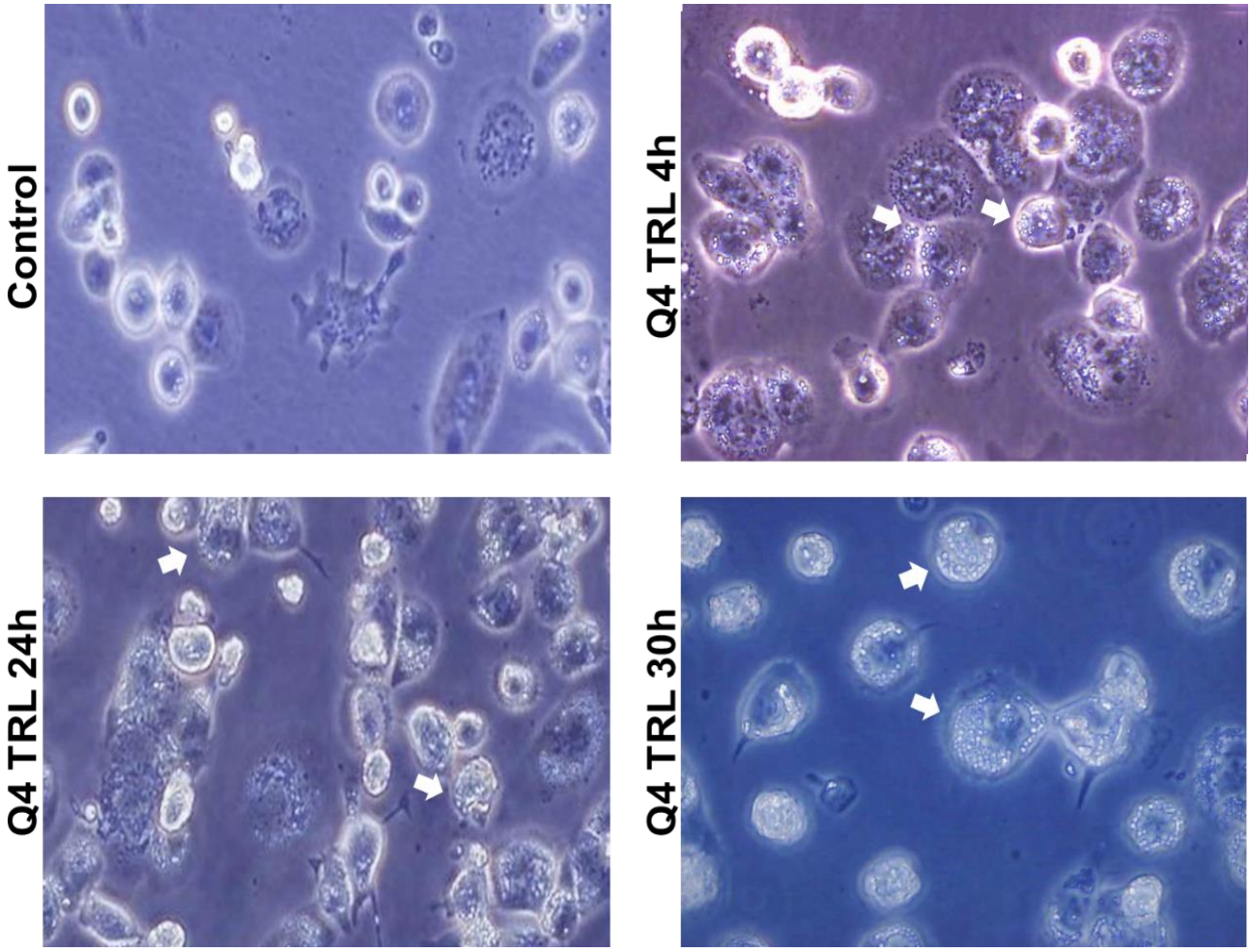
A) Time-course of THP-1 monocytes differentiation at 24 h, 48h and 72 h with PMA (200nM). The cells were photographed at 200X magnification with an inverted microscope. **B)** Cell morphology of THP-1 changes to an adherent phenotype after PMA treatment for 72h (right). Morphological changes were visualized under 400X magnifications. Arrows indicate a monocyte (left) and differentiated macrophage (right).

Treatment of THP-1 macrophages with quartiles of the TRL fraction from patients

TRL uptake by THP-1 macrophages

The effects of triglycerides in TRL fraction by THP-1 macrophage cell model were investigated. After differentiation, macrophages were treated with 10% v/v TRL pools at different time conditions (4-30h). TRL were observed as cytosolic lipid droplet after 4 hours of Q4 TRL treatment as shown in Figure 8. Treatment of THP-1 macrophages with TRL quartiles (Q1-Q4) were uptaken by macrophages. The effect of quartile four (Q4) on THP-1 macrophages is observed in Figure 8.

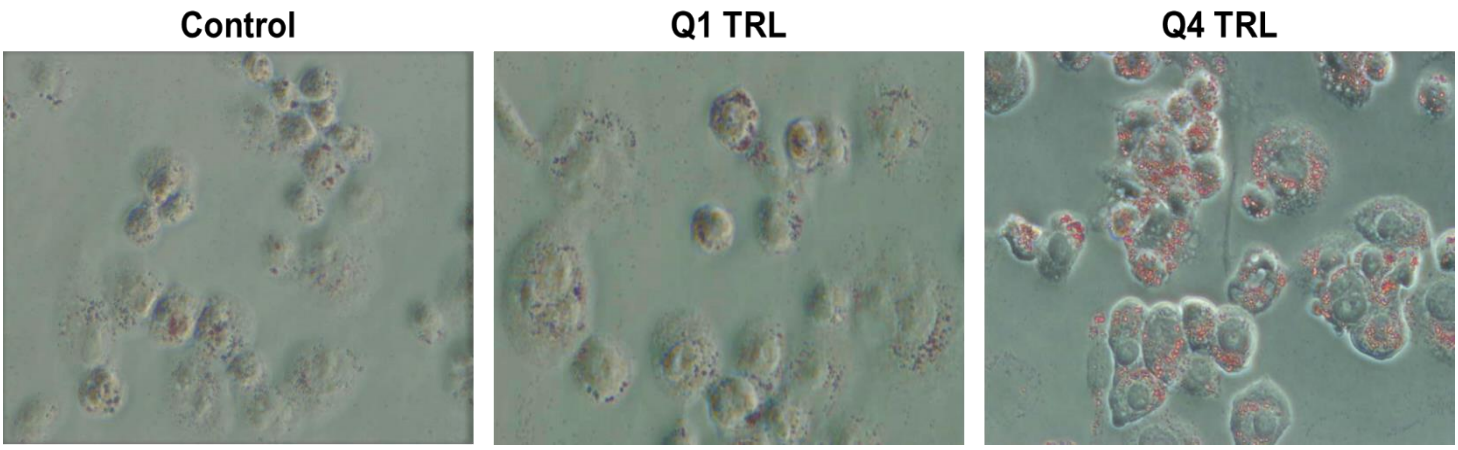
Figure 8: TRL uptake by macrophages.



Time-course of TRL (10% v/v) uptake by macrophages after 4h, 24h and 30 h. Control refers without treatment. Cells were imaged at different incubation times under 400X magnifications. Arrows represent lipid droplets within the macrophages cytoplasm.

Oil and Nile red staining were performed at 8 hours after TRL treatment to observe differences between the fat uptake of macrophages incubated with TRL and control macrophages. Figure 9 shows the Oil red staining of control, Q1 and Q4 TRL. Q2 and Q3 TRL lipid droplets were also stained (not shown).

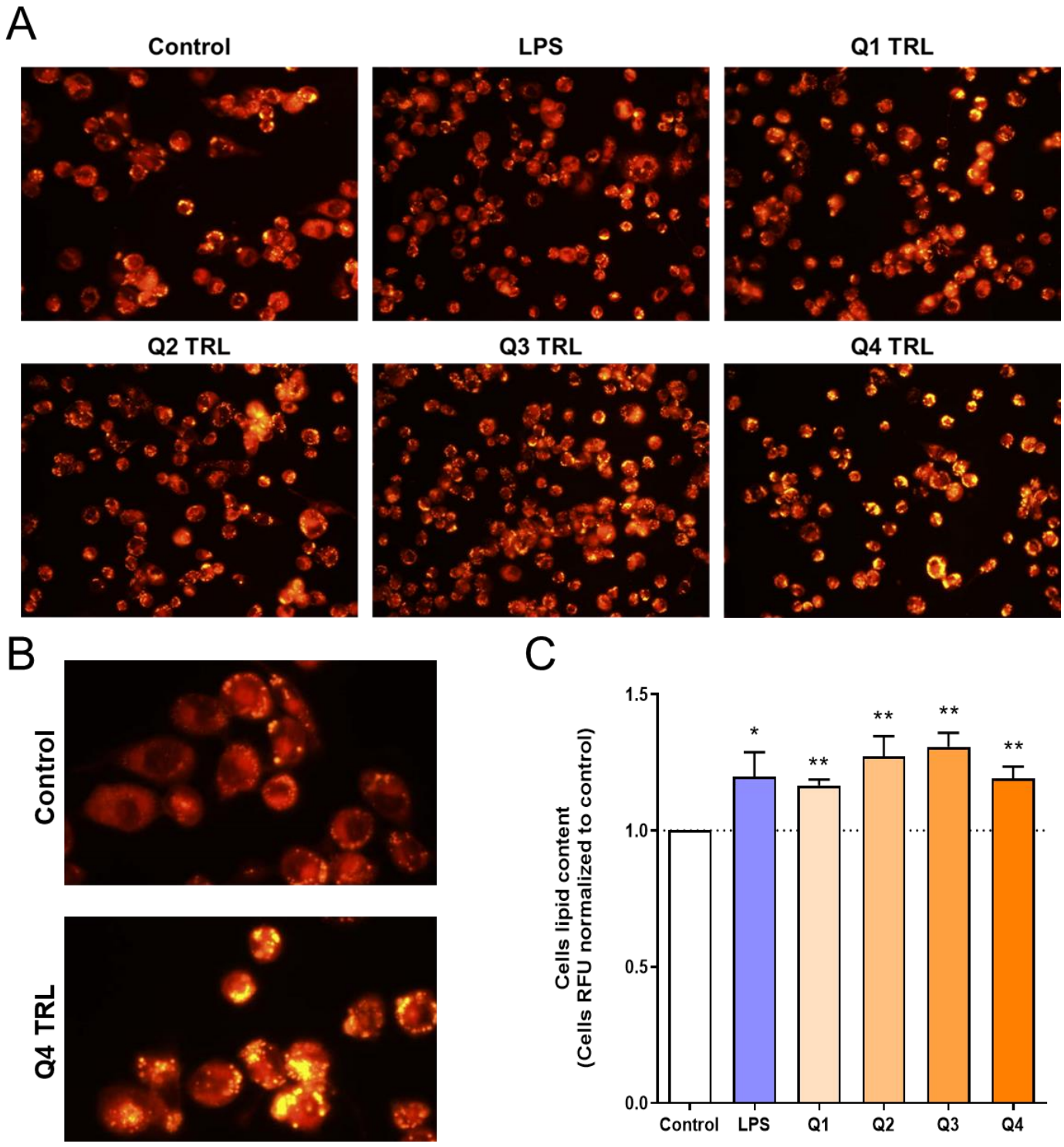
Figure 9: Oil red staining of THP-1 macrophages treated with TRL quartiles.



Oil red staining of control macrophages, Q1 and Q4 TRL incubated (10 % v/v) with THP-1 macrophages at 8 hours. Lipid droplets are stained in red. Control refers without treatment. Cells were photographed at 400x magnification in an inverted microscope.

Figure 10A represents the Nile red staining of THP-1 macrophages incubated with LPS (10 ng/mL), Q1-Q4 TRL (10% v/v) for 8h. Quartiles showed a higher fluorescence intensity compared with control (without treatment). In Figure 10B we can observe a higher magnification differences between control and Q4 TRL whose lipid droplets showed higher fluorescence intensity. In order to measure the lipid content, Nile red dye extraction was performed using cholic acid. Then fluorescence was quantified and represented in the Figure 10C. LPS, and all TRL treatments were significantly higher with respect to control.

Figure 10: Nile red staining of THP-1 macrophages treated with TRL quartiles.

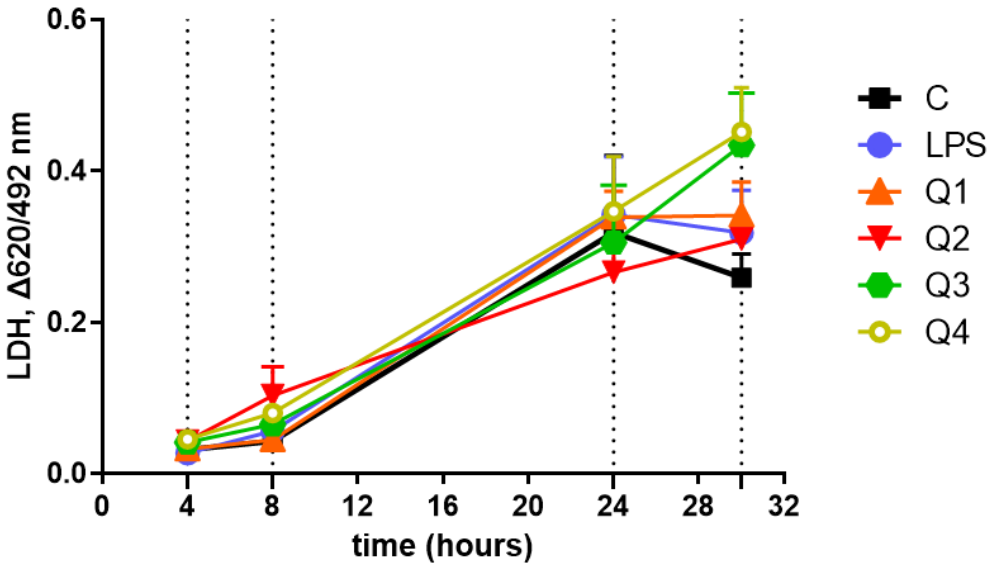


A) Nile red staining of macrophages after 8h TRL treatment in control (without treatment), LPS (10 ng/ml), Q1-Q4 TRL (10% v/v). Cells were photographed at 200X magnification using an inverted microscope. Lipid droplets were stained in red-yellow depending on the lipid content. **B)** Control and Q4 TRL treatment at 400X magnification. **C)** Lipid content quantification after dye extraction with cholic acid. Relative Fluorescence Units (λex 485/20, λem 590/35) were normalized to control. Data represent the mean of two independent experiments each one done for duplicate.
p-values from Mann-Whitney U test. *p<0.05 vs control; **p<0.01 vs control.

Effect of TRL treatment on THP-1 macrophages cytotoxicity

To study the cytotoxic effect of TRL in THP-1 macrophages, LDH assay was performed. Cells were treated with 10% v/v of TRL pools during 4h, 8h, 24h and 30h. The time-course of LDH released to culture media is represented in Figure 11. LDH release increased with time and no differences were observed between control, LPS and TRL treated cells. However, after 24 hours Q3 and Q4 showed a weak increase in the release of LDH compared with control.

Figure 11: LDH release in THP-1 macrophages treated with quartiles of TRL.



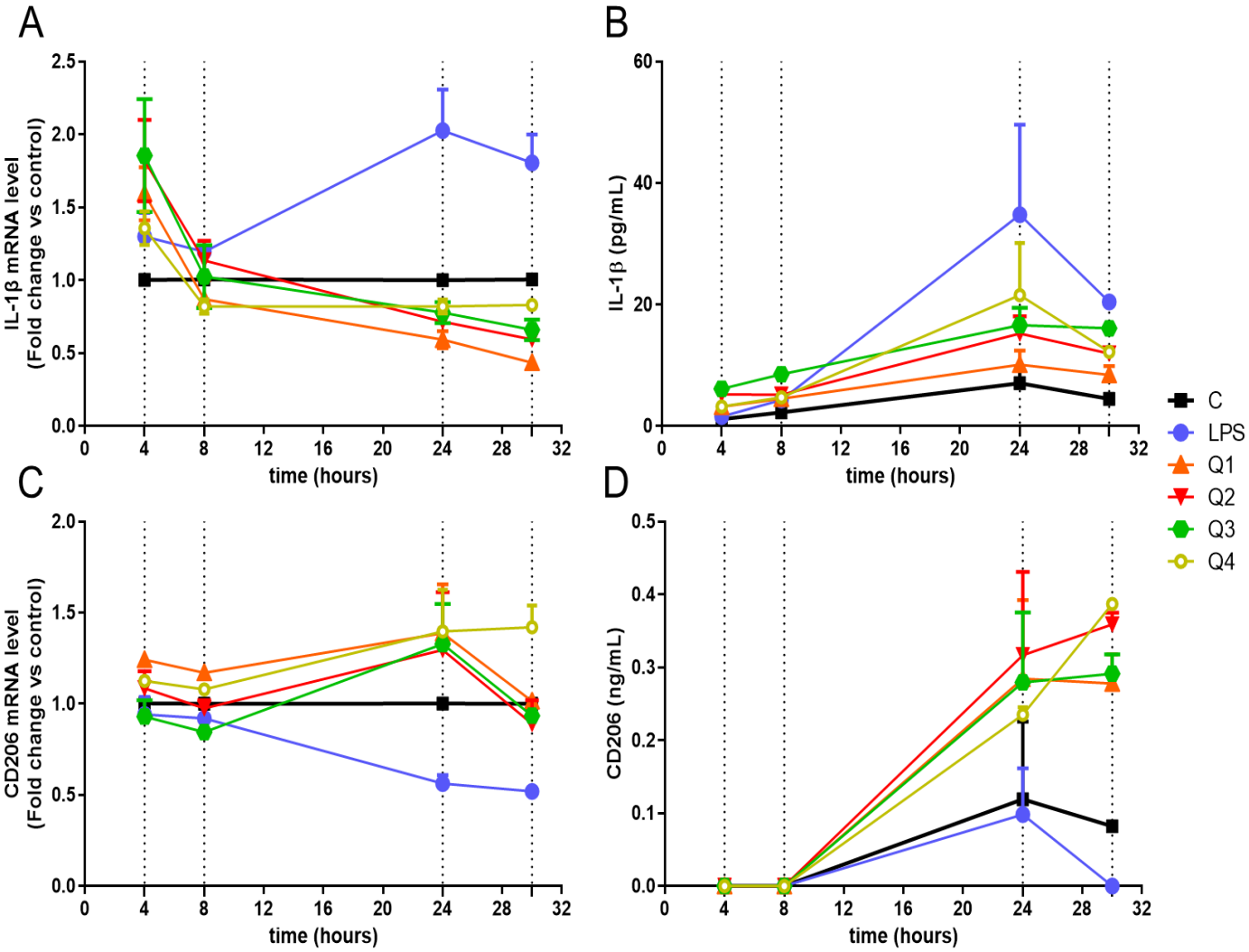
LDH cytotoxicity assay of macrophages after TRL treatment with LPS (10 ng/mL) and Q1-Q4 TRL (10% v/v) at 4h, 8h, 24h and 30h. C, control without treatment. The graph shows the mean absorbance ± SEM (Δ620/492 nm) of LDH release to the media. Data represent the mean of three independent experiments each one done for duplicate.

Inflammatory effects of TRL treatment on THP-1 macrophages

In order to study the inflammatory effects of TRL on THP-1 macrophages we measured the expression of a classical M1 polarization marker IL-1β as a pro-inflammatory gene, and CD206 as anti-inflammatory M2 polarization gene (19).

Figure 12 shows the time-course of the expression (mRNA) (Figure 12A) and secretion (protein) (Figure 12B) of IL-1β by THP-1 macrophages treated with TRL. We show that at 4h, LPS (positive control) and all the quartiles of TRL increases the expression of IL-1β mRNA compared with respect to control ($p<0.05$) (Figure 12A). After 4 hours we show that TRL treatment decreases the mRNA expression until 30h with respect to control. This decrease in the mRNA expression was accompanied with an increase of IL-1β protein (Figure 12B). At protein level we observed that LPS and TRL increases IL-1β secretion until 24h with respect to control (Figure 12B). In addition, we determined the time-course of the expression (Figure 12C) and secretion (Figure 12D) of the anti-inflammatory protein CD206. At mRNA level we observed that TRL did not change the expression of CD206 compared with control until 24 hours. At 24h we observed an increase of CD206 by quartiles of TRL. As expected, LPS decreases the expression of CD206 compared with control and was time dependent (Figure 12C). At secretion level of CD206 we observe no detectable values until 8 hours. THP-1 macrophages treatment with TRL increases the CD206 secretion at 24 hours with respect to control and treatment with LPS decreases the expression compared with control (Figure 12D).

Figure 12: Time-course of the mRNA expression and protein secretion of IL-1β and CD206 by THP-1 macrophages.



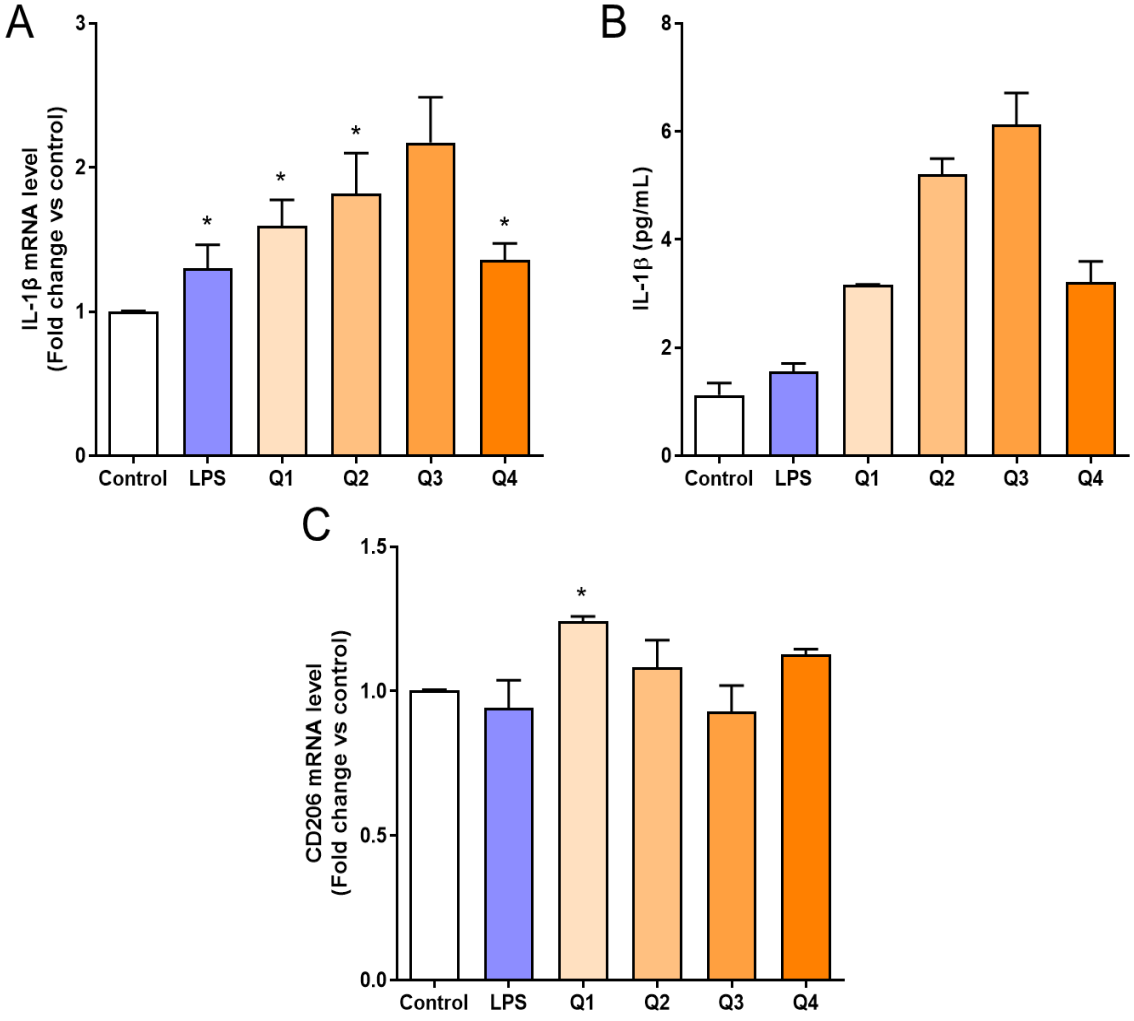
A) Time-course of gene expression of IL-1β at 4h, 8h, 24h and 30h. Cells were treated with LPS (10 ng/mL) and Q1-Q4 of TRL pools (10%v/v). **B)** Time-course of secretion of IL-1β at 4h, 8h, 24h and 30h. The graph shows the mean ± SEM (pg/mL). **C)** Time-course of gene expression of CD206 at 4h, 8h, 24h and 30h. Cells were treated with LPS and Q1-Q4 of TRL pools. **D)** Time-course of secretion of CD206 at 4h, 8h, 24h and 30h. The graph shows the mean ± SEM (ng/mL). Data represent the mean of three independent experiments each one done for duplicate.

Figure 13 shows the expression and secretion levels of IL-1β and CD206 at 4 hours.

At mRNA level at 4 hours of TRL treatment, we observed that Q1, Q2 and Q3 of TRL gradually increases the expression of IL-1β compared to control (60% to 117%, $p<0.05$). LPS increases 30% ($p<0.05$) with respect to control. TRL have a high effect on IL-1β expression than the positive control LPS (Figure 13A). At secretion level we showed that LPS and TRL induce a weak increase of IL-1β compared to control and this secretion increases between quartiles (182% to 446%) except Q4 TRL (Figure 13B). At CD206 mRNA level, Q1 of TRL induced a higher expression of CD206 compared with control

(24%, p<0.05). We observed a decrease of the expression of CD206 along TRL quartiles until reaching control CD206 expression levels (Q2=8% and Q3=-7%), except Q4 TRL. As expected, LPS showed a lower expression of CD206 compared with control (-6%) (Figure 13C). Similarly to the Figure 12D we could see no detectable values of CD206 secretion at 4 hours of LPS or TRL treatment (not shown).

Figure 13: Expression and secretion levels of IL-1β and CD206 after 4h of TRL and LPS treatment.

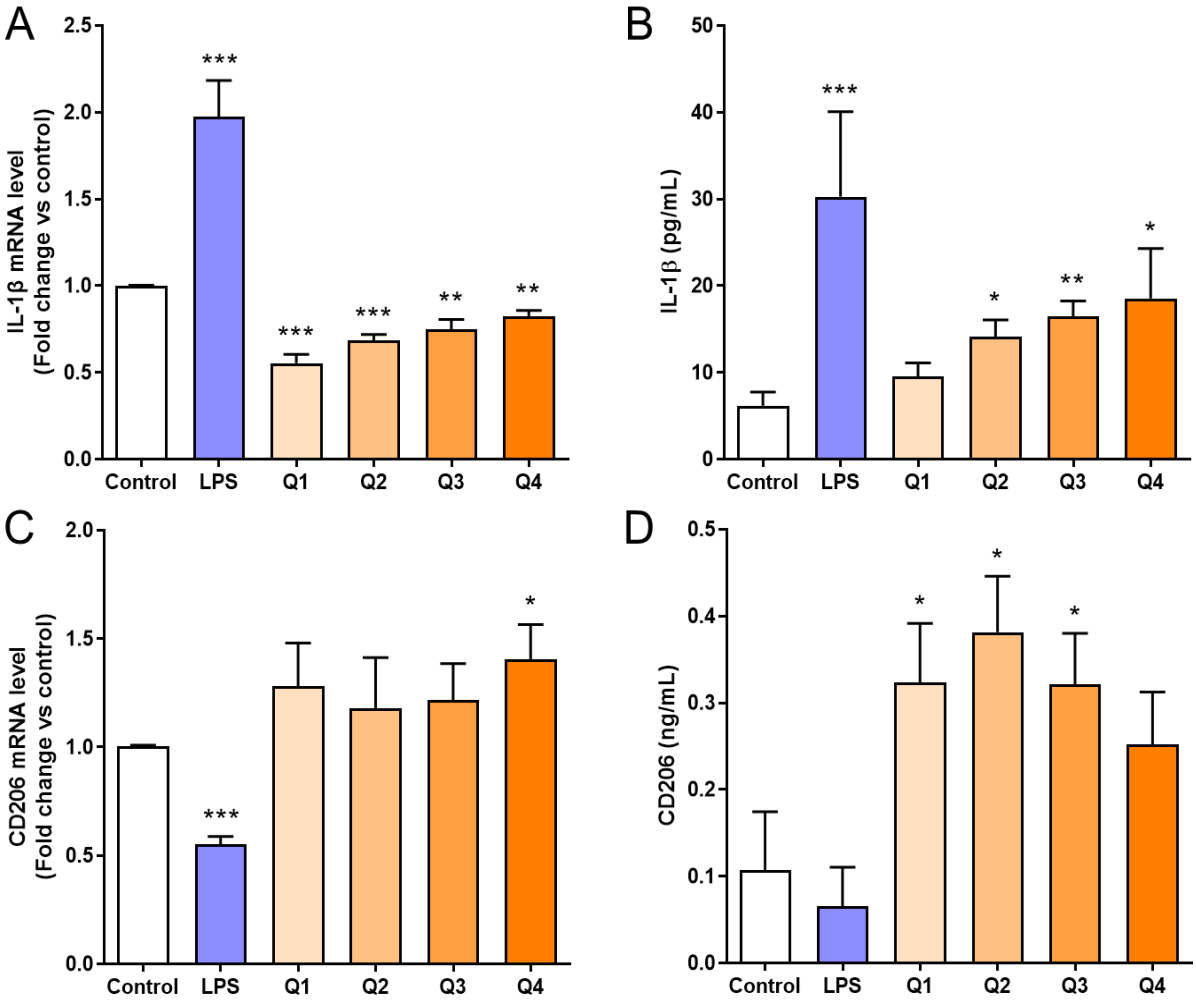


A) Bar graphs of gene expression of IL-1β after 4 hours. Cells were treated with LPS (10 ng/mL) and Q1-Q4 of TRL (10% v/v) pools. **B)** Bar graphs of secretion of IL-1β after 4 hours. Cells were treated with LPS and Q1-Q4 of TRL pools. **C)** Bar graphs of gene expression of CD206 after 4 hours. Cells were treated with LPS and Q1-Q4 of TRL pools.
Data were represented as mean ± SEM of 2 independent experiment.
p-values from Mann-Whitney U test. *p<0.05 vs control

The more consistent results were at 24h (Figure 14). We observed that TRL decreases the expression of IL-1β mRNA compared with control (Q1=-45% p<0.001, Q2=-32% p<0.001, Q3=-26% p<0.01, Q4=-18% p<0.01). Interestingly, TRL induced a gradual increase along increases quartiles. As expected, LPS showed a higher expression of IL-

1β compared with control (97%, p<0.001) (Figure 14A). At secretion level it is shown that quartiles of TRL increases gradually the levels with respect to control (Q1=54%, Q2=129% p<0.05, Q3=166% p<0.01, Q4=200% p<0.05). In addition, LPS showed a higher secretion levels compared to control (390%, p<0.001) (Figure 14B). At mRNA level of CD206, Q4 of TRL increases by 40% (p<0.05) with respect to control. As expected, LPS decreased its expression (-45%, p<0.001) respect to control (Figure 14C). At secretion level of CD206, TRL induced a higher expression compared with control with statistical differences except Q4 TRL (Q1=220% p<0.05, Q2=280% p<0.05, Q3=220% p<0.05, Q4=150%). Moreover, LPS secretion decreases -40% the CD206 secretion without reaching statistical significance.

Figure 14: Expression and secretion levels of IL-1β and CD206 after 24h of TRL and LPS treatment.



A) Bar graphs of gene expression of IL-1β after 24 hours. Cells were treated with LPS (10 ng/mL) and Q1-Q4 of TRL (10% v/v) pools. **B)** Bar graphs of secretion of IL-1β after 24 hours. Cells were treated with LPS and Q1-Q4 of TRL pools. **C)** Bar graphs of gene expression of CD206 after 24 hours. Cells were treated with LPS and Q1-Q4 of TRL pools. **D)** Bar graphs of secretion of CD206 after 24 hours. Cells were treated with LPS and Q1-Q4 of TRL pools.
Data were represented as mean ± SEM of 3 independent experiment done in duplicates.
p-values from Mann-Whitney U test. *p<0.05 vs control;** p<0.01 vs control;*** p<0.001 vs Control

DISCUSSION

In the present study we divided 72 patients with different metabolic disorders into four growing quartiles of triglycerides concentration in isolated TRL. By ^{1}H-NMR we have demonstrated that TRL particle numbers increased in each quartile of TG-TRL. After TRL isolation, we have biochemically detected significant higher percentage of triglycerides in the composition of TRL in the growing quartiles. Particular attention is paid to determine the inflammatory effects of the growing quartiles of TG-TRLs in macrophages. We have detected an increased intracellular TRL uptake. Additionally, we have observed a peak expression of IL-1β at 4 hours while an increased secretion of IL-1β through time. Moreover, a slight increased expression of CD206 during time was seen while we could only detect lower levels of secretion at 24 hours.

In our patients, ^{1}H-NMR determination of TRL particle number and size, showed that concentrations of large, medium, small and total TRL particles increased with each TG quartile. We next isolated by ultracentrifugation of the plasma the TRL fractions, and generated 3 different pools for each quartile of patients. We studied the biochemical composition of these TRL pools (cholesterol, triglycerides, phospholipids and proteins), and detected how the mean percentage of TG within the fractions increased in each quartile whereas the percentage of proteins decreased. These observations could help us to elucidate how the increase of TG within the pools could affect cells in vitro.

To investigate the effects of isolated TRL on macrophage inflammatory processes, we used THP-1 cell line and 10% v/v quartile pools of TRL at 4h, 8h, 24h and 30h. First of all, THP-1 monocytes were differentiated into macrophages by 72 hours of PMA incubation followed by 24 hours of supplemented medium incubation. It has been shown that PMA induces an upregulation of pro-inflammatory cytokines (like IL-1β), but their expression decreases after 24 hours in supplemented medium in order to become unstimulated M0 macrophages (19). This phenotype allows to investigate the possible polarizing effects of TRL.

Previous studies have demonstrated that macrophages engulf TRL whole particle by different receptor-mediated pathways (VLDL receptor or apolipoprotein receptor), although exact mechanisms are not yet known (25,26). In order to test the TRL uptake, cells were stained with Oil and Nile red (Figure 9 and Figure 10) after 8 hours of TRL treatment. The images obtained were consistent with literature (25). Our results showed that TRL uptake by macrophages had a strong and gradual red staining in growing TRL quartiles, being Q4, with the highest TG-TRL, the major red stained condition (Figure 10B). We could confirm that TRL induce macrophage foam cell formation as other

studies suggest (11,12,25,26). As expected, the incubation of macrophages with TRL significant increases the amount intracellular lipid droplets by mean of Nile Red staining (Figure 10C). However, we couldn't observe a gradual cell lipid content in growing quartiles. This could be an experimental consequence, since the number of cells/well could not be exactly the same or maybe higher amounts of TRL induce a slight cytotoxic effect on THP-1 macrophages.

To study these possible cytotoxic effects of TRL in macrophages, we performed a LDH assay at different TRL treated times (4h, 8h, 24h and 30h). LDH release to media increased with time showing no differences between untreated TRL macrophages (control), quartiles of TRL and LPS. Based on literature LPS at the concentration used in this work (10 ng/mL) does not cause cytotoxicity, while higher concentrations have been reported to induce cell death (19). Nevertheless, after 24 hours of TRL treatment, cells showed a higher cytotoxicity compared with control (Figure 11), specially the quartiles with a higher concentration of TG-TRL (Q3 and Q4). This data led us to treat cells up to 24 hours.

Recently, TRL effect in macrophages is getting a higher importance in the inflammation field (11,12,25,26). It is well known that inflammation play an important role in the pathogenesis of atherosclerosis (7,8). It has been described that TRL induce inflammatory processes, increasing leucocyte activation and vascular sensitivity (25). At this moment, the main focus of our project was to study the inflammatory effects of macrophages incubated with TRL and its polarization into M1 or M2. Classical M1 (pro-inflammatory) and alternative M2 (immunosuppressive) markers were assessed by measuring the gene expression and protein secretion (20,21).

In the present study, we evaluated the time-course mRNA expression of IL-1β after TRL treatment compared with control (untreated cells) and LPS, a positive control that induce M1 polarization (19). Our results showed that TRL induced a peak of IL-1β mRNA expression at 4 hours compared to control and the expression decreased with time. Nevertheless, TRL treatment increased IL-1β secretion through time compared with control and remained increased until 24 hours. Moreover, our positive control (LPS) demonstrated that macrophages responded to a pro-inflammatory stimulus, with IL-1β mRNA expression and secretion peak at 24 hours. These observations suggest that after TRL treatment there is a transcriptional regulation of IL-1β expression at mRNA level after 4 hours, but its secretion remains and increases with time up to 24h.

The time-course evolution of IL-1β expression and secretion was determinant to focus our research at 4 and 24 hours of TRL treatment. At first sight the pattern of IL-1β mRNA

expression and protein secretion at 4 hours is similar: increasing quartiles of TG-TRL induced a higher and gradual expression of IL-1β between quartiles compared with control, except Q4. These findings advise that Q4 TRL may cause higher cytotoxicity on treated cells, or the elevated TG content on this quartile of TRL could promote the saturation of molecular mechanisms implicated in the pro-inflammatory response responsible of IL-1β secretion. At 24h, the expression of IL-1β mRNA in TRL-treated macrophages was lower than control and LPS-treated cells. On contrary, IL-1β secretion levels of both LPS and TRL-treated macrophages were higher than control. Therefore, IL-1β transcription after 4 hours is regulated. Interestingly, growing quartiles of TRL treatment show a similar and gradual pattern of mRNA expression and secretion of IL-1β. These results could suggest that increased triglycerides within TRL promote higher pro-inflammatory effects.

Furthermore, we evaluated the time-course mRNA expression of CD206 in order to assess the expression of a M2 marker. The time-course analysis showed that TRL treatment followed a slight increase in CD206 mRNA levels in time compared to control, on contrary to IL-1β mRNA. In fact, CD206 protein secretion was no detectable until 24 hours in TRL-treated conditions. As described, LPS induces pro-inflammatory M1 polarization (19). For this reason, we show how LPS treatment didn't induce the mRNA expression and secretion of CD206, whose levels were lower than control. These observations suggested that while the peak of IL-1β mRNA expression after TRL treatment is detected at 4 hours, the secretion levels of CD206 at this time point are not detectable. These observations led us to elucidate that 4 hours treatment with TRL induces a M1 phenotype on treated macrophages; however, the slight increased CD206 mRNA expression and protein secretion at 24 hours propose an autoregulation of the pro-inflammatory effect exerted by TRL treatment at 4 hours, via M2 polarization.

Next, we focussed on CD206 at mentioned times of 4h and 24h TRL treatment. After 4 hours treatment, CD206 secretion levels were no detectable. However, its mRNA expression was higher in Q1 TRL treatment compared with control. In addition, we observed a non-statistically significant decreasing trend along quartiles, except Q4, until reaching the same expression levels as control. These observations are in accordance with the observed IL-1β expression patterns at this time point: while higher TG-TRL quartiles showed higher IL-1β mRNA expression, CD206 mRNA expression is higher at lower TRL quartiles (Q1). At this time point we had detected the highest IL-1β mRNA expression levels, while later in time we observed how levels decreased although the effect exerted by growing TG-TRL quartiles in maintained. These effects may be a consequence of the fact that M1 macrophage polarization inhibits the expression of M2

markers as TG concentrations within TRL increase. Nevertheless, at 24 hours we have observed that the expression of CD206 mRNA on TRL-treated cells remains lineal and higher than control; furthermore, at 24 hours we detected protein secretion, time point at which IL-1β mRNA expression is lower than control. Overall, these findings suggest that the reduced expression of IL-1β mRNA detected at 24 hours may be affected because of an increase in CD206 secretion, again, as a possible regulatory mechanism to control the pro-inflammatory effects caused by TRL and the TG within these lipoproteins.

Some limitations of our work should be pointed out. First of all, our findings are based on the study of a single classical M1 marker (IL-1β) and a single M2 marker (CD206), limiting the research of the inflammatory effects of TRL. The study of more genes as TNF-α, IL-6, CXCL10, or IL-10 (among others) could complete the study of the macrophages phenotype. As well, we cannot confirm the exact molecular mechanism implicated in our findings. We should point out that the study at the time point of 4 hours for IL-1β and CD206 mRNA expression could not be done in triplicate, which could affect the statistical study. For this research, we have used THP-1 cell line as an in vitro model, and it has provided us a wide range of different assays possibilities; nonetheless, this cell line presents some difficulties, since the maintenance of the cell culture must be controlled extensively as they become pro-inflammatory when a maximum density is reached. For this reason, we believe that this fact strengthens the results obtained. Finally, the TRL patients' samples were limited but despite that fact, we could perform three independent experiments, most of them done in duplicates.

Altogether, these results describe that growing quartiles of TG-TRL induce a regulated M1 polarization of macrophages, via IL-1β mRNA expression which is accompanied by a progressive increase of its protein secretion, suggesting that TRL have pro-inflammatory effects early in time. The reduction of the IL-1β expression levels after 4 hours of TRL treatment and the later secretion of CD206 protein have led us to propose that this transcriptional regulatory effect may be consequence of a M2 polarization, which appears later in time to counteract the pro-inflammatory effects caused by TRL. From the data of this study we could affirm that the content of TG in the TRL fraction have a pro-inflammatory effect that could add a more proatherogenic profile in patients at high risk of cardiovascular disease such as obesity, type 2 diabetes and metabolic syndrome. New pharmacological approaches leading to decreases the TG amount in the TRL fraction and inflammation could be important to prevent cardiovascular disease.

CONCLUSION

From our results we can conclude that:

- ^{1}H-NMR of TRL showed that concentration of large, medium, small and total particles increased with each TG-TRL quartile. Biochemical composition of TRL detected an increase of the percentage of TG within the fraction in each quartile whereas the percentage of protein decrease.
- THP-1 macrophages engulf TRL particles becoming foam cells.
- Macrophages treated with TRL showed a peak expression of IL-1β at 4 hours after TRL treatment while an increased secretion of IL-1β through time. Treated macrophages showed a slight increased expression of CD206 during time while we could only detect CD206 secretion levels at 24 hours.
- TRL could have pro-inflammatory effects early in time.

In this work, we have repeatedly emphasized that triglyceride-rich lipoproteins and triglycerides could be an important causal risk factor for atherosclerotic cardiovascular disease. In turn macrophages could engulf TRL particles becoming foam cells and playing and important pro-inflammatory role in atherosclerosis process. Much more attention must be given to the molecular mechanism implicated in the uptake of TRL by macrophages as well as TRL pro-inflammatory effect for additional reduction of atherosclerotic cardiovascular disease events.

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SELF-ASSESSMENT

Understand biotechnology is a process in which you examine yourself in attempt to discover and learn more about biological systems, living organisms and, in short, molecular mechanisms of life.

When I started in college I have noticed that study science was a slow and difficult process. But as I have continued in my studies I felt more tentative to get more knowledge and laboratory's skills. But the idea of being a science future researcher would not have been possible without the internship I realized in URLA's research unit.

I believe that my skills and my abilities to work in a lab team have improved during the last months working as a researcher. Moreover, I have gained an amazing experience being in contact with a researchers day-to-day.

I have successfully learnt new strategies based on my development in different situations in which I have to put into practice all the knowledge I have learned, practiced, and applied. Furthermore, I have improved my scientific divulge vocabulary.

Thanks to this project, I have been able to challenge myself in order to communicate and write an entire experiment process in English. On one hand, I gained skills using computer programs, doing graphics and analyzing data. On the other hand, I have learnt how to treat and maintain cells, new techniques as mRNA extraction, RT-PCR and q-PCR between others.

Overall, this project has been an amazing opportunity to initiate myself as a researcher. I feel I have faced the entire project and I am sure that this experience has only been the beginning.

ANNEX

ANNEX 1: Lipoprotein isolation by ultracentrifugation

ULTRACENTRIFUGATION IN A KONTRON 45.6 ROTOR

1
2 ml of plasma-EDTA (1 mg/mL)
Add 2 mL of 1.006 g/mL
Add 1 mL of 1.071 g/mL and mix
Spin a7 37000 rpm, 20 h, 4ºC
Aspirate 2 mL from the top = VLDL +IDL (<1.006-1.019 g/mL)

2
Make the infranate up to 4 mL with 1.019 g/mL
Add 1 mL of 1.239 g/mL and mix
Spin at 37000 rpm, 20h, 4ºC
Aspirate 2 mL from the top = LDL (1.019-1.063 g/mL)

3
Make the infranate up to 4 mL with 1.063 g/mL
Add 1.1g of NaBr
Spin at 37000 rpm, 40h, 4ºC
Aspirate 2 mL from the top =HDL (1.063-1.210)

ANNEX 2: Lowry method

LOWRY METHOD / SYNERGY

PROTOCOL:

0.- Defrost or prepare the standard calibration line and samples.
Prepare the alkaline copper solution (solution A:C:B, 100:1:1) with the adjusted quantity for well/plates and protect it from the light.
The solution D must be prepared just before use.

1.- Put 40µl of the standard line, sample (and controls) on the plate. If dilution is required, the final volume should be 40µl.
2.- Add 200µl of the alkaline copper solution (solution A:C:B, 100:1:1)
3.- Mix / shake, 30seg-1min with the plate shaker.
4.- Incubate for 10min at room temperature (in the dark)
5.- Add 20µl of solution D (Folin: H_2O, 1:1)
6.- Mix /shake, 30seg-1min with the plate shaker.
7.- Incubate at room temperature in the dark.
8.- Read the spectrophotometer (Synergy) at 650nm

BSA STANDARD LINE:

0µg/µl o mg/ml ; 0.1µg/µl ; 0.3µg/µl ; 0.5µg/µl ; 0.7µg/µl ; *1µg/µl*
BSA LINE (stock 2mg/ml o µg/µl) [stock and line in use may be frozen]

0mg/ml (o µg/µl) → 500µl H_2OmQ
0.1mg/ml (o µg/µl) → 25µl BSA stock + 475µl H_2OmQ
0.3mg/ml (o µg/µl) → 75µl BSA stock + 425µl H_2OmQ
0.5mg/ml (o µg/µl) → 125µl BSA stock + 375µl H_2OmQ
0.7mg/ml (o µg/µl) → 175µl BSA stock + 325µl H_2OmQ
1mg/ml (o µg/µl) → 250µl BSA stock + 250µl H_2OmQ

STOCK SOLUTIONS:

SOLUTION A: 2% Na_2CO_3 , 0.1M NaOH (10g + 2g + 500ml H_2Od)
SOLUTION B: 1% $CuSO_4 \cdot 5H_2O$ (1g + 100ml H_2Od)
SOLUTION C: 1% Sodium Potassium Tartrate (1g + 100ml H_2Od)
SOLUTION D: Folin reagent + H_2Od (1:1)

ANNEX 3: mRNA extraction

Purifying RNA from Animal and Plant Cells

Introduction

This section provides instructions for purifying total RNA from animal and plant cells. Separate protocols are provided for ≤5 × 10^6 cells (suspension and monolayer) and for 5 × 10^6–5 × 10^7 cells.

Materials Needed

You will need the following items in addition to the kit components:

- 2–mercaptoethanol
- 70% ethanol (in RNase-Free Water)
- Microcentrifuge capable of centrifuging 12,000 × *g*
- 1.5 mL RNase-free microcentrifuge tubes
- 15 mL RNase-free tubes (>10^7 cells per sample)
- PBS (>10^7 cells per sample)
- RNase-free pipette tips
- *Optional:* PureLink® DNase (page 67)

For ≤5 × 10^6 cells:

- Homogenizer (see page 67 and page 7) **or,**
- RNase-free syringe (1 mL) with 18-21 gauge needle **or,**
- Rotor-stator homogenizer (page 8)

For 5 × 10^6–5 × 10^7 cells:

Rotor-stator homogenizer (page 8)

Amount of Lysis Buffer Needed

Before beginning the lysis and homogenization steps, prepare a fresh amount of Lysis Buffer containing 1% 2-mercaptoethanol for each purification procedure. Add 10 µL 2–mercaptoethanol for each 1 mL Lysis Buffer.

Using the table below, determine the correct amount of Lysis Buffer needed for your sample type and amount.

Note: For larger than average samples, or if using a rotor-stator, additional Lysis Buffer may be required. See page 12 for details.

Number of cells in your sample	Amount of Lysis Buffer Needed (prepared with 2-mercaptoethanol)
≤1 × 10^6	0.3 mL*
1 × 10^6–5 × 10^6	0.6 mL
5 × 10^6–5 × 10^7	0.6 mL per 5 × 10^6 cells For example: use 1.2 mL for 1 × 10^7 cells and 6.0 mL for 5 × 10^7 cells

*Use 0.6 mL if using rotor-stator for lysis or homogenization.

Continued on next page

Binding, Washing, and Elution

Follow the steps below to bind, wash, and elute the RNA from your sample:

1. Add one volume 70% ethanol to each volume of cell homogenate (prepared as described in the sample-specific protocols (pages 15–18).

   Note: If part of the sample was lost during homogenization, adjust the volume of ethanol accordingly.

2. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol.
3. Transfer up to 700 µL of the sample (including any remaining precipitate) to the Spin Cartridge (with the Collection Tube).
4. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through, and reinsert the Spin Cartridge into the same Collection Tube.

   Note: If you are processing the maximum starting amount of sample, you may centrifuge for up to 10 minutes to completely pass the lysate through the Spin Cartridge.

5. **Repeat** Steps 3–4 until the entire sample is processed.

   ***Optional*:** If DNA-free total RNA is required, proceed to **On-column PureLink® DNase Treatment Protocol** (page 63).

6. Add 700 µL Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through **and** the Collection Tube. Place the Spin Cartridge into a **new** Collection Tube.
7. Add 500 µL Wash Buffer II with ethanol (page 11) to the Spin Cartridge.
8. Centrifuge at 12,000 × *g* for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into the same Collection Tube.
9. Repeat Steps 7–8 **once.**
10. Centrifuge the Spin Cartridge at 12,000 × *g* for 1-2 minutes to dry the membrane with attached the RNA. **Discard** the Collection Tube and insert the Spin Cartridge into a Recovery Tube.
11. Add 30 µL–3 × 100 µL RNase–Free Water to the center of the Spin Cartridge (see **Elution Parameters**, page 13).
12. Incubate at room temperature for 1 minute.
13. Centrifuge the Spin Cartridge for 2 minutes at ≥12,000 × *g* at room temperature to elute the RNA from the membrane into the Recovery tube.

    Note: If you are performing sequential elutions, collect all elutes into the same tube (see page 13 for **Elution Parameters**).

14. Store your purified RNA or proceed to **Analyzing RNA Yield and Quality** (page 53) or to **DNase I Treatment After RNA Purification** (page 65).

ANNEX 4: Diagram of the patients included in the study