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High triglycerides and low HDL-c lipid profile in rheumatoid arthritis: a potential link among inflammation, oxidative status and dysfunctional HDL

Javier Rodríguez-Carrio, Mercedes Alperi-López, Patricia López, Raquel López-Mejías, Sara Alonso-Castro, Francisco Abal, Francisco J. Ballina-García, Miguel Á. González-Gay, Ana Suárez

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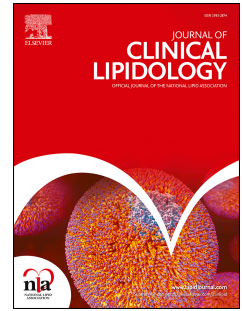
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1 **High triglycerides and low HDL-c lipid profile in rheumatoid arthritis:**
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9 Javier Rodríguez-Carrio¹, Mercedes Alperi-López², Patricia López¹, Raquel López-Mejías³,
10 Sara Alonso-Castro^{4,5}, Francisco Abal⁶, Francisco J. Ballina-García², Miguel Á. González-
11 Gay^{3,7,8} and Ana Suárez^{1#}

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14 ¹ Area of Immunology, Department of Functional Biology, University of Oviedo, Asturias, Spain

15 ² Department of Rheumatology, Hospital Universitario Central de Asturias, Asturias, Spain

16 ³ Epidemiology, Genetics and Atherosclerosis Research Group on Systemic Inflammatory Diseases,
17 Rheumatology Division, Hospital Universitario Marqués de Valdecilla, IDIVAL, Santander, Spain

18 ⁴ Servicio de Reumatología, Hospital de Cabueñes

19 ⁵ University of León, Castilla y León, Spain

20 ⁶ Centro de Salud Sariego, Servicio de Salud del Principado de Asturias, Asturias, Spain

21 ⁷ Department of Medicine, University of Cantabria, Santander, Spain

22 ⁸ Cardiovascular Pathophysiology and Genomics Research Unit, School of Physiology, Faculty of Health
23 Sciences, University of the Witwatersrand, Johannesburg, South Africa

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32
33 **# Corresponding author:** Dr. Ana Suárez
34 Area of Immunology, Department of Functional Biology,
35 Faculty of Medicine, University of Oviedo
36 Campus El Cristo
37 C/ Julián Clavería s/n
38 33006 – Oviedo
39 Spain

40
41 E-mail: anasua@uniovi.es

42 Phone number: +34 98510 2789

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46 **Short title:** high TG and low HDL-c lipid profile in RA

1 **ABSTRACT**

2

3 **Background:** the interactions between inflammation and lipid profile in rheumatoid arthritis
4 (RA) are poorly understood. The lipid profile study in RA has been biased towards lipoprotein
5 levels, whereas those of triglycerides (TG) and lipoprotein functionality have been
6 underestimated.

7 **Objetives:** since recent findings suggest a role for TG and TG-rich lipoproteins (TRL) on
8 inflammation, we aimed to evaluate a combined lipid profile characterized by high TG and low
9 HDL-cholesterol levels (TG^{high}HDL^{low}) in RA.

10 **Methods:** lipid profiles were analyzed in 113 RA patients, 113 healthy controls (HC) and 27
11 dyslipemic (DL) subjects. Levels of inflammatory mediators, paraoxonase-1 (PON1) activity
12 and Total Antioxidant Capacity (TAC) were quantified in serum. PON1-rs662 status was
13 evaluated by RT-PCR.

14 **Results:** the TG^{high}HDL^{low} profile was detected in 29/113 RA patients. Although no differences
15 in prevalence compared to HC or DL subjects were observed, this profile was associated with
16 increased TNF α (p=0.004), MCP-1 (p=0.004), IP-10 (p=0.018) and leptin (p<0.001) serum
17 levels in RA, where decreased PON1 activity and TAC were found. TG^{high}HDL^{low} prevalence
18 was lower among anti-TNF α -treated patients (p=0.004). When RA patients were stratified by
19 PON1-rs662 status, these associations remained in the low-activity genotype (QQ). Finally, a
20 poor clinical response upon TNF α -blockade was related to an increasing prevalence of the
21 TG^{high}HDL^{low} profile over treatment (p=0.021) and higher TRL levels at baseline (p=0.042).

22 **Conclusions:** the TG^{high}HDL^{low} profile is associated with systemic inflammation, decreased
23 PON1 activity and poor clinical outcome upon TNF α -blockade in RA, suggesting a role of TRL
24 and HDL dysfunction as the missing link between inflammation and lipid profile.

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27 **Keywords:** high density lipoproteins, triglycerides, triglyceride-rich lipoproteins, paraoxonase,
28 inflammation, oxidative status, rheumatoid arthritis.

1 INTRODUCTION

2 Blood lipid abnormalities are a frequent hallmark of active rheumatoid arthritis (RA). Many
3 authors have reported an alteration of the lipid profile in RA patients, especially those of
4 lipoproteins during high disease activity states¹⁻³. Accordingly, disease activity control by
5 different immunomodulatory treatment strategies is related to a restoration of the lipid profile to
6 different degrees, although certain controversy exists¹. Due to the fluctuation of some
7 lipoproteins in the context of a chronic inflammation, and taking into account that a single lipid
8 compound cannot provide enough information on its own, there is a need for more reliable
9 biomarkers related to the lipid profile in RA.

10 The classical clinical management of blood lipids focused on lipoproteins is currently
11 challenged by a compelling body of evidence highlighting an important contribution of other
12 lipid compounds, such as triglycerides (TG), lipoprotein A or cholesterol remnants⁴⁻⁶. Different
13 panels of experts have raised the point that individual lipoproteins alone may not be accurate for
14 patient stratification and treatment recommendations in the clinical setting (reviewed in⁵).
15 Actually, reducing LDL-cholesterol (LDL-c) levels as a therapeutic target is now put into
16 question and there is a recommendation to consider other lipid fractions or surrogate markers as
17 they can be more informative⁷. TG exhibit important differences compared to lipoproteins in
18 their origin, metabolic pathways and downstream effects in different cell types. Therefore, it is
19 tempting to speculate that their inclusion in the clinical management will bring valuable
20 information which can complement that of provided by lipoproteins. In fact, several
21 epidemiological studies have found an attenuation of the protective effect of HDL-cholesterol
22 (HDL-c) levels on cardiovascular disease (CVD) outcomes when adjusted for TG⁸⁻¹⁰, thus
23 suggesting the existence of interactive effects among lipid classes. Hence, there is a growing
24 body of evidence supporting the study of combined indices, especially when TG are included¹¹.
25 Consequently, in recent years the relevance of a lipid profile characterized by decreased HDL-c
26 levels and elevated TG has emerged⁵. This profile has been linked to inflammation and
27 atherosclerosis development, although some knowledge gaps remain⁴. Recently, this profile has
28 been shown to affect the leukocyte gene expression in dyslipidemia subjects¹². However, the
29 clinical and immunological relevance of this profile in RA is unknown.

30 On the other hand, current epidemiological and experimental studies have stated that the only
31 analysis of lipid levels is a too simplistic approach that does not reflect lipoprotein functionality.
32 Lipoproteins can develop a broad range of enzymatic activities, apart from cholesterol transport
33¹³. Interestingly, lipoprotein functionality seems to have a better clinical relevance than levels
34^{14,15}. Among lipoprotein functions, anti-oxidant properties are emerging as a pivotal player to
35 understand the interaction between lipid profiles and inflammation¹⁶, compromised anti-oxidant

1 activity being associated with a number of conditions. The most important determinant of the
2 anti-oxidant function of lipoproteins is the enzyme paraoxonase 1 (PON1), whose activity is
3 regulated at the genetic level by the PON1 rs662 polymorphism^{17,18}. Again, although the role of
4 genetic variants of different genes on lipid levels has been evaluated, their influence on
5 lipoprotein functionality and its clinical relevance is merely started to be appreciated.

6 Taken together, these lines of evidence point to the fact that lipid profiles are much more
7 complex than initially thought. Not only different blood lipid species should be considered
8 together with lipoproteins, but also it may be important to consider the lipoprotein functionality
9 in order to obtain a more realistic insight into the significance of the lipid profile. Therefore, in
10 the present report, we aim to evaluate the relevance of the lipid profile characterized by a
11 combination of deleterious levels of HDL-c and TG in RA patients, with a special focus on their
12 interaction with the inflammatory burden and the role of oxidative status. Moreover, the role of
13 PON1 rs662 as genetic determinant of the HDL functionality was also analyzed to provide a
14 more in-depth insight into this complex scenario.

1 MATERIAL AND METHODS

2 Patients

3 This cross-sectional study involved 3 groups of individuals recruited (Table 1). RA patients,
4 fulfilling 2010 ACR/EULAR classification criteria, were enrolled from the Department of
5 Rheumatology at Hospital Universitario Central de Asturias. A complete clinical examination,
6 including Disease Activity Score 28-joints (DAS28) calculation, was performed on all patients.
7 Clinical records were revised so as to register traditional CV risk factors. An additional group of
8 13 biological-naïve RA patients (12 women, median age 43 (range: 30 – 65), DAS28
9 5.08(1.93), 38.5% RF+, 46.1% ACPA+), candidates for TNF α -blockers was prospectively
10 followed for 3 months. RA patients must have experienced failure to methotrexate and/or
11 conventional synthetic DMARDs and no previous exposure to any biological DMARD. A blood
12 sample was obtained immediately before (baseline, pre-treatment) as well as 3-months after
13 initiation of TNF α -blockade therapy (post-treatment). Clinical response was evaluated by
14 EULAR criteria.

15 Simultaneously, 113 gender- and age-matched healthy volunteers (HC) were recruited from the
16 same population and a group of 27 individuals with dyslipidemia (DL) was recruited from their
17 primary healthcare center. Dyslipidemia diagnosis was performed according to national
18 guidelines¹⁹. Exclusion criteria was the previous diagnosis of any immune-mediated condition.
19 Exclusion criteria for all study groups were: recent (<3 months) infections or surgeries, cancer
20 diagnosis or pregnancy.

21 Automated serum lipids analysis was performed on all the participants in fresh blood samples
22 after an overnight fast. TRL levels were calculated according to the equation provided by
23 Hermans *et al.*²⁰. Serum samples were stored at -80°C until laboratory measurements were
24 carried out. Approval for the study was obtained from the Institutional Review Board (Comité
25 de Ética Regional de Investigación Clínica), in compliance with the Declaration of Helsinki. All
26 the participants gave written informed consent prior to their inclusion in the study.

27 Quantification of inflammatory mediators' serum levels

28 TNF α , MCP-1, sICAM-1, EGF, IP-10, leptin and resistin serum levels were measured by means
29 of a Mini ELISA Development Kits (PeproTech), following the instructions provided by the
30 manufacturer (detection limits were: 3.9 pg/ml, 8 pg/ml, 23.4 pg/ml, 3.9 pg/ml, 3.8 pg/ml, 63
31 pg/ml and 24 pg/ml, respectively). IFN γ serum levels were quantified using an OptEIA kit (BD)
32 following the manufacturer's protocol (detection limit: 0.58 pg/ml).

1 Levels of IL-8 and GM-CSF were quantified using a Cytometric Bead Array Flex Set (BD) in a
2 FACS Canto II flow cytometer using FCAP Array v.1.0.1, following the manufacturer's
3 instructions. The detection limits were 1.2 pg/ml and 0.2 pg/ml, respectively.

4 **Assessment of PON1 activity**

5 PON1 activity was measured in serum samples using paraoxon (Sigma Aldrich, Germany) as
6 substrate, as previously described ²¹. PON1 activity was expressed as units (U), where one U
7 represent the micromoles of p-nitrophenol formed per minute and per ml of serum.

8 **Analysis of Total Antioxidant Capacity**

9 A spectrophotometric method based on the cupric reducing antioxidant capacity (CUPRAC
10 method) using a commercial kit (TAC Assay Kit, Sciencell Research Laboratories) was used to
11 quantify the Total Antioxidant Capacity of serum samples. Serum TAC was expressed as mM
12 Trolox equivalent units (mM T-Eq).

13 **Analysis of Free Fatty Acids**

14 The levels of total Free Fatty Acids (FFA) were quantified in serum samples using an
15 enzymatic, colorimetric assay using a commercial kit (NEFA kit, Roche) according to the
16 protocol provided by the manufacturer. The detection limit was 0.02 mM.

17 **PON1 rs662 genotyping**

18 DNA was isolated from peripheral blood using conventional methods. The PON1 rs662
19 polymorphism was genotyped with TaqMan predesigned single-nucleotide polymorphism
20 (SNP) genotyping assays (C__2548962_20) in a 7900 HT Real-Time polymerase chain
21 reaction (PCR) system, as previously described ²¹.

22 **Statistical analyses**

23 Continuous variables were expressed as median (interquartile range) or mean \pm standard
24 deviation, whereas n(%) was used for categorical ones. Differences among groups were
25 analyzed by Mann Withney U, Kruskal-Wallis (with Dunn-Bonferroni correction for multiple
26 comparisons), χ^2 or Fisher exact tests, as appropriate. Wilcoxon test was used for paired
27 samples. Correlations were assessed by Spearman ranks test. The association of categorical
28 variables adjusted for confounders was analyzed by multivariate logistic regression models, and
29 odds ratios (OR) with 95% confidence intervals (CI) were computed. When required, variables
30 were log-transformed to achieve a normal distribution. With an $\alpha=0.05$, and assuming a
31 prevalence of the TG^{high}HDL^{low} profile in HC of 0.20, our case-control study was able to detect
32 an exposure in the case population up to 0.482. A p-value>0.050 was considered as statistically

- 1 significant. Hedges' g statistic was used to estimate size effect, $g > 0.8$ being considered as a large
- 2 effect. Statistical analyses were performed in SPSS 22.0 and GraphPad Prism 5.0 for Windows.

ACCEPTED MANUSCRIPT

1 RESULTS

2 1. High triglyceride-low HDL-c profile (TG^{high}HDL^{low}) in RA

3 Given the controversy on the blood lipid levels and their functionality in RA, and taking into
4 account the heterogeneity among lipid classes, we decided to evaluate the impact of a combined
5 altered lipid profile in RA. To this aim, we focused on the simultaneous presence of decreased
6 HDL-c levels and elevated triglycerides (TG^{high}HDL^{low}) by evaluating this combined profile in
7 113 RA patients, 27 non-autoimmune dyslipidemic patients and 113 age- and gender-matched
8 HC. No differences in the lipid profile between HC and RA patients were observed. The cut-off
9 points for the combined lipid profile were obtained from the HC group¹². Thus, by splitting the
10 HC group into tertiles, 102 mg/dl (upper tertile) and 52 mg/dl (lower tertile) were established as
11 high triglycerides and low HDL-c cut-offs, respectively. Of note, when these cut-offs were
12 applied to patients and controls, no significant differences in the prevalence of the TG^{high}HDL^{low}
13 profile were observed among groups (Table 1).

14 Then, we evaluated whether the TG^{high}HDL^{low} profile could be associated with clinical or
15 immunological parameters in RA patients. Overall, no differences in clinical features, disease
16 duration or severity were observed (Table 2). Similarly, no differences in RF or ACPA
17 positivity were found. However, the TG^{high}HDL^{low} group was associated with higher CRP
18 levels, suggesting a link with the inflammatory burden. When traditional CV risk factors were
19 analyzed, the TG^{high}HDL^{low} profile was increased in, but not restricted to, RA patients with a
20 previous diagnosis of dyslipidemia. Similarly, this profile was more frequent among males and
21 obese patients. Surprisingly, those patients under TNF α -blockade were less likely to exhibit the
22 TG^{high}HDL^{low} profile, whereas the opposite effect was observed for tocilizumab. No effect was
23 observed for other treatments, including statins and glucocorticoids.

24 2. Association between the TG^{high}HDL^{low} profile, inflammatory mediators and oxidative 25 status in RA

26 As expected, RA patients exhibited increased levels of a number of inflammatory mediators
27 compared to the other groups analyzed, as well as a reduced serum PON1 activity and TAC
28 (Supplementary Table 1).

29 Interestingly, TNF α , MCP-1, IP-10 and leptin serum levels were increased in RA patients with
30 the TG^{high}HDL^{low} profile, compared to their normal lipid profile-counterparts (Table 3). The
31 differences in inflammatory mediators remained after excluding those patients with a previous
32 CV event (CRP: p= 0.022, TNF α : p=0.041, MCP-1: p<0.001, IP-10: p=0.096 and leptin:
33 p=0.004) or those under statin treatment (p=0.028, p=0.049, p=0.004, p=0.014 and p=0.022,

1 respectively). Equivalent findings were observed for glucocorticoid usage. Importantly, these
2 differences were not observed in the DL nor in the HC group.

3 Interestingly, the TG^{high}HDL^{low} profile was associated with increased TRL levels in RA patients
4 (41.33(19.45) vs 17.09(15.64) mg/dl, $p<0.001$; $g=1.60$), as well as in HC (28.91(7.69) vs
5 16.56(12.39) mg/dl, $p<0.001$; $g=1.94$) and DL (40.93(25.90) vs 24.55(11.88) mg/dl, $p<0.001$
6 $g=1.15$) subjects. Moreover, TRL were found to be positively correlated with the levels of CRP
7 ($r=0.204$, $p=0.042$), TNF α ($r=0.351$, $p<0.0001$), MCP-1 ($r=0.409$, $p<0.0001$), IP-10 ($r=0.239$,
8 $p=0.018$) and leptin ($r=0.257$, $p=0.008$), and negatively with PON1 activity ($r=-0.203$, $p=0.036$)
9 in RA patients. However, TRL were not related to these mediators in the HC or DL groups.
10 TRL levels were not influenced by treatments. Finally, the serum levels of FFA in RA patients
11 were similar between lipid profiles (TG^{high}HDL^{low}: 0.56 ± 0.32 vs normal: 0.53 ± 0.30 mM,
12 $p=0.646$). No differences in FFA between RA and HC were found (0.55 ± 0.31 vs 0.48 ± 0.24
13 mM, $p=0.418$).

14 All these results revealed that the TG^{high}HDL^{low} profile and TLR levels were associated with an
15 enhanced pro-inflammatory milieu in RA patients, whereas no effect was observed in healthy
16 individuals or patients with dyslipidemia alone. Furthermore, TNF α seem to have a prominent
17 role.

18 **3. Effects of TG^{high}HDL^{low} profile are dependent on the PON1 rs662 genotype**

19 In addition to their levels, a growing body of evidence highlights the relevance of HDL
20 functionality. Due to the significance of the PON1 rs662 genetic variants on the HDL-PON1
21 antioxidant activity, we further examined whether the presence of the TG^{high}HDL^{low} profile in
22 RA patients could have a different effect depending on the rs662 status.

23 As expected, a gene-dosage effect was observed on serum PON1 activity, patients harboring the
24 QQ genotype exhibiting the lowest levels (Supplementary Figure 1A). However, the prevalence
25 of the TG^{high}HDL^{low} profile in RA was similar among rs662 variants ($p=0.719$). No effect of this
26 polymorphism on clinical parameters or serum levels of inflammatory mediators was registered.
27 Frequency of the different treatments did not differ among genotypes (all $p>0.050$). Similarly,
28 FFA serum levels and TAC were not affected by the rs662 status in RA (Supplementary Figure
29 1B-C). Interestingly, the association between the TG^{high}HDL^{low} profile and the inflammatory
30 mediators was restricted to patients harboring the QQ genotype, being absent in their QR- or
31 RR-counterparts (Table 4). Moreover, TRL were correlated with TNF α ($r=0.340$, $p=0.071$) and
32 MCP-1 ($r=0.604$, $p<0.001$) levels in QQ-patients but not in those QR or RR. The altered lipid
33 profile did not influence the FFA levels in any of the rs662 variants (QQ: $p=0.318$, QR: $p=0.538$
34 and RR: $p=0.864$). Equivalent results were obtained for PON1 activity and TAC (Table 4).

1 Overall, these findings revealed a link between the TG^{high}HDL^{low} lipid profile and inflammation
2 in RA, a rs662-driven effect regulating these associations. Moreover, a decreased antioxidant
3 milieu as a consequence of the QQ rs662 status, but not an increased release of FFA, seem to
4 underlie this effect.

5 **4. TG^{high}HDL^{low} profile and inflammation upon TNF α -blockade**

6 TNF α -blockade has been reported to be able to down-regulate several inflammatory mediators
7 as well as to impact the lipid profile in RA patients. The negative association observed between
8 anti-TNF α treatment and the TG^{high}HDL^{low} profile (Table 2) suggests that this lipid profile
9 could be used as a feasible serum biomarker of clinical response. Further analyses allowed us to
10 confirm that TNF α -blockers usage was related to a decreased prevalence of the TG^{high}HDL^{low}
11 profile even after adjusting for age, gender, dyslipidemia, disease duration, disease activity and
12 obesity (OR [95% CI], p: 0.164[0.037, 0.725], p=0.017). Thus, we decided to analyze the effect
13 of the TNF α -blockade on inflammatory mediators, lipid profile and PON1 activity in a group of
14 RA patients prospectively followed for three months.

15 None of the patients who achieved an EULAR good clinical response exhibited the
16 TG^{high}HDL^{low} profile (Table 5), whereas it was present in 6 patients within the non-responder
17 group (p=0.021). Similarly, TNF α -blockade was associated with decreasing serum levels of
18 TNF α and MCP-1 in responders, but not in their non-responder counterparts. TNF α -blockade
19 had no effect on serum FFA levels (p=0.221), whereas a slight increase in serum PON1 activity
20 was detected in the whole group (333.49 \pm 127.86 vs 269.46 \pm 122.73, p=0.062), not depending on
21 the clinical outcome. Finally, non-responders exhibited increased TRL levels at baseline
22 (23.44 \pm 12.34 mg/dl, p=0.042) and after treatment (25.44 \pm 14.38 mg/dl, p=0.045) compared to
23 responders (10.23 \pm 3.72 and 11.48 \pm 5.18 mg/dl, respectively).

24 Our findings showed increased TRL levels and an overrepresentation of the TG^{high}HDL^{low}
25 profile in patients with a poor clinical response upon TNF α -blockade. These changes were
26 paralleled to those of serum TNF α , hence suggesting a link between altered lipid profile and
27 clinical outcome.

1 DISCUSSION

2 The links between the altered blood lipid profile and inflammation in RA are still poorly
3 understood. Although several studies have focused on individual lipid classes, less attention has
4 been paid to combined lipid approaches, lipoprotein functionality as well as their impact on
5 surrounding mediators. In the present study, we show the presence of a combined lipid profile in
6 RA, characterized by altered levels of TG and HDL-c and related to the TRL levels. Although
7 no differences in prevalence compared to HC were detected, this profile was associated with
8 systemic inflammation and a poor clinical response upon TNF α -blockade. A decreased
9 antioxidant status seems to underlie these effects. Overall, these findings emphasize the
10 relevance of the HDL dysfunction in RA.

11 Most of studies on lipid profiles in RA were focused on lipoprotein levels, whereas the role of
12 TG, and its clinical relevance beyond lipid metabolism, in RA have been ill-defined. As in other
13 conditions, the use of lipid ratios has started to become used in RA. The EULAR consensus for
14 cardiovascular risk management in inflammatory arthritis encourages the use of the total- to
15 HDL-c ratio²². However, several concerns need to be underlined. On the one hand, this lipid
16 ratio still underestimates the use of TG levels, as they are not included. However, important
17 divergences in the association between TG and inflammatory markers compared to lipoproteins
18 or cholesterol composite indices arise in RA²³. On the other hand, lipid ratios imply, at least in
19 part, certain stoichiometry between the lipid classes considered. Although this may not be a
20 problem when similar species (for instance, lipoproteins) are studied, this approach may yield
21 inconclusive results when different compounds are analyzed. This may account for the lack of
22 appropriate results when the TG/HDL ratio was studied in other conditions. Similarly, since
23 non-linear associations between blood lipids and clinical outcomes have been reported in RA¹,
24 simple ratios could not be adequate. It is important to note that interesting findings arise even
25 within normal ranges of individual lipid classes, thus strengthening the relevance of the study of
26 the combined profile and the need for different cut-offs than those used for single lipid classes
27 alone. Therefore, a different combined approach as the one herein reported may provide more
28 reliable results.

29 Our findings revealed an association between the TG^{high}HDL^{low} profile with elevated TRL, thus
30 supporting the deleterious effect of this combined profile. High TG and TRL levels can impact
31 the HDL-c levels, composition and thus, functional status. Elevated TG and TRL may lead to a
32 greater cholesteryl ester exchange via Cholesteryl Ester Transfer Protein (CETP) between TRL
33 and nascent HDL, resulting in TG-rich small, dense HDL particles with reduced anti-oxidant
34 and anti-inflammatory activities and decreased cholesterol-accepting properties^{24,25}.
35 Importantly, increased CETP has been reported in RA²⁶. Under these circumstances, LDL

1 hepatic metabolism turns these lipoproteins into smaller and denser particles, with reduced
2 avidity for their liver receptors. Then, these particles exhibit a longer half-life and are more
3 susceptible to oxidization and to subsequent monocyte/macrophages uptake ^{4,27}. Interestingly, it
4 has been demonstrated that HDL particles from individuals with low HDL-c and high TG levels
5 exhibit a reduced capacity to promote cholesterol efflux (CE) ¹⁴. Decreased CE has been also
6 found in RA patients linked to disease activity ²⁸, and being partially restored upon TNF α -
7 blockade ²⁹⁻³¹, although certain controversy exists ³². These lines of evidence align with the
8 decreased prevalence of the TG^{high}HDL^{low} profile in RA patients undergoing anti-TNF α
9 treatment found in our study. Moreover, these results may suggest certain degree of causality of
10 the TNF α pathway in the altered lipid profile in RA. The fact that an effective TNF α blockade
11 was associated with a normal lipid profile, whereas the lack of a beneficial effect related to the
12 presence of this altered profile, is also in accordance with this hypothesis. Although our findings
13 may suggest the use of the TG^{high}HDL^{low} profile as a biomarker of therapy response, the low
14 sample size of our study is an important limitation. Larger and long-term clinical studies are
15 needed.

16 The altered CE may account, at least in part, for the associations between the altered lipid
17 profile and the inflammatory burden. CE by HDL can reduce the raft-like regions in the
18 membrane ⁴. Higher levels of cholesterol in plasma membranes of leukocytes are linked to
19 inflammatory responses ³³⁻³⁵. Hence, decreased CE and reduced HDL-c levels cannot counteract
20 the pro-inflammatory activities of TRL, including upregulation of adhesion molecules and the
21 promotion of monocyte recruitment and activation ^{36,37}. Interestingly, we have found strong
22 associations between TRL levels and those of inflammatory mediators, most of them related to
23 monocyte activation. Therefore, our findings provide new insight into the lipid profile–
24 monocytes–systemic inflammation axis in RA, which can be of outstanding relevance for the
25 clinical outcome of this condition.

26 A key result from our study is the interaction between the lipid profile and the oxidative status.
27 The clinical relevance of the TG^{high}HDL^{low} profile was not uniform among individuals, but it
28 seemed to be dependent on the oxidative status. Thus, a profound effect on the inflammatory
29 burden was observed in RA, where PON1 activity and TAC were strongly diminished, but not
30 in HC or DL groups. This notion is strengthened by the association with the PON1 rs662
31 genetic variants. Among RA patients, only in those with the lowest PON1-mediated antioxidant
32 activity (that is, those harboring the QQ status) showed that association. Therefore, these results
33 disclose a link between the altered lipid profile and the oxidant status, which are reinforced by
34 the negative association between TRL levels and PON1 activity. In this sense, it is important to
35 note that the impaired CE in RA has been related to decreased PON1 functionality and
36 increased MPO activity ²⁸. A causative role of MPO in the HDL dysfunction has been also

1 proposed in other scenarios³⁸. Overall, these results expand the current knowledge on the
2 clinical relevance of the PON1 rs662 polymorphism, strongly determined by gene-environment
3 interactions. Environmental factors can critically impair the antioxidant activity of individuals
4 the low activity genotype^{21,39,40}, hence explaining their increased susceptibility to different
5 clinical outcomes. Interestingly, loss of PON-1 activity in knockout mice models was associated
6 with increased lipid oxidation and inflammation⁴¹, oxidized lipid species playing a crucial role.
7 Surprisingly, Wang and colleagues have revealed that TRL can release oxidized lipid species
8 which in turn can elicit pro-inflammatory responses, including TNF α secretion and upregulation
9 of adhesion molecules, in a more potent fashion than native FFA⁴². These results may explain
10 the strong association between TRL and inflammatory mediators, but not FFA, in RA patients
11 harboring the QQ genotype. Moreover, TRL can also produce reactive oxidative species on their
12 own⁴³. Additionally, TRL have been also reported to promote NF κ B expression⁴⁴, which can
13 control MCP-1 production.

14 Taken together, these lines of evidence may delineate a bidirectional interaction between lipid
15 profile and inflammation. Classically, inflammation was known to influence the lipid profile.
16 However, it has been recently reported that inflammation can marginally explain lipid
17 disturbances in RA⁴⁵, and the idea that lipids indeed influence inflammation is emerging.
18 Actually, the altered lipid profile can appear in the preclinical stage of RA^{45,46}. Furthermore,
19 this double interaction aligns with the existence of a cross-talk between traditional and non-
20 traditional risk factors in RA^{47,48}. In fact, the anti-atherogenic functionality of HDL is known to
21 be diminished in RA and other chronic inflammatory conditions^{32,49}. Inflammation-driven
22 protein composition shifts towards a decreased antioxidant and pro-inflammatory profile may
23 cause these findings⁵⁰.

24 It is tempting to speculate that the findings herein reported may unravel new perspectives for the
25 CV risk in RA. However, this should be interpreted with caution. On the one hand, some
26 controversy exists regarding the altered CE in RA³². On the other hand, whether impaired HDL
27 function is associated with increased CV risk in RA has to be elucidated in prospective studies
28³². However, our results may shed some light into these connections, as a role for specific
29 mediators of inflammation (TNF α , MCP-1, EGF and leptin) and those of lipid metabolism
30 (TRL) is reported. Additionally, inflammation and increased oxidative status are known to
31 impact vascular repair by directly impairing circulating progenitors⁵¹, hence bridging abnormal
32 lipid profile, vascular repair and CV risk. Taken together, these lines of evidence warrants
33 further studies addressing the interaction among HDL dysfunction, endothelial homeostasis and
34 CV risk in RA.

1 Our findings provide valuable insight for the clinical setting and personalized medicine.
2 Although lipid ratios are recommended in the clinical management of RA, its current use may
3 be revised and additional biomarkers are needed. Moreover, whether lipoprotein ratios can
4 provide information on the functional status of HDL is unknown. Taking into account the
5 relevance of a disturbed lipoprotein functionality^{4,14,15}, it may be advisable to include this
6 pathological finding in the clinical setting. However, routine analyses for HDL functional status
7 are lacking, due to its time and methodological limitations. Taking into account our findings, the
8 TG^{high}HDL^{low} profile can be considered as a surrogate biomarker of an altered HDL functional
9 status. Thus, our results provide a new rationale for patient stratification and treatment decision
10 in this sense, as the TG^{high}HDL^{low} profile can be used to identify RA patients with an enhanced
11 systemic response and increased pro-oxidative status, linked to HDL dysfunction. These
12 patients may be considered for anti-TNF α treatment and/or some therapeutic drugs able to
13 counteract oxidative stress linked to inflammation⁵².

14 In conclusion, our results revealed that the TG^{high}HDL^{low} lipid profile in RA patients is
15 associated with a number of inflammatory mediators and negatively related to anti-TNF α
16 therapy usage. More importantly, the effect of this profile seems to be related to a decreased
17 antioxidant activity, a negative link between TRL and PON1 activity being observed. Finally, a
18 good clinical outcome upon TNF α -blockade was associated with prevention of this altered
19 profile. To the best of our knowledge, this is the first study emphasizing the relevance of this
20 lipid profile in RA patients and addressing a comprehensive analysis of the altered lipid profile,
21 oxidative status, inflammation and genetic determinants of HDL-PON1 functionality.

22

1 CONFLICT OF INTEREST

2 The authors declared no conflicts of interest. Funders have no role in study conception and
3 design, data analysis and interpretation or decision to publish.

4

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10

11 AUTHOR CONTRIBUTIONS

12 JR-C performed most of the experimental procedures, carried out the statistical analyses and
13 drafted and edited the manuscript. PL and RL-M performed some experimental procedures.
14 MA-L, SA-C, FJB-G and FA were in charge of patients’ recruitment and clinical data collection
15 and management. MAG-G made important contributions to the interpretation and discussion of
16 the results. AS conceived the study, designed the protocols and drafted and edited the
17 manuscript. All authors read and approved the final version of the manuscript.

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1 TABLES

2

3 **Table 1: Demographic features and lipid blood measurements in the individuals recruited**
 4 **for this study.** Continuous variables are summarized as median (interquartile range), whereas
 5 categorical variables are expressed as n, unless otherwise specified. Differences between groups
 6 were assessed by Kruskal-Wallis tests (p-values from Dunn-Bonferroni correction for multiple
 7 comparisons tests are indicated in superscripts) or χ^2 tests, as appropriated. DL (dyslipidemia),
 8 HC (healthy controls), TRL (triglyceride-rich lipoproteins), RA (rheumatoid arthritis).

9

	HC (n= 113)	RA (n= 113)	DL (n=27)	p-value
Age, years; median (range)	53.83 (23.17 – 80.00)	53.43 (22.00 – 87.00)	57.50 (44.00 – 68.67)	0.331
Gender, f/m	82/31	92/21	17/10	0.131
Lipid profile				
Total-cholesterol, mg/dl	199.00 (46.50)	209.00 (52.50)	211.00 (95.50)	0.389
HDL-cholesterol, mg/dl	61.00 (21.50)	62.00 (19.00)	52.00 (19.00) ^a	0.014^b
LDL-cholesterol, mg/dl	122.00 (44.00)	118.50 (45.75)	133.5 (65.00)	0.260
Triglycerides (TG), mg/dl	98.00 (17.50)	106.00 (74.00)	145.50 (102.75) ^c	<0.001^d
Total/HDL-cholesterol ratio	3.56 (1.16)	3.36 (1.40)	3.97 (1.54) ^e	0.032
TRL, mg/dl	20.91 (13.53)	22.00 (23.44)	30.50 (20.22) ^f	<0.001^f
TG ^{high} , n(%)	45 (37.1)	59 (52.2)	20 (74.0)	0.002^g
HDL ^{low} , n(%)	39 (34.5)	35 (30.9)	11 (40.7)	0.438
TG ^{high} HDL ^{low} , n(%)	30 (26.5)	29 (25.6)	11 (40.7)	0.215

10

11 ^a DL vs HC: p=0.035, DL vs RA: p=0.01112 ^b Hedges' g statistic (DL vs HC): g=0.5013 ^c DL vs HC: p<0.001, DL vs RA: p<0.00614 ^d Hedges' g statistic (DL vs HC): g=1.3115 ^e DL vs RA: p=0.02916 ^d DL vs HC: p<0.0001, DL vs RA: p<0.00717 ^f Hedges' g statistic (DL vs HC): g=1.2218 ^g power=0.952 (a=0.05)

19

1 **Table 2: Clinical and immunological features of RA patients according to the lipid profiles**
 2 **studied in RA patients.** Continuous variables are expressed as median (interquartile range),
 3 whereas categorical ones are summarized as n(%), unless otherwise specified. Differences
 4 between groups were assessed by Mann-Whitney U, χ^2 or Fisher exact tests, as appropriated.
 5 ACPA (anti-citrullinated proteine antibodies), CRP (C-reactive protein), DAS28 (disease
 6 activity score 28-joints), ESR (erythrocyte sedimentation rate), HAQ (health assessment
 7 questionnaire), RF (rheumatoid factor)

8

	Normal lipid profile (n=84)	TG ^{high} HDL ^{low} (n=29)	p-value
<i>Demographical features</i>			
Age, years; median (range)	53.29 (22.00 – 82.50)	54.91 (31.50 – 76.17)	0.765
Gender, f/m	74/10	18/11	0.002
<i>Disease features</i>			
Disease duration, years	5.00 (9.08)	3.87 (6.25)	0.183
Age at diagnosis, years; median (range)	46.00 (18.00 – 78.50)	50.12 (21.25 – 70.33)	0.206
Recruited at onset, n(%)	9 (10.7)	6 (20.7)	0.207
Disease activity (DAS28)	3.66 (1.99)	3.63 (1.53)	0.479
Tender Joint Count	3.00 (6.00)	2.00 (6.50)	0.470
Swollen Joint Count	2.00 (5.00)	2.00 (4.50)	0.685
Patient Global Assessment (0-100)	46.00 (40.75)	35.00 (40.00)	0.138
ESR, mm/h	16.50 (23.25)	12.00 (30.50)	0.918
CRP, mg/l	1.70 (3.50)	3.00 (6.53)	0.012
HAQ (0-3)	1.00 (1.13)	0.75 (1.25)	0.495
RF (+), n(%)	48 (60.0)	20 (71.4)	0.281
ACPA (+), n(%)	53 (66.3)	16 (57.1)	0.388
Erosive disease, n(%)	32 (39.0)	8 (29.6)	0.380
<i>Traditional CV risk factors, n(%)</i>			
Dyslipidemia	21 (25.3)	19 (65.5)	<0.001
Hypertension	26 (31.3)	12 (41.4)	0.325
Diabetes	4 (4.8)	4 (13.8)	0.106
Obesity (BMI>30)	13 (16.0)	10 (34.5)	0.036
Smoking habit	24 (28.6)	13 (44.8)	0.108

<i>Treatments, n(%)</i>			
Glucocorticoids	50 (60.2)	13 (44.8)	0.150
Methotrexate	61 (73.5)	20 (60.0)	0.639
TNF α blockers	35 (43.2)	4 (13.8)	0.004
Tocilizumab	4 (4.8)	7 (24.1)	0.006
Statins	13 (15.9)	8 (27.5)	0.104

1

2

1 **Table 3: Serum levels of inflammatory mediators and oxidative status parameters according to the lipid profiles studied in HC, RA and DL patients.** Continuous
 2 variables are expressed as mean \pm standard deviation, or median (interquartile range). Differences between groups were assessed by Mann-Whitney U tests. DL
 3 (dyslipidemia), HC (healthy controls), TRL (triglyceride-rich lipoproteins), PON1 (paraoxonase 1), RA (rheumatoid arthritis), TAC (total antioxidant capacity).

	HC			RA			DL		
	Normal lipid profile (n=83)	TG ^{high} HDL ^{low} (n=30)	p-value	Normal lipid profile (n=84)	TG ^{high} HDL ^{low} (n=29)	p-value	Normal lipid profile (n=16)	TG ^{high} HDL ^{low} (n=11)	p-value
<i>Cytokines and inflammatory mediators</i>									
TNF α (pg/ml)	158.14 \pm 193.97	136.89 \pm 173.87	0.916	282.92 \pm 243.78	582.64 \pm 753.27	0.004	141.19 \pm 187.76	191.49 \pm 193.81	0.397
IFN γ (pg/ml)	3.00 \pm 9.68	5.89 \pm 27.27	0.195	8.23 \pm 14.22	7.93 \pm 12.95	0.884	3.40 \pm 61.07	6.20 \pm 52.01	0.148
IL-8 (pg/ml)	25.74 \pm 37.54	18.46 \pm 6.72	0.930	50.69 \pm 31.40	58.40 \pm 40.64	0.156	14.96 \pm 8.26	13.59 \pm 6.04	1.000
GM-CSF (pg/ml)	1.34 \pm 1.68	0.99 \pm 1.09	0.659	32.61 \pm 19.18	49.00 \pm 58.14	0.655	4.20 \pm 3.21	4.10 \pm 25.07	0.343
MCP-1 (pg/ml)	289.74 \pm 220.18	242.74 \pm 159.43	0.765	419.18 \pm 418.34	639.83 \pm 538.32	0.004	336.64 \pm 208.26	500.45 \pm 327.24	0.148
sICAM-1 (pg/ml)	219.31 \pm 147.80	368.24 \pm 240.53	0.146	256.01 \pm 144.26	302.36 \pm 171.94	0.154	213.77 \pm 100.47	326.61 \pm 90.78	0.008
EGF (pg/ml)	119.95 \pm 87.57	111.21 \pm 65.85	0.948	133.35 \pm 79.92	225.80 \pm 255.86	0.137	156.75 \pm 169.89	138.81 \pm 119.28	0.959
IP-10 (pg/ml)	87.27 \pm 103.37	89.70 \pm 121.67	0.866	106.10 \pm 89.25	165.19 \pm 124.00	0.018	74.79 \pm 55.01	100.97 \pm 51.25	0.087
Leptin (ng/ml)	9.21 \pm 7.80	11.70 \pm 11.10	0.664	12.31 \pm 10.53	27.27 \pm 37.97	<0.001	12.98 \pm 11.92	12.01 \pm 10.99	0.878
Resistin (pg/ml)	7.32 \pm 3.32	8.54 \pm 2.51	0.120	9.32 \pm 3.55	11.40 \pm 4.90	0.084	8.19 \pm 2.44	8.36 \pm 2.58	0.799
<i>Oxidative status parameters</i>									
PON1 (U)	336.36 \pm 134.19	392.53 \pm 135.80	0.097	251.43 \pm 112.45	220.50 \pm 97.50	0.223	329.47 \pm 145.16	378.63 \pm 171.13	0.507
TAC (mM T-Eq)	4.43 \pm 0.86	5.01 \pm 1.21	0.060	3.82 \pm 0.81	4.00 \pm 0.86	0.236	4.57 \pm 0.91	5.08 \pm 1.46	0.697

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1 **Table 4: Differential effect of the TG^{high}HDL^{low} profile on the inflammatory milieu and oxidative status parameters depending on the PON1 rs662 genetic variants**
 2 **in RA patients.** DNA was available from 103/113 (91.1%) RA patients. Variables are summarized as mean \pm standard deviation. Differences between lipid profiles were
 3 assessed by Mann-Whitney U tests. PON1 (paraoxonase 1), TAC (total antioxidant capacity).

PON1 rs662 genotype	QQ			QR			RR		
	Normal lipid profile (n=36)	TG ^{high} HDL ^{low} (n=14)	p-value	Normal lipid profile (n=31)	TG ^{high} HDL ^{low} (n=10)	p-value	Normal lipid profile (n=9)	TG ^{high} HDL ^{low} (n=3)	p-value
<i>Cytokines and inflammatory mediators</i>									
TNF α (pg/ml)	235.15 \pm 161.49	783.23 \pm 804.37	0.002	329.91 \pm 323.54	260.61 \pm 136.31	1.000	337.23 \pm 227.96	368.88 \pm 193.13	0.864
IFN γ (pg/ml)	5.93 \pm 6.22	5.36 \pm 3.77	1.000	11.58 \pm 22.08	6.14 \pm 2.69	0.782	4.95 \pm 2.81	2.98 \pm 0.45	0.133
IL-8 (pg/ml)	48.83 \pm 30.58	60.03 \pm 50.73	0.253	54.55 \pm 33.70	52.95 \pm 19.70	0.932	43.45 \pm 15.76	42.10 \pm 4.61	0.600
GM-CSF (pg/ml)	30.82 \pm 19.28	43.75 \pm 52.70	0.905	34.42 \pm 22.59	27.75 \pm 2.29	0.483	32.98 \pm 13.68	23.25 \pm 2.88	0.100
MCP-1 (pg/ml)	344.65 \pm 330.65	685.71 \pm 622.92	0.013	479.01 \pm 515.64	593.41 \pm 319.59	0.073	387.53 \pm 256.55	240.93 \pm 108.99	0.482
sICAM-1 (pg/ml)	247.20 \pm 142.73	292.37 \pm 170.25	0.310	294.31 \pm 152.05	293.99 \pm 133.02	0.905	239.06 \pm 149.74	472.26 \pm 285.06	0.209
EGF (pg/ml)	113.69 \pm 63.91	286.36 \pm 336.35	0.047	159.80 \pm 83.24	223.33 \pm 162.52	0.449	159.08 \pm 122.14	102.91 \pm 93.45	0.482
IP-10 (pg/ml)	94.11 \pm 96.81	194.77 \pm 142.96	0.018	121.57 \pm 88.88	142.35 \pm 111.57	0.621	116.20 \pm 60.33	135.33 \pm 141.87	0.727
Leptin (ng/ml)	9.32 \pm 7.00	29.71 \pm 43.44	0.002	15.27 \pm 12.63	18.84 \pm 8.27	0.195	14.65 \pm 9.87	14.29 \pm 8.87	1.000
Resistin (pg/ml)	9.37 \pm 3.74	10.51 \pm 3.71	0.389	9.83 \pm 3.44	9.11 \pm 2.87	0.591	8.91 \pm 2.77	15.07 \pm 3.55	0.058
<i>Oxidative status parameters</i>									
PON1 (U)	175.42 \pm 63.49	177.09 \pm 58.18	0.947	316.68 \pm 104.29	258.35 \pm 112.88	0.183	363.30 \pm 91.85	374.97 \pm 86.37	1.000
TAC (mM T-Eq)	3.86 \pm 4.11	10.51 \pm 3.71	0.181	3.52 \pm 0.79	3.83 \pm 0.52	0.171	4.20 \pm 1.00	3.49 \pm 0.84	0.282

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Table 5: Changes in lipid profiles, inflammatory mediators, oxidative status parameters and blood lipid classes in RA patients upon TNF α -blockade. RA patients are stratified according to the clinical response achieved based on EULAR criteria after three months of anti-TNF α treatment. Variables are summarized as mean \pm standard deviation. Categorical data are expressed as n. Differences were analyzed by Wilcoxon paired tests. BL (baseline), FFA (free fatty acids), PON1 (paraoxonase 1), PT (post-treatment), TAC (total antioxidant capacity).

	Responders (n=5)			Non-responders (n=8)		
	BL	PT	p-value	BL	PT	p-value
<i>Lipid profile</i>						
TG ^{high} HDL ^{low} , n	0	0		4	6	
HDL-c, mg/dl	69.40 \pm 9.31	71.80 \pm 1.92	0.465	49.85 \pm 33.00	55.42 \pm 15.79	0.344
TG, mg/dl	74.80 \pm 23.22	83.00 \pm 22.56	0.892	109.14 \pm 40.60	137.33 \pm 59.13	0.116
TRL, mg/dl	10.23 \pm 3.72	11.48 \pm 5.18	0.699	23.44 \pm 12.34	25.44 \pm 14.38	0.389
<i>Inflammatory mediators and oxidative status parameters</i>						
TNF α (pg/ml)	451.05 \pm 265.98	150.14 \pm 153.47	0.045	352.02 \pm 197.97	394.95 \pm 179.31	0.484
MCP-1 (pg/ml)	225.38 \pm 72.72	145.11 \pm 63.31	0.043	250.37 \pm 60.01	213.02 \pm 72.14	0.161
PON1 (U)	296.62 \pm 141.02	377.49 \pm 142.30	0.225	252.51 \pm 116.62	305.99 \pm 119.20	0.161
TAC (mM T-Eq)	3.64 \pm 0.78	2.95 \pm 0.68	0.138	4.07 \pm 0.99	3.69 \pm 0.35	0.327
FFA (mM)	0.56 \pm 0.28	0.54 \pm 0.37	0.893	0.54 \pm 0.30	0.31 \pm 0.20	0.161

HIGHLIGHTS

- TG^{high}HDL^{low} lipid profile is associated with inflammatory mediators in RA
- High TRL levels may underlie the effect of the TG^{high}HDL^{low} profile
- The association between TG^{high}HDL^{low} and inflammation depends on oxidative status
- TG^{high}HDL^{low} profile is related to a poor clinical outcome upon TNF α -blockade in RA