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# PON activity is modulated by rs662 polymorphism and IgG anti-HDL antibodies in Rheumatoid Arthritis patients: potential implications for CV disease

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## ABSTRACT

**Objective:** Paraoxonase 1 (PON1) is a High Density Lipoprotein (HDL)-associated antioxidant enzyme which plays an important role in HDL-mediated cardioprotection. Although genetic polymorphisms are known to modulate PON1 activity, its involvement in cardiovascular disease (CVD) in Rheumatoid Arthritis (RA) is controversial, suggesting that other factors may modulate its function. Since anti-HDL antibodies have been related to impaired lipid profile and CVD in RA, we aimed to examine the associations between PON1 activity, anti-HDL antibodies and CVD in RA according to PON1 genetic variants.

**Methods:** serum PON1 activity, using paraoxon as substrate, and IgG anti-HDL antibodies were quantified in 212 RA patients and 110 healthy controls (HC). The PON1 rs662 genotype (Q>R) was determined with TaqMan probes. An additional group of 13 biologic-naïve RA patients was prospectively followed for three months.

**Results:** PON1 activity was decreased in RA compared to HC ($p=0.005$) and an effect of the genotype was noted in both groups, QQ homozygotes exhibiting the lowest activity. Distribution of genotypes did not differ between groups ($p=0.215$). Anti-HDL antibodies were associated with an impaired PON1 activity ($p=0.010$), decreasing HDL levels ($r=-0.680$, $p<0.001$) and higher prevalence of CV events in univariate and multivariate models in patients carrying the QQ genotype. Furthermore, change in anti-HDL antibodies upon TNFα-blockade independently predicted improved PON1 activity (β[95% CI], p: -0.369 [-0.669, -0.069], $p=0.024$).

**Conclusions:** PON1 activity is impaired in RA in association with rs662 status and anti-HDL antibodies, the latter being a pivotal player to understand the link between rs662 and CVD.


**Keywords:** paraoxonase 1, anti-HDL antibodies, High Density Lipoproteins, cardiovascular, rheumatoid arthritis

## INTRODUCTION

Rheumatoid Arthritis (RA) has been consistently associated with an increased cardiovascular (CV) risk and mortality compared to the general population. Conventional algorithms fail to fully predict the occurrence of CV disease (CVD) in these patients (1), probably because of the involvement of non-traditional CV risk factors (2). Therefore, CV risk stratification is an unmet need in the clinical setting in RA.

Although the role of blood lipids in CVD development in the general population is clear, their association with CVD in RA is more complex. Decreased levels of total cholesterol and low density lipoproteins (LDL)-cholesterol are found in RA patients with active disease, whereas successful therapy is related to increasing lipid levels (3;4). This contradictory situation, termed as "lipid paradox" (5;6), suggest that not only levels but also function of these lipoproteins are relevant in CV homeostasis, especially in inflammatory conditions. Actually, inflammation is accompanied by changes in HDL composition, which are related to an impaired HDL function (7). Recently, the concept of HDL dysfunction has emerged as a pivotal player in this scenario (8), and other functions than cholesterol efflux have been reported for HDL particles (9). Antioxidant function of HDL seems to be crucial for HDL protective properties. The main component responsible for HDL antioxidant effect is Paraoxonase 1 (PON1) (10). PON1 activity is under genetic control by a number of polymorphisms at the PON1 locus in humans. A functional single nucleotide polymorphism at position 192 (rs662), which accounts for most of its variation, confers an amino acid change (Q>R), the R isoform being associated with a higher PON1 activity (11). Inconsistent results about the association between CVD susceptibility and PON1 rs662 variants were reported in different conditions (12-14), thereby suggesting that gene-environment interactions could modulate the association between PON1 polymorphism and CV disease. Interestingly, although decreased PON1 activity has been found in RA patients (15;16), little is known about its relevance for CVD development.

In this scenario, our group has recently revealed that IgG anti-HDL antibodies are associated with an impaired blood lipid profile, inflammatory milieu and CVD occurrence in RA (17). However, whether the anti-HDL antibodies can interfere with HDL function in RA or if the association between anti-HDL antibodies and HDL lipoproteins as well as CV disease depends on PON1 polymorphism and function remains unclear. Therefore, in the present report we aimed to study (i) the association between anti-HDL antibodies and PON1 function in RA patients, (ii) the effect of PON1 rs662 on the association between

anti-HDL antibodies and CVD, and (iii) the effect of anti-HDL antibodies on PON1 activity upon TNFα blockade.

## MATERIAL AND METHODS

### Patients and controls

This was a cross-sectional study involving 212 RA patients consecutively recruited from the Department of Rheumatology at Hospital Universitario Central de Asturias. All of them fulfilled the 2010 American College of Rheumatology classification criteria for RA. A complete clinical examination, including Disease Activity Score 28-joints (DAS28) calculation, was performed on each patient during their clinic appointment and a blood sample was drawn by venipuncture. Clinical records were retrospectively revised in order to register traditional CV risk factors and CV events occurrence. Definition of CV events and traditional CV risk factors was performed as previously described (18). Additionally, a group of 13 biologic-naïve RA patients (12 women, median age 43 (range: 30 – 65), DAS28 5.08(1.93), 38.5% RF+, 46.1% ACPA+), candidates for anti-TNFα therapy, was prospectively followed for three months. Blood samples were collected immediately before and 3 months after TNFα-blocking therapy. RA patients were classified in those recruited at onset (less than 3 months since RA definite diagnosis, not receiving treatments when recruited, n=47) and established disease (>3 months of disease duration, n=165) (Supplementary Table 1). Clinical response was evaluated by EULAR criteria (19).

Simultaneously, 110 age- and gender matched healthy individuals (HC) were recruited from the general population. Automated serum lipids analysis was carried out on all the participants from fresh blood samples. Serum samples were stored at -80ºC until analyses.

Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, in compliance with the Declaration of Helsinki. All the participants gave written informed consent prior to their inclusion in the study.

### Analysis of PON1 activity

PON1 activity was quantified in previously unthawed serum samples according to Eckerson et al. (20) with slight modifications. Briefly, 300 µl of freshly prepared 1mM paraoxon (Sigma Aldrich) in 50 mM glycine buffer containing 1 mM $CaCl_2$ (pH 10.5) was incubated with 7.5 µl of serum samples in 96-well Maxisorp plates (Nunc) for 20 minutes at 37ºC protected from light. Formation of p-nitrophenol was monitored at 405 nm. A unit (U) of PON1 activity was expressed as micromoles of p-nitrophenol formed per minute per ml of serum. The molar extinction coefficient of p-nitrophenol was 18690 $mol^{-1} \cdot cm^{-1}$.

Quality controls were included in each plate to correct for interassay variations. Inter- and intraassay coefficients of variation were 9.03% and 7.09%, respectively.

### Quantification of anti-HDL antibodies

Serum levels of IgG anti-HDL antibodies were measured by ELISA as previously described (17) using 96-well plates half-coated overnight at 4º C with 20 μg/ml human HDL-cholesterol (Sigma) in 70% ethanol (test half) or ethanol alone (control half). Blocking with PBS 1% BSA was performed for 1 hour at room temperature. Plates were then washed with PBS and 1:50-diluted serum samples were incubated for 2 hours at room temperature. After three washing steps with TBS, alkaline phosphatase-conjugated anti-human IgG was added. Finally, the plate was washed twice with TBS followed by addition of p-nitrophenylphosphate (Sigma) in dietanolamine buffer. Absorbance at 405 nm was recorded and signal from the control half of the plate was subtracted to that of the test half. IgG anti-HDL Arbitrary Units (AU) were calculated for each sample according to the standard curves from pooled sera.

Similarly, total IgG was quantified by conventional ELISA techniques and AU values obtained from the IgG anti-HDL ELISA were corrected using total IgG levels (anti-HDL/IgG). Positivity for anti-HDL antibodies was defined using the 90$^{th}$ percentile of anti-HDL/IgG in healthy controls as cutoff (169.80), as was previously determined in a large cohort from the same population (17).

### PON1 rs662 genotyping

DNA from 105 controls (95.4%) and 186 patients (87.7%) was obtained from whole peripheral blood using standard 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, according to the conditions recommended by the manufacturer (Applied Biosystems, Foster City). PCR was carried out in a total reaction volume of 4 μl with the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 92°C for 15 seconds and then annealing and extension at 60°C for 1 minute.

Negative controls and duplicate samples were included to check the accuracy of genotyping.

### Statistical analysis

Continuous variables were summarized as median (interquartile range), unless otherwise stated. Categorical variables were expressed as n(%) and analyzed using chi-square tests. Differences between groups were assessed by Mann-Withney U, Kruskal Wallis tests or bivariate ANOVA, as appropriate. Spearman rank's test was used for assessing correlations. Paired T test were used for prospective analyses. Variables were log-transformed to achieve normal distribution prior to multiple regression analyses. The association between CV disease and anti-HDL antibodies was analyzed by binary logistic regression, and adjusted OR and 95% confidence intervals (CI) were calculated. p-value $<0.050$ was considered as statistically significant. Statistical analyses were performed under SPSS 19.0 and Graph Pad 5.0.

## RESULTS

### 1. PON1 activity is decreased in RA patients

PON1 activity was analyzed in serum samples from 212 RA patients (Table 1) (supplementary Table 1) and 110 sex- and age-matched healthy individuals (HC). As indicated in Figure 1A, decreased PON1 activity was found in the whole RA group (309.75(136.78) U) compared with HC (226.98(208.39) U). Although no association was observed with disease activity ($r=0.021$, $p=0.778$), PON1 activity was negatively correlated with disease duration ($r= -0.253$, $p<0.001$). Actually, no differences between RA patients at onset (n=47) and HC were seen (306.22 (252.13) vs 309.75 (136.78), $p=0.451$). Conversely, no associations were observed with CRP ($r= 0.029$, $p=0.716$) or ESR ($r= -0.036$, $p=0.622$).

Stratification according to rs662 genotype showed that frequency of these gene variants was comparable between patients and HC (QQ: 95 (51.0%) vs 60 (55.5%), QR: 69 (37.0%) vs 39 (35.7%), RR: 22 (11.8%) vs 6 (5.5%); p (chi-square) = 0.215), and distribution of genotypes fit Hardy-Weinberg equilibrium in both groups. As expected, homozygotes for the wild-type allele (QQ) exhibited the lowest PON1 activity in both patients and controls, and a dosage-dependent effect was registered in both groups (Figure 1B). Overall, there results suggest that additional factors other than genetic ones should explain the decreased PON1 activity found in RA.

### 2. Anti-HDL antibodies and rs662 polymorphism are related to decreased PON1 activity in RA patients

Since the presence of anti-HDL antibodies has been related to an impaired blood lipid profile and inflammatory mediators in RA patients (17), we wondered whether they may be involved in the impairment of PON activity.

We observed that patients classified as anti-HDL-positive (n=40, 18.8%) exhibited a trend towards decreased PON1 activity compared with their negative counterparts (224.34 (198.15) vs 276.28 (204.27) U, $p=0.060$). Interestingly, an equivalent trend was observed in anti-HDL-positive HC (n=9) (231.54(158.13) vs 314.76(134.90) U, $p=0.080$). No associations between rs662 variants and anti-HDL positivity ($p=0.184$), ESR ($p=0.895$) or CRP levels ($p=0.619$) were noted in RA patients. However, when patients were stratified according to rs662 status, a differential effect of anti-HDL antibodies on PON1 activity was observed (Figure 2), the effect of anti-HDL antibodies being stronger in patients with the QQ genotype. Moreover, QQ homozygotes without anti-HDL antibodies did not exhibit

diminished PON1 activity compared with HC, although the effect was different in QR and RR groups. A multivariate ANOVA analysis confirmed an independent role of both rs662 ($p<0.0001$) and presence of anti-HDL antibodies ($p=0.026$) on PON1 activity (interaction: $p=0.267$). Since differences in PON1 activity between patients at onset and their established-disease counterparts were observed, the effect of anti-HDL antibodies was studied in both groups. Interestingly, among patients with established disease, a negative association between IgG anti-HDL levels and PON1 activity was found in those carrying the QQ genotype ($r= -0.364$, $p=0.002$; $n=70$), but not in QR ($r= -0.210$, $p=0.119$; $n=56$) or RR carriers ($r= 0.130$, $p=0.619$; $n=17$). In order to exclude an effect of inflammation on PON1 activity, we carried out a regression analysis including ESR and CRP levels as covariates, thereby revealing that anti-HDL levels predicted PON1 activity (β[95% CI], p: -0.065[-0.127, -0.004], $p=0.039$) whereas no effect of ESR ($p=0.690$) or CRP ($p=0.519$) were noted, in QQ patients. Subsequent analyses adjusting for treatments (glucocorticoids, methotrexate, TNFα-blockers and statins) did not change the effect of anti-HDL antibodies (-0.063[-0.126, -0.001], $p=0.047$). However, this effect was not found in QR or RR patients, in line with the results of the univariate tests. Moreover, no association was found in patients at onset ($r=0.001$, $p=0.998$). Interestingly, a multivariate regression analysis adjusted for disease duration, CRP, ESR and treatments usage performed in the whole group of patients showed a significant effect of rs662 status (β[95% CI], p: 0.174[0.130, 0.218], $p<0.0001$) and anti-HDL antibodies (-0.056[-0.096, -0.015], $p=0.007$) on PON1 activity, thus confirming an independent role of these parameters after controlling for the inflammatory burden.

Next, as anti-HDL antibodies were found to be associated with CV disease occurrence and controversial results have been reported for rs662, a combined analysis of both parameters was conducted. RA patients positive for anti-HDL antibodies were more likely to have suffered from a CV event than anti-HDL negative patients (16/40 vs 22/172, $p<0.0001$). When patients were stratified according to rs662 status, this association was restricted to QQ carriers (9/14 vs 9/82, $p=1\cdot10^{-6}$), being absent in QR (4/18 vs 8/50, $p=0.553$) and RR patients (2/4 vs 1/18, $p=0.124$). Logistic regression analyses confirmed these results, even after adjusting for age, gender and traditional CV risk factors (hypertension, dyslipidemia, diabetes, smoking and obesity) (Table 2). Interestingly, patients carrying the QQ genotype who suffered a CV event (n=18/78) exhibited lower PON1 activity compared to their CVD-free counterparts (157.89 (139.19) vs 212.80 (113.60) U, $p=0.037$). No differences in CV disease occurrence were noted among rs662 genotype ($p=0.860$).

These results suggest that anti-HDL antibodies have detrimental effects on PON1 activity only in patients carriers of the low activity genotype (QQ), whereas higher PON1 activity seems to counteract this effect in patients with QR and RR variants. The association with CV disease occurrence supports these findings.

**3. Anti-HDL-related lipid profile is dependent on PON1 rs662 polymorphism**

Since we have reported that anti-HDL antibodies may underlie the decreased HDL levels in RA patients at onset, we aimed to analyze whether rs662 could influence this association. IgG anti-HDL levels were negatively correlated with circulating HDL in RA patients at onset (r= -0.502, p=0.002). However, when rs662 genotype was taken into account, only carriers of the QQ genotype exhibited this association (r= -0.680, p<0.001; n=22), being absent in the rest of patients (r= -0.161, p=0.567; n=15). This association remain significant after controlling for ESR and CRP levels in a multivariate model (β[95% CI], p: -0.210[-0.326, -0.076], p=0.005). Finally, statin usage was found to have no effect on PON1 activity (p=0.633) and did not differed among genotype groups (p=0.719).

These results again point to a crucial role of these genetics variants in the effect of anti-HDL antibodies in RA patients.

**4. Anti-HDL antibodies and PON1 activity upon TNFα blockade**

Since anti-HDL antibodies seem to have a role in the normalization of HDL levels after anti-TNFα therapy, we aimed to analyze the effect on PON1 activity in addition to HDL level as well as the potential effect of rs662 polymorphism in this scenario. To this aim, a group of 13 biologic-naïve RA patients, candidates for anti-TNFα therapy was recruited and followed up for three months.

A trend towards increased PON1 activity after anti-TNFα treatment was observed (Figure 3A) in the whole group. No differences were registered when patients were categorized according to EULAR clinical response (responders: p=0.263, n=5; no responders: p=0.115, n=8) or rs662 genotype (QQ: p=0.320, n=7; QR: p=0.140, n=5). Importantly, neither differences were observed after 3 months of treatment in HDL (p=0.170) nor in anti-HDL levels (p=0.194). However, an inverse correlation was found between the change in IgG anti-HDL antibodies and that of PON1 activity (Figure 3B), thus highlighting that the stronger the decrease in anti-HDL antibodies, the bigger the increase in PON1 activity. In order to exclude a role of potential confounders, the effect of the change in anti-HDL antibodies, as well as EULAR clinical response, change in ESR, change in HDL levels and rs66s status were included in a multiple regression analysis. Interestingly, the change

in anti-HDL antibodies was the only predictor of PON1 activity improvement (β[95% CI], p: -0.369 [-0.669, -0.069], p=0.024).

These results confirm that anti-TNFα-mediated anti-HDL decrease underlie PON1 activity improvement in RA patients.

## DISCUSSION

Despite the important advances in the last years, the link between the altered lipid blood profile and CVD development in RA patients is poorly understood. Several pieces of evidence suggest that, in addition to the levels, the function of lipoproteins (mainly HDL) should also be taken into account. The present article addresses a multilevel analysis of this situation, where levels of HDL and anti-HDL antibodies as well as PON1 activity and rs662 polymorphism are studied together in order to gain more insight into the complex scenario of HDL dysfunction in RA patients with and without CVD.

In line with the relevance of PON1 function for HDL-mediated cardioprotection, decreased PON1 activity has been related to increased risk of CV events in the general population (12). Similarly, previous studies have revealed decreased PON1 activity in RA patients (15;16;21). However, factors underlying this finding remain unclear. Although some authors have proposed an overrepresentation of PON1 rs662 QQ genotype in RA patients compared to controls (16), this study was performed with a relatively low sample size and was not confirmed in further studies (15;21;22). Moreover, discrepancies on the rs662-related CVD susceptibility were found in the general population even within the same ethnic population (13;14;23;24). Additionally, PON1 activity but not genetic variants have been found to be associated with CV events in two large cohorts (25). Overall, these results suggest that gene-environment interactions may modulate the association between this polymorphism and CVD.

Apart from inflammation, the presence of IgG antibodies against HDL lipoproteins has been reported in a number of conditions (26). These antibodies are suggested to interfere with HDL functionality in Systemic Lupus Erythematosus (27), but little is known about their role in RA. The results herein presented expand the previous knowledge on the role of anti-HDL antibodies in RA. Our findings revealed that the association between PON1 genetic variants and CVD is dependent on anti-HDL antibodies in RA patients. Moreover, the effect of these antibodies was different among genotypes and it seems to be mediated by a detrimental effect on PON1 activity, this finding being more evident in subjects with genetically low PON1 activity.

Since a growing body of evidence suggest a genetic component in the risk of CVD in RA (reviewed in (28)), the potential association of rs662 variants with CV disease in RA patients was further examined in some studies. Charles-Schoeman and colleagues (29) revealed that high PON1 activity (considered as either RR genotype or serum PON1 activity) protected from subclinical atherosclerosis development in RA. Nevertheless, our

group did not find such an association when this polymorphism was analyzed in a larger cohort (30). However, the findings herein presented could be the key to explain these discrepancies, thereby being anti-HDL antibodies the missing link. Although anti-HDL antibodies were associated with CVD in the whole RA group, when rs662 variants were taken into account, this association only remained in the QQ genotype, even after adjusting for traditional CV risk factors. It is interesting to note that among individuals carrying QQ genotype, only patients with anti-HDL antibodies exhibited an impaired PON1 activity compared to their rs662 QQ healthy referrals, thus highlighting a link between anti-HDL antibodies, PON activity and CVD. This effect was not observed in patients with genotypes associated with higher PON1 activity. All these findings lead us to hypothesize that despite being lower than that of QR or RR genotype, QQ PON1 activity may be adequate to maintain an acceptable level of antioxidant HDL-mediated protection. However, when additional environmental factors which can impair PON1 activity are present, such as decreased HDL levels, increased oxidative stress, enhanced inflammatory response or the presence of blocking anti-HDL antibodies, QQ-determined PON1 activity decreased below a critical level that would render it unable to counteract these traits. Under these circumstances, lipid oxidation and inflammation could promote the CVD development. Actually, loss of PON1 activity in knockout mice is associated with accelerated atherosclerosis (31;32). Conversely, QR or RR individuals would not achieve this "threshold" of PON1 activity because of their natively higher enzyme activity. Interestingly, a study of 280 patients of myocardial infarction found no association of PON1 rs662 polymorphism with MI occurrence in the entire population, but QQ genotype was associated with a higher risk when HDL levels were decreased and a dose-dependent association was observed according to gene dosage (33). These findings are in accordance with our current results, since the presence of anti-HDL antibodies could be associated with lower HDL levels in RA (17), and this effect was stronger in patients harboring the QQ variant. In a similar way, QQ genotype was associated with CV events in smokers (34), smoking being a traditional CV risk factor prone to lipid oxidation (35).

In addition to anti-HDL antibodies, other factors may impact PON1 activity, since decreased PON1 activity was found in patients with established disease, IgG anti-HDL levels and rs662 variant not differing between disease duration groups. It is tempting to hypothesize that accumulated exposure to oxidative stress as a consequence of chronic activation of the immune system in the context of autoimmunity may result in decreased PON1 activity. Actually, oxidization or chlorination of lipoproteins have been described to impair HDL functionality (36). Additionally, disease mechanisms are clearly different in early stages compared to established disease, cytokines exhibiting diverse patterns (37).

Previous findings of our group revealed different associations of cytokines, like IL-8, depending on disease stage, although no differences in levels were observed in some cases (38). IL-8 is known to promote neutrophil chemotaxis and activation, neutrophils being known to promote oxidative stress and enhance lipids oxidization (39) which can in turn enhance the inflammatory response, thus promoting a positive feedback loop. Therefore, a crosstalk between cytokine milieu and oxidative stress could progressively impair HDL functionality. Alternatively, whether chronic exposure to inflammatory burden could impair HDL composition (and thus, functionality) apart from chemical modifications remains unknown (40).

Another important conclusion of our work is the association of anti-HDL antibodies and PON1 activity during anti-TNFα therapy. RA patients with similar degree of disease control, and thus similar level of immunosuppression, may exhibit differences in lipid levels (41). Since disease control can be considered as protective for CV risk in RA (42), these lines of evidence again support the relevance of lipid function rather than levels when HDL protective ability is considered. Popa and colleagues (43) reported that TNFα blockade promotes improvements in PON1 activity, not being strictly related to increasing HDL levels. However, the mechanisms that underlie this finding remained unknown. We found that anti-HDL reduction can independently predict a favorable change in PON1 activity after adjusting by clinical response, acute-phase reactants, change in HDL and genetic variants. Then, TNFα-blockade may be considered as an advisable strategy in patients with genetically determined low PON1 activity, especially in patients with anti-HDL antibodies. Due to the low number of patients included in our prospective group, larger sample size studies covering all the rs662 status and longer-term prospective studies need to be performed.

All these results are of special interest not only from the basic point of view but also for the clinical setting. Currently, the CV risk stratification of RA patients is known to be suboptimal because of the lack of adequate biomarkers (44;45). A combined anti-HDL determination and PON1 rs662 typing would be of outstanding interest in the CV risk stratification of RA patients, as strict both preventive and therapeutic strategies should be focused on those anti-HDL-positive and QQ homozygotes, whereas the clinical meaning of anti-HDL antibodies in QQ or QR RA patients remain to be established. This would provide a better identification of patients at risk and avoid unnecessary interventions in other patients. Similarly, as anti-HDL antibodies can be detected in a small fraction of HC, and some effect was noted on PON1 activity, its potential relevance as CV risk biomarkers needs to be further carefully examined in larger prospective cohort studies in the general

population or in other "at risk" groups, especially in subjects carrying the QQ genotype. Due to the frequency of this genotype, the potential benefit of such an intervention could be considerable. Nevertheless, our findings are based on prevalent CV events, which may represent a limitation of the present study. Therefore, the use of anti-HDL antibodies and rs662 typing for incident CVD prediction needs to be explored in prospective cohorts.

In summary, we report that impaired HDL function is found in RA patients, PON1 rs662 polymorphism and IgG anti-HDL antibodies having a pivotal role. The presence of anti-HDL antibodies in patients with the low activity genotype was correlated with a decreased PON1 function and associated with a higher prevalence of CV events in these patients and related to gene dosage. Interestingly, these associations remain significant after controlling for inflammatory markers, thus pointing to an independent role of anti-HDL antibodies in this scenario. Moreover, decreasing anti-HDL antibodies upon TNFα-blockade independently predicted a favorable change in PON1 activity. To the best of our knowledge, this is the first work that carried out a global analysis of the HDL, anti-HDL and PON1 axis involvement in CV disease occurrence in RA, thereby providing a more real insight into the HDL biology in this condition.

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## AUTHOR CONTRIBUTIONS

All authors have read the journal's policy on disclosure of potential conflicts of interest. All authors were involved in drafting the manuscript or revising it critically for important intellectual content and all the authors gave their approval of the final version of the manuscript to be published. The authors have not financial conflict of interest.

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## TABLES

**Table 1: Demographic and clinical parameters of individuals entered in this study.**

| | HC (n=110) | RA (n=212) | p |
|---|---|---|---|
| Age (years) | 57.79 (16.13) | 53.93 (16.92) | 0.711 |
| Gender (female/male) | 81/29 | 175/37 | 0.060 |
| Total cholesterol (mg/dl) (mean±SD) | 205.55±31.63 | 208.36±35.03 | 0.955 |
| HDL-cholesterol (mg/dl) (mean±SD) | 58.36±14.07 | 60.68±17.58 | 0.535 |
| LDL-cholesterol (mg/dl) (mean±SD) | 126.97±26.59 | 122.60±32.38 | 0.167 |
| Anti-HDL/IgG | 13.87 (38.48) | 35.78 (113.88) | <0.0001 |
| Anti-HDL (+) | 9 (8.1) | 40 (18.8) | 0.009 |
| **Disease features** | | | |
| Disease duration (years) (range) | | 2.75 (0.00 – 30.00) | |
| CRP (mg/dl) | | 1.00 (2.63) | |
| ESR (mm) | | 18.00 (23.00) | |
| DAS28 score | | 3.73 (2.24) | |
| RF (+) | | 119 (56.1) | |
| ACPA (+) | | 121 (57.0) | |
| Age at diagnosis (years) | | 50.00 (17.00) | |
| Shared Epitope (+) (n=141) | | 83 (58.8) | |
| **Treatments, n(%)** | | | |
| None or NSAIDs | | 47 (22.1) | |
| Glucocorticoids | | 103 (48.5) | |
| Methotrexate | | 139 (65.5) | |
| TNFα-blockers | | 48 (22.6) | |
| Tocilizumab | | 12 (5.6) | |
| Statins | | 22 (10.3) | |
| **Traditional CV risk factors, n(%)** | | | |
| Hypertension | | 65 (30.6) | |
| Dyslipidemia | | 52 (34.5) | |
| Diabetes | | 22 (10.3) | |
| Obesity (n=129) | | 29 (13.6) | |
| Smoking | | 74 (34.9) | |
| **History of previous CV events, n(%)** | | 38 (17.9) | |

Data are expressed as median (interquartile range) for continuous variables and n(%) for categorical ones, unless otherwise stated. Differences between patients and controls were assessed by Mann Withney U or chi-square tests, as appropriate.

**Table 2: Logistic regression analyses between anti-HDL antibodies and CV disease in RA patients.**

| | OR | 95% CI | p (univariate) | p (adjusted)ǂ |
|---|---|---|---|---|
| anti-HDL (+) | | | | |
| All patients | 4.485 | 2.066 – 9.735 | <0.001 | <0.001 |
| QQ (n=96) | 14.600 | 4.003 – 53.254 | <0.0001 | 0.0004 |
| QR (n=68) | 1.500 | 0.391 – 5.752 | 0.554 | 0.615 |
| RR (n=22) | 17.000 | 1.021 – 283.011 | 0.060 | - |

ǂ Adjusted by age, gender and traditional CV risk factors in a multivariate model (not performed in RR individuals because of the low sample size obtained).

## FIGURE LEGENDS

**Figure 1: PON1 activity in RA patients and healthy controls.** PON1 activity (enzyme units) was quantified in serum samples from RA patients and healthy controls. Differences were analyzed between the whole groups (A) and according to PON1 rs662 polymorphism (B) (HC: white bars, RA: gray bars) using Mann-Withney U tests. Sample size for each group is indicated between parentheses.

**Figure 2: PON1 activity and anti-HDL antibodies according to PON1 rs662 status.** PON1 activity was compared among HC (white bars), RA patients without (clear gray) and with anti-HDL antibodies (dark gray) stratified according to PON1 rs662 polymorphism. Differences were assessed by Kruskal Wallis and Dunn's post hoc tests, and only comparisons found to be significant are indicated (p-values from Dunn's post hoc tests). Sample size for each group is indicated between parentheses (total of individuals genotyped: HC: n=105, RA n=186).

**Figure 3: PON1 activity and anti-HDL antibodies upon TNFα blockade.** (A) PON1 activity was compared between at baseline and 3 months after initiation of anti-TNFα therapy in 13 biologic-naïve RA patients. (B) Change in PON1 activity upon TNFα blockade was associated to that of anti-HDL antibodies. Spearman rank's test was performed.

SUPPLEMENTARY MATERIAL

**Supplementary Table 1: Clinical parameters of RA patients classified according to disease duration groups**

| | Onset (n=47) | Established disease (n=165) | p |
|---|---|---|---|
| **PON1 rs662 status** | | | |
| QQ/QR/RR (n) | 25/12/5 | 71/56/17 | 0.448 |
| **Disease features** | | | |
| Disease duration (years) (range) | 0.00 (0.00 – 0.25) | 3.42 (0.50 – 30.00) | <0.0001 |
| CRP (mg/dl) | 1.10 (3.57) | 0.98 (2.50) | 0.090 |
| ESR (mm) | 26.00 (31.00) | 15.00 (21.75) | 0.128 |
| DAS28 score | 5.10 (1.98) | 3.59 (1.81) | <0.0001 |
| RF (+) | 26 (55.3) | 93 (56.3) | 0.646 |
| ACPA (+) | 27 (57.4) | 94 (56.9) | 0.915 |
| Age at diagnosis (years) | 52.08 (20.00) | 49.08 (14.00) | 0.031 |
| Shared Epitope (+) (n=141) | 17 (60.7) | 66 (58.4) | 0.983 |
| **Treatments, n(%)** | | | |
| None or NSAIDs | 47 (0.0) | 0 (0.0) | - |
| Glucocorticoids | | 103 (62.4) | - |
| Methotrexate | | 139 (84.2) | - |
| TNFα-blockers | | 48 (29.0) | - |
| Tocilizumab | | 12 (7.2) | - |
| Statins | 2 (4.2) | 20 (12.1) | 0.689 |
| **Traditional CV risk factors, n(%)** | | | |
| Hypertension | 13 (27.6) | 52 (31.5) | 0.638 |
| Dyslipidemia | 7 (14.8) | 45 (27.2) | 0.065 |
| Diabetes | 4 (8.5) | 18 (10.9) | 0.655 |
| Obesity (n=129) | 6 (12.7) | 23 (13.9) | 0.359 |
| Smoking | 19 (40.4) | 55 (33.3) | 0.105 |
| **History of previous CV events, n(%)** | 0 (0.0) | 38 (23.0) | - |

Data are expressed as median (interquartile range) for continuous variables and n(%) for categorical ones, unless otherwise stated. Differences between patients and controls were assessed by Mann Withney U or chi-square tests, as appropriate.

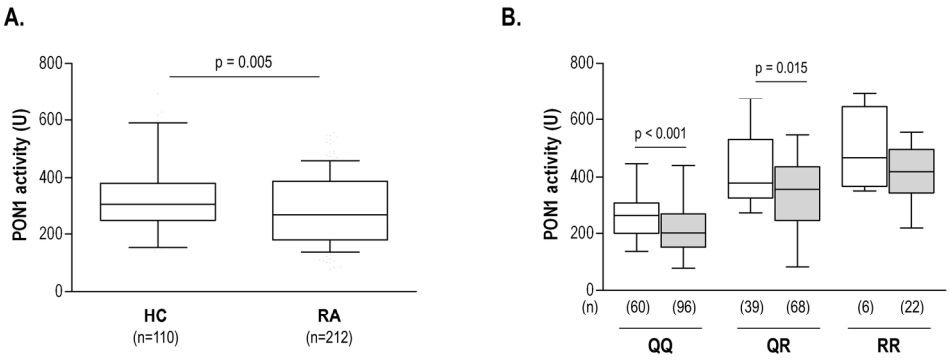


Figure 1
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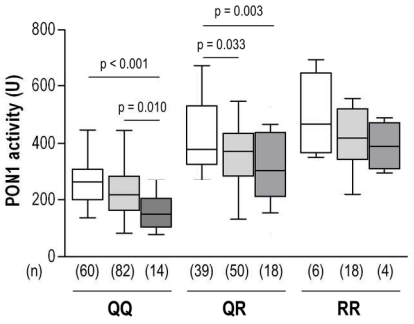


Figure 2
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