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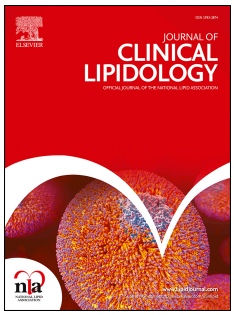

High triglycerides and low HDL-c lipid profile in rheumatoid arthritis: a potential link among inflammation, oxidative status and dysfunctional HDL

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PII: S1933-2874(17)30335-5

DOI: 10.1016/j.jacl.2017.05.009

Reference: JACL 1121

To appear in: *Journal of Clinical Lipidology*

Received Date: 13 March 2017

Revised Date: 1 May 2017

Accepted Date: 8 May 2017

Please cite this article as: Rodríguez-Carrio J, Alperi-López M, López P, López-Mejías R, Alonso-Castro S, Abal F, Ballina-García FJ, González-Gay MÁ, Suárez A, High triglycerides and low HDL-c lipid profile in rheumatoid arthritis: a potential link among inflammation, oxidative status and dysfunctional HDL, *Journal of Clinical Lipidology* (2017), doi: 10.1016/j.jacl.2017.05.009.

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

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

ABSTRACT

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

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

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

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

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

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

INTRODUCTION

Blood lipid abnormalities are a frequent hallmark of active rheumatoid arthritis (RA). Many authors have reported an alteration of the lipid profile in RA patients, especially those of lipoproteins during high disease activity states [1–3]. Accordingly, disease activity control by different immunomodulatory treatment strategies is related to a restoration of the lipid profile to different degrees, although certain controversy exists [1]. Due to the fluctuation of some lipoproteins in the context of a chronic inflammation, and taking into account that a single lipid compound cannot provide enough information on its own, there is a need for more reliable biomarkers related to the lipid profile in RA.

The classical clinical management of blood lipids focused on lipoproteins is currently challenged by a compelling body of evidence highlighting an important contribution of other lipid compounds, such as triglycerides (TG), lipoprotein A or cholesterol remnants [4–6]. Different panels of experts have raised the point that individual lipoproteins alone may not be accurate for patient stratification and treatment recommendations in the clinical setting (reviewed in [5]). Actually, reducing LDL-cholesterol (LDL-c) levels as a therapeutic target is now put into question and there is a recommendation to consider other lipid fractions or surrogate markers as they can be more informative [7]. TG exhibit important differences compared to lipoproteins in their origin, metabolic pathways and downstream effects in different cell types. Therefore, it is tempting to speculate that their inclusion in the clinical management will bring valuable information which can complement that of provided by lipoproteins. In fact, several epidemiological studies have found an attenuation of the protective effect of HDL-cholesterol (HDL-c) levels on cardiovascular disease (CVD) outcomes when adjusted for TG [8–10], thus suggesting the existence of interactive effects among lipid classes. Hence, there is a growing body of evidence supporting the study of combined indices, especially when TG are included [11]. Consequently, in recent years the relevance of a lipid profile characterized by decreased HDL-c levels and elevated TG has emerged [5]. This profile has been linked to inflammation and atherosclerosis development, although some knowledge gaps remain [4]. Recently, this profile has been shown to affect the leukocyte gene expression in dyslipidemia subjects [12]. However, the clinical and immunological relevance of this profile in RA is unknown.

On the other hand, current epidemiological and experimental studies have stated that the only analysis of lipid levels is a too simplistic approach that does not reflect lipoprotein functionality. Lipoproteins can develop a broad range of enzymatic activities, apart from cholesterol transport [13]. Interestingly, lipoprotein functionality seems to have a better clinical relevance than levels [14,15]. Among lipoprotein functions, anti-oxidant properties are emerging as a pivotal player to understand the interaction between lipid profiles and inflammation [16], compromised anti-oxidant

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

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

MATERIAL AND METHODS

Patients

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

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

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

Quantification of inflammatory mediators' serum levels

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

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

Assessment of PON1 activity

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

Analysis of Total Antioxidant Capacity

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

Analysis of Free Fatty Acids

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

PON1 rs662 genotyping

DNA was isolated from peripheral blood using conventional methods. The PON1 rs662 polymorphism was genotyped with TaqMan predesigned single-nucleotide polymorphism (SNP) genotyping assays (C___2548962_20) in a 7900 HT Real-Time polymerase chain reaction (PCR) system, as previously described [21].

Statistical analyses

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

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

RESULTS

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

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

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

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

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

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

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

Interestingly, the $TG^{high}HDL^{low}$ profile was associated with increased TRL levels in RA patients (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 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 g=1.15) subjects. Moreover, TRL were found to be positively correlated with the levels of CRP (r=0.204, p=0.042), TNFα (r=0.351, p<0.0001), MCP-1 (r=0.409, p<0.0001), IP-10 (r=0.239, p=0.018) and leptin (r=0.257, p=0.008), and negatively with PON1 activity (r=-0.203, p=0.036) in RA patients. However, TRL were not related to these mediators in the HC or DL groups. TRL levels were not influenced by treatments. Finally, the serum levels of FFA in RA patients were similar between lipid profiles ($TG^{high}HDL^{low}$: 0.56±0.32 vs normal: 0.53±0.30 mM, p=0.646). No differences in FFA between RA and HC were found (0.55±0.31 vs 0.48±0.24 mM, p=0.418).

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

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

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

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

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

4. $TG^{high}HDL^{low}$ profile and inflammation upon TNFα-blockade

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

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

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

DISCUSSION

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

Most of studies on lipid profiles in RA were focused on lipoprotein levels, whereas the role of TG, and its clinical relevance beyond lipid metabolism, in RA have been ill-defined. As in other conditions, the use of lipid ratios has started to become used in RA. The EULAR consensus for cardiovascular risk management in inflammatory arthritis encourages the use of the total- to HDL-c ratio [22]. However, several concerns need to be underlined. On the one hand, this lipid ratio still underestimates the use of TG levels, as they are not included. However, important divergences in the association between TG and inflammatory markers compared to lipoproteins or cholesterol composite indices arise in RA [23]. On the other hand, lipid ratios imply, at least in part, certain stoichiometry between the lipid classes considered. Although this may not be a problem when similar species (for instance, lipoproteins) are studied, this approach may yield inconclusive results when different compounds are analyzed. This may account for the lack of appropriate results when the TG/HDL ratio was studied in other conditions. Similarly, since non-linear associations between blood lipids and clinical outcomes have been reported in RA [1], simple ratios could not be adequate. It is important to note that interesting findings arise even within normal ranges of individual lipid classes, thus strengthening the relevance of the study of the combined profile and the need for different cut-offs than those used for single lipid classes alone. Therefore, a different combined approach as the one herein reported may provide more reliable results.

Our findings revealed an association between the $TG^{high}HDL^{low}$ profile with elevated TRL, thus supporting the deleterious effect of this combined profile. High TG and TRL levels can impact the HDL-c levels, composition and thus, functional status. Elevated TG and TRL may lead to a greater cholesteryl ester exchange via Cholesteryl Ester Transfer Protein (CETP) between TRL and nascent HDL, resulting in TG-rich small, dense HDL particles with reduced anti-oxidant and anti-inflammatory activities and decreased cholesterol-accepting properties [24,25]. Importantly, increased CETP has been reported in RA [26]. Under these circumstances, LDL

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

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

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

proposed in other scenarios [38]. Overall, these results expand the current knowledge on the clinical relevance of the PON1 rs662 polymorphism, strongly determined by gene-environment interactions. Environmental factors can critically impair the antioxidant activity of individuals the low activity genotype [21,39,40], hence explaining their increased susceptibility to different clinical outcomes. Interestingly, loss of PON-1 activity in knockout mice models was associated with increased lipid oxidation and inflammation [41], oxidized lipid species playing a crucial role. Surprisingly, Wang and colleagues have revealed that TRL can release oxidized lipid species which in turn can elicit pro-inflammatory responses, including TNFα secretion and upregulation of adhesion molecules, in a more potent fashion than native FFA [42]. These results may explain the strong association between TRL and inflammatory mediators, but not FFA, in RA patients harboring the QQ genotype. Moreover, TRL can also produce reactive oxidative species on their own [43]. Additionally, TRL have been also reported to promote NFκB expression [44], which can control MCP-1 production.

Taken together, these lines of evidence may delineate a bidirectional interaction between lipid profile and inflammation. Classically, inflammation was known to influence the lipid profile. However, it has been recently reported that inflammation can marginally explain lipid disturbances in RA [45], and the idea that lipids indeed influence inflammation is emerging. Actually, the altered lipid profile can appear in the preclinical stage of RA [45,46]. Furthermore, this double interaction aligns with the existence of a cross-talk between traditional and non-traditional risk factors in RA [47,48]. In fact, the anti-atherogenic functionality of HDL is known to be diminished in RA and other chronic inflammatory conditions [32,49]. Inflammation-driven protein composition shifts towards a decreased antioxidant and pro-inflamamatory profile may cause these findings [50].

It is tempting to speculate that the findings herein reported may unravel new perspectives for the CV risk in RA. However, this should be interpreted with caution. One the one hand, some controversy exists regarding the altered CE in RA [32]. On the other hand, whether impaired HDL function is associated with increased CV risk in RA has to be elucidated in prospective studies [32]. However, our results may shed some light into these connections, as a role for specific mediators of inflammation (TNFα, MCP-1, EGF and leptin) and those of lipid metabolism (TRL) is reported. Additionally, inflammation and increased oxidative status are known to impact vascular repair by directly impairing circulating progenitors [51], hence bridging abnormal lipid profile, vascular repair and CV risk. Taken together, these lines of evidence warrants further studies addressing the interaction among HDL dysfunction, endothelial homeostasis and CV risk in RA.

Our findings provide valuable insight for the clinical setting and personalized medicine. Although lipid ratios are recommended in the clinical management of RA, its current use may be revised and additional biomarkers are needed. Moreover, whether lipoprotein ratios can provide information on the functional status of HDL is unknown. Taking into account the relevance of a disturbed lipoprotein functionality [4,14,15], it may be advisable to include this pathological finding in the clinical setting. However, routine analyses for HDL functional status are lacking, due to its time and methodological limitations. Taking into account our findings, the $TG^{high}HDL^{low}$ profile can be considered as a surrogate biomarker of an altered HDL functional status. Thus, our results provide a new rationale for patient stratification and treatment decision in this sense, as the $TG^{high}HDL^{low}$ profile can be used to identify RA patients with an enhanced systemic response and increased pro-oxidative status, linked to HDL dysfunction. These patients may be considered for anti-TNFα treatment and/or some therapeutic drugs able to counteract oxidative stress linked to inflammation [52].

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

CONFLICT OF INTEREST

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

FUNDING

This work was supported by European Union FEDER funds, "Fondo de Investigación Sanitaria (FIS, PI12/00523 and PI16/00113; ISCIII, Spain) and SER/FER funds (Sociedad Española de Reumatología, FER043/2016). J.R.-C. is supported by a postdoctoral contract from the "Juan de la Cierva" program (FJCI-2015-23849; MICINN, Spain).

AUTHOR CONTRIBUTIONS

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

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TABLES

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

	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

[a] DL vs HC: p=0.035, DL vs RA: p=0.011
[b] Hedges'g statistic (DL vs HC): g=0.50
[c] DL vs HC: p<0.001, DL vs RA: p<0.006
[d] Hedges'g statistic (DL vs HC): g=1.31
[e] DL vs RA: p=0.029
[d] DL vs HC: p<0.0001, DL vs RA: p<0.007
[f] Hedges'g statistic (DL vs HC): g=1.22
[g] power=0.952 (a=0.05)

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

	Normal lipid profile (n=84)	**TG^{high} HDL^{low} (n=29)**	**p-value**
Demographical features			
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**
Disease features			
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
Traditional CV risk factors, n(%)			
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

Treatments, n(%)			
Glucocorticoids	50 (60.2)	13 (44.8)	0.150
Methotrexate	61 (73.5)	20 (60.0)	0.639
TNFα 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

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Table 3: Serum levels of inflammatory mediators and oxidative status parameters according to the lipid profiles studied in HC, RA and DL patients. Continuous variables are expressed as mean ± standard deviation, or median (interquartile range). Differences between groups were assessed by Mann-Withney U tests. DL (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**
Cytokines and inflammatory mediators									
TNFα (pg/ml)	158.14±193.97	136.89±173.87	0.916	282.92±243.78	582.64±753.27	**0.004**	141.19±187.76	191.49±193.81	0.397
IFNγ (pg/ml)	3.00±9.68	5.89±27.27	0.195	8.23±14.22	7.93±12.95	0.884	3.40±61.07	6.20±52.01	0.148
IL-8 (pg/ml)	25.74±37.54	18.46±6.72	0.930	50.69±31.40	58.40±40.64	0.156	14.96±8.26	13.59±6.04	1.000
GM-CSF (pg/ml)	1.34±1.68	0.99±1.09	0.659	32.61±19.18	49.00±58.14	0.655	4.20±3.21	4.10±25.07	0.343
MCP-1 (pg/ml)	289.74±220.18	242.74±159.43	0.765	419.18±418.34	639.83±538.32	**0.004**	336.64±208.26	500.45±327.24	0.148
sICAM-1 (pg/ml)	219.31±147.80	368.24±240.53	0.146	256.01±144.26	302.36±171.94	0.154	213.77±100.47	326.61±90.78	**0.008**
EGF (pg/ml)	119.95±87.57	111.21±65.85	0.948	133.35±79.92	225.80±255.86	0.137	156.75±169.89	138.81±119.28	0.959
IP-10 (pg/ml)	87.27±103.37	89.70±121.67	0.866	106.10±89.25	165.19±124.00	**0.018**	74.79±55.01	100.97±51.25	0.087
Leptin (ng/ml)	9.21±7.80	11.70±11.10	0.664	12.31±10.53	27.27±37.97	**<0.001**	12.98±11.92	12.01±10.99	0.878
Resistin (pg/ml)	7.32±3.32	8.54±2.51	0.120	9.32±3.55	11.40±4.90	0.084	8.19±2.44	8.36±2.58	0.799
Oxidative status parameters									
PON1 (U)	336.36±134.19	392.53±135.80	0.097	251.43±112.45	220.50±97.50	0.223	329.47±145.16	378.63±171.13	0.507
TAC (mM T-Eq)	4.43±0.86	5.01±1.21	0.060	3.82±0.81	4.00±0.86	0.236	4.57±0.91	5.08±1.46	0.697

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 in RA patients. DNA was available from 103/113 (91.1%) RA patients. Variables are summarized as mean ± standard deviation. Differences between lipid profiles were assessed by Mann-Withney 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**
Cytokines and inflammatory mediators									
TNFα (pg/ml)	235.15±161.49	783.23±804.37	**0.002**	329.91±323.54	260.61±136.31	1.000	337.23±227.96	368.88±193.13	0.864
IFNγ (pg/ml)	5.93±6.22	5.36±3.77	1.000	11.58±22.08	6.14±2.69	0.782	4.95±2.81	2.98±0.45	0.133
IL-8 (pg/ml)	48.83±30.58	60.03±50.73	0.253	54.55±33.70	52.95±19.70	0.932	43.45±15.76	42.10±4.61	0.600
GM-CSF (pg/ml)	30.82±19.28	43.75±52.70	0.905	34.42±22.59	27.75±2.29	0.483	32.98±13.68	23.25±2.88	0.100
MCP-1 (pg/ml)	344.65±330.65	685.71±622.92	**0.013**	479.01±515.64	593.41±319.59	0.073	387.53±256.55	240.93±108.99	0.482
sICAM-1 (pg/ml)	247.20±142.73	292.37±170.25	0.310	294.31±152.05	293.99±133.02	0.905	239.06±149.74	472.26±285.06	0.209
EGF (pg/ml)	113.69±63.91	286.36±336.35	**0.047**	159.80±83.24	223.33±162.52	0.449	159.08±122.14	102.91±93.45	0.482
IP-10 (pg/ml)	94.11±96.81	194.77±142.96	**0.018**	121.57±88.88	142.35±111.57	0.621	116.20±60.33	135.33±141.87	0.727
Leptin (ng/ml)	9.32±7.00	29.71±43.44	**0.002**	15.27±12.63	18.84±8.27	0.195	14.65±9.87	14.29±8.87	1.000
Resistin (pg/ml)	9.37±3.74	10.51±3.71	0.389	9.83±3.44	9.11±2.87	0.591	8.91±2.77	15.07±3.55	0.058
Oxidative status parameters									
PON1 (U)	175.42±63.49	177.09±58.18	0.947	316.68±104.29	258.35±112.88	0.183	363.30±91.85	374.97±86.37	1.000
TAC (mM T-Eq)	3.86±4.11	10.51±3.71	0.181	3.52±0.79	3.83±0.52	0.171	4.20±1.00	3.49±0.84	0.282

Table 5: Changes in lipid profiles, inflammatory mediators, oxidative status parameters and blood lipid classes in RA patients upon TNFα-blockade. RA patients are stratified according to the clinical response achieved based on EULAR criteria after three months of anti-TNFα treatment. Variables are summarized as mean ± 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**
Lipid profile						
$TG^{high}HDL^{low}$, n	0	0		4	6	
HDL-c, mg/dl	69.40±9.31	71.80±1.92	0.465	49.85±33.00	55.42±15.79	0.344
TG, mg/dl	74.80±23.22	83.00±22.56	0.892	109.14±40.60	137.33±59.13	0.116
TRL, mg/dl	10.23±3.72	11.48±5.18	0.699	23.44±12.34	25.44±14.38	0.389
Inflammatory mediators and oxidative status parameters						
TNFα (pg/ml)	451.05±265.98	150.14±153.47	**0.045**	352.02±197.97	394.95±179.31	0.484
MCP-1 (pg/ml)	225.38±72.72	145.11±63.31	**0.043**	250.37±60.01	213.02±72.14	0.161
PON1 (U)	296.62±141.02	377.49±142.30	0.225	252.51±116.62	305.99±119.20	0.161
TAC (mM T-Eq)	3.64±0.78	2.95±0.68	0.138	4.07±0.99	3.69±0.35	0.327
FFA (mM)	0.56±0.28	0.54±0.37	0.893	0.54±0.30	0.31±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 TNFα-blockade in RA