Anti-carbamylated protein autoantibodies associated with mortality in Spanish rheumatoid arthritis patients

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Abstract

Patients with rheumatoid arthritis (RA) have an increased mortality rate that is associated with the presence of RA-specific autoantibodies in many studies. However, the relative role of rheumatoid factor (RF), anti-CCP antibodies and the most recently established RA-autoantibodies, directed against carbamylated proteins (anti-CarP antibodies), is unclear. Here, we have assessed the role of these three antibodies in 331 patients with established RA recruited from 2001 to 2009 and followed until November 2015. During this time, 124 patients died (37.5%). This death rate corresponds to a mortality rate 1.53 (95% CI 1.26 to 1.80) folds the observed in the reference population. We used for analysis of all-cause mortality the Cox proportional hazard regression model with adjustment for age, sex and smoking. It showed a trend for association with increased mortality of each of the three RA autoantibodies in antibody-specific analysis (hazards ratio (HR) from 1.37 to 1.79), but only the HR of the anti-CarP antibodies was significant (HR = 1.79, 95% CI 1.23 to 2.61, p = 0.002). In addition, the multivariate analysis that included all autoantibodies showed a marked decrease in the HR of RF and of anti-CCP antibodies, whereas the HR of anti-CarP remained significant. This increase was specific of respiratory system causes of death (HR = 3.19, 95% CI 1.52 to 6.69, p = 0.002). Therefore, our results suggest a specific relation of anti-CarP antibodies with the increased mortality in RA, and drive attention to their possible connection with respiratory diseases.

Introduction

Rheumatoid arthritis (RA) is an autoimmune systemic disease that affects primarily diarthrosis (joints with a wide range of movement and covered with synovial tissue) with inflammation, pain, disability and deformity [1,2]. It can also include extra-articular manifestations involving the lungs, the blood vessels or the skin. Other characteristics of the disease are systemic
inflammation and alterations of the immune system, including the production of specific auto-
antibodies. RA is also associated with a notable increase in the death rate that has been quanti-
fied at about double the standard mortality rate of the population [3–6]. This increase has dire 
consequences as it shortens the life expectancy of patients with RA by about 10 years. The 
excess mortality is due to multiple comorbidities, which include cardiovascular and cerebro-
vascular diseases, infections, lymphoma, and gastrointestinal diseases, as well as, a group of 
less common causes of death [3,4,6,7]. The mechanisms leading to increased mortality are not 
completely understood, although inflammation is associated with cardiovascular disease, and 
the cumulative burden of disability, decrepitude, pain and treatment side effects is suspected 
to cooperate with other comorbidities [3,4,7,8]. Accordingly, mortality is associated with RA 
disease activity and severity. These associations could, in turn, account for the increased mor-
tality observed in patients with RA-specific autoantibodies [3,4,6,7,9–13], because they show a 
more severe disease [14–17]. However, a more direct effect of the anti-CCP antibodies could 
be also involved [18].

The first identified RA-specific autoantibody is rheumatoid factor (RF) [1,2]. It consists of 
IgM antibodies directed against the Fc portion of IgG. It is present in about 70% of the RA 
patients, but also in a small fraction of patients with other inflammatory diseases or healthy 
subjects. More recently, several antibodies directed against post-translational modifications of 
proteins have been identified as RA-specific. The first antibodies of this type that were charac-
terized recognize citrullinated proteins. They are analyzed as anti-cyclic citrullinated peptide 
antibodies (anti-CCP). These antibodies are very specific of RA, participate in its pathogenesis 
and are useful diagnostic, prognostic and treatment biomarkers [1,2]. Subsequently, the anti-
carbamylated protein antibodies (anti-CarP) were discovered [19–23]. They are rarely assessed 
because we do not have yet a standardized assay for these antibodies. However, there is already 
evidence of their involvement in RA pathogenesis and of their possible utility as biomarkers.

As mentioned, the presence of RA autoantibodies has been associated with mortality in 
multiple studies [3,4,6,7,9–13]. However, most studies have included only one antibody or 
have not compared their relative roles. In the minority of reports comparing antibodies, the 
results are often discordant [9–13]. This heterogeneity of results is evident for RF and anti-
CCP antibodies, which are the most frequently analyzed. The relative association with mortal-
ity has been reported as a significant association that is exclusive or dominated by the presence 
of RF [9,12,13], or the reverse, a significant association dominated by the presence of anti-CCP 
antibodies [11], or in between, with the two antibodies contributing additively to a significant 
association [10]. A similar situation was observed in the single study addressing the anti-CarP 
antibodies, where the relative weight of the antibodies was different in each of the three patient 
cohorts, one showed an association with mortality dominated by RF, a second cohort an associa-
tion dominated by anti-CarP antibodies, and in the third the association with mortality 
involved the additive contribution of the three antibodies [9].

With these antecedents and considering the need of more information, we have addressed 
the relative association of the three RA autoantibodies with mortality in our RA patients. In 
particular, we wanted to address the anti-CarP antibodies, as the less studied of the three anti-
bodies. In addition, we were specifically interested in testing if the associations with the differ-
ent autoantibodies were statistically different, as opposed to simply of different strength, and if 
they showed an additive effect irrespective of the autoantibody type. After completing the anal-
ysis, we have found that only the anti-CarP antibodies were significantly associated with mor-
tality, but this association was not statistically different from the trends observed with the 
other two autoantibodies. In addition, the anti-CarP antibodies association with mortality was 
dominant over the association with the number of antibodies, and showed specificity for 
Deaths attributed to respiratory diseases.
Materials and methods

We have included patients with established RA meeting the 1987 American College of Rheumatology classification criteria in this study [24]. Their recruitment took place from 2001 to 2009 at the Rheumatology Unit in our Hospital. We also obtained samples, demographic data and smoking information, together with the written informed consent. The Ethics Committee for Clinical Research of Galicia approved the study. All patients were Spanish Caucasians. Information on survival since recruitment until November 2015 was collected from the electronic medical records of the patients, which are kept across all levels, from primary care to tertiary level hospitals, of the public and private health centers of Galicia in the IANUS system [25]. The last entry for each patient was the date of death or of the last follow-up visit. Causes of death were obtained from the same IANUS system in 68 patients and from the Galician Death Registry in the remaining 56 patients according with the International Classification of Diseases (ICD) 9 and 10 revisions. They were grouped in four categories: circulatory system, respiratory system, neoplasms and other, which included the remaining causes of death of less frequency.

Autoantibodies were determined in serum samples taken at the time of recruitment. IgM RF was measured by rate nephelometry (IMMAGE Immunochemistry System; Beckman Coulter). Anti-CCP antibodies were assayed by ELISA (Euro-Diagnostica, Malmö, Sweden) applying a cutoff level of 5 units/ml. Anti-CarP antibodies were assessed by homemade ELISA with in vitro carbamylated fetal bovine serum following the protocol described by Shi et al. [19], as we have previously reported in detail [23]. The cutoff level was determined using 208 healthy controls at 138.61 arbitrary units against serial dilutions of a pool of positive sera.

We calculated standardized mortality ratios (SMR) of the patients with RA relative to the sex- and age-specific mortality rates observed in the province of A Coruña, where most of the patients in this cohort have their residence. The year of reference was 2008, the mid-year of follow-up. Mortality rates of the population were obtained from the Spanish National Institute of Statistics that provides detailed actuarial tables [26]. For the association analysis, we used Cox proportional hazard regression models for all-cause mortality or for specific causes of death considering age, sex and smoking status as covariates. Hazard ratios (HR) of RF, anti-CCP and anti-CarP positivity were obtained in antibody-specific and, antibody-combination models. In addition, number of autoantibodies and the logarithm-transformed antibody titers were considered in separate analyses. For graphical representation, we obtained survival curves with the Kaplan-Meier product limit analysis, which is a univariate analysis (not adjusted for any confounding variable). In addition, proportional Venn diagrams were drawn with eulerAP [27]. Finally, concordance in status between antibodies was assessed with the Goodman and Kruskal gamma ($\gamma$). All analyses were done with Statistica 7.0 (Stat Soft, Inc).

Results

The 331 patients included in this study were recruited from the outpatient Rheumatology clinic without any selection except fulfilling the ACR classification criteria. They showed characteristics of established RA as shown in Table 1, with an excess of women, advanced age and long time since the start of the first disease symptoms (Data in S1 Table). They were also typical in the fraction showing RA-specific autoantibodies: RF = 60.1%, anti-CCP = 64.7% and anti-CarP = 32.9%, and in the overlap between them (Fig 1), corresponding with concordances of $\gamma = 0.82$ between anti-CCP and RF, 0.66 between anti-CCP and anti-CarP, and 0.64 between RF and anti-CarP (p for all of them $< 2.0 \times 10^{-16}$). The frequency of ever smokers was low (Table 1), but characteristic of the smoking habit in the area covered by our hospital. Smoking
showed a notable difference between women and men (5.3% of the women, 95% CI 3.1 to 8.9%, vs. 52.3% of the men, 95% CI 41.9 to 62.6%).

Recruitment was done from 2001 to 2009 and survival data were collected until November 2015. This corresponds to a mean follow-up of 9.0 years (SD = 3.5 years) and to 2991 person-years of survival data. In this period, 124 patients have died (Table 1), which correspond to a crude mortality rate of 41.5 ‰ (95% CI 34.9 to 49.3 ‰). This number of deaths reflects an increased mortality as shown by the SMR of 1.53 (95% CI 1.26 to 1.80), which is the excess observed mortality among the patients relative to the observed in the reference population. The main causes of death (individual level supporting information provided in S1 Table) were diseases of the circulatory and of the respiratory system (36 deaths in each), followed by neoplasms (18 deaths) and infections (13 deaths).

Known risk factors were associated with increased mortality in the univariate analyses (Fig 2), old age (HR = 1.13 expressed in years, 95% CI 1.11 to 1.16, p = 2.5 x 10^{-24}), smoking (HR = 1.59, 95% CI 1.04 to 2.42, p = 0.03) and male sex (HR = 1.56, 95% CI 1.07 to 2.27, p = 0.02). However, only old age and smoking remained significant in multivariate analysis (old age HR = 1.15, 95% CI 1.12 to 1.18, p = 8.6 x 10^{-25}; smoking HR = 2.66, 95% CI 1.58 to 4.48, p = 0.0002; and male sex HR 0.88, 95% CI 0.56 to 1.37, P = 0.6). In contrast, bone erosions (HR = 0.81, 95% CI 0.58 to 1.22, p = 0.4), year of recruitment (HR = 0.98, 95% CI 0.87 to 1.09, p = 0.7) and duration of RA since symptom onset accounting for differences in age (HR = 1.01, 95% CI 0.99 to 1.02, p = 0.2) were not associated with increased deaths. Most patients have already experienced RA before the arrival of the biologic drugs of increased efficacy, around the year 2000, and no differences in HR were observed between those starting before and after this year (HR = 0.94, 95% CI 0.52 to 1.71, p = 0.9).

In the antibody-specific analyses, only the anti-CarP antibodies showed a significant association with decreased survival (Fig 3). This result from the unadjusted analysis was reflected in increased HR for anti-CarP positive patients (HR = 1.79, 95% CI 1.23 to 2.61, p = 0.002), with age, sex and smoking as covariates in the adjusted analysis. RF and anti-CCP positive status showed a trend to higher HR in similarly adjusted analyses, but none of them was statistically significant (HR = 1.37, 95% CI 0.94 to 2.00, p = 0.10; and HR = 1.38, 95% CI 0.93 to 2.04, p = 0.11, respectively).

The differences between the HR observed with the three antibodies were not significantly different (Fig 4A). However, in a conditional analysis considering the status for the three antibodies (Fig 4B), the trend showed by RF and anti-CCP was notably decreased (HR = 1.11 and
1.05, respectively), whereas the HR of the anti-CarP antibodies remained significantly increased (HR = 1.69, 95% CI 1.11 to 2.58, p = 0.01).

In addition, we assessed the number of positive autoantibodies, either 0, 1, 2 or 3. This variable showed a significant association with mortality (HR = 1.25, 95% CI 1.06 to 1.48, p = 0.008), but it was dependent on the presence of the anti-CarP antibodies, not on the contribution of the other two autoantibodies. These results were obtained by conditional analysis: the number of autoantibodies did not remain associated in analysis conditional on anti-CarP status (HR = 1.08, 95% CI 0.84 to 1.39, p = 0.6), whereas it remained significantly associated, and was nominally more marked, conditional on RF, or on anti-CCP status (Fig 5). Finally, we addressed the autoantibody titers, which were only available for the anti-CCP and anti-CarP antibodies. None of them was associated with a higher HR of death.

When the specific causes of death were considered, only the deaths attributed to diseases of the respiratory system were associated with the anti-CarP antibodies (HR = 3.19, 95% CI 1.52 to 6.69, p = 0.002). This HR was considerably larger than the observed in the all-cause of mortality analysis reported above. The respiratory system group of causes of death included 16 pneumonias and other respiratory infections, 8 interstitial lung diseases that could be attributed to RA, 3 respiratory insufficiencies attributed to COPD, and 9 patients with only disease group level information corresponding to the J00-J99 codes of the ICD10, which we received without detailing. The small number of patients with each disease did not allow to distinguish if any of them was disproportionately contributing to the increased association. Even so,

Fig 1. Distribution of patients with RA positive for the different autoantibodies. A proportional Venn diagram representing percentages (%) of patients in each of the antibody strata: anti-CCP positive in the lower left oval, RF positive in the lower right oval and anti-CarP positive in the upper one. Percentage of triple negatives is shown outside the Venn diagram in the upper left corner.

https://doi.org/10.1371/journal.pone.0180144.g001
Fig 2. Kaplan-Meier survival curves of all patients with RA stratified by demographic factors. (A) Stratified by age in relation with the median age at the time of recruitment (red > 69 years, blue < 69 years); (B) stratified by smoking habit (red = ever smoker, blue = never smoker), and (C) stratified by gender (red = men, blue = women). Dots = deaths, crosses = censored data.

https://doi.org/10.1371/journal.pone.0180144.g002
Fig 3. Kaplan-Meier survival curves of all patients with RA stratified by the status of the RA specific antibodies. (A) anti-CarP antibodies, (B) RF, and (C) anti-CCP antibodies. Red = antibody positive, blue = antibody negative, dots = deaths, crosses = censored data. Note that these are unadjusted analyses.

https://doi.org/10.1371/journal.pone.0180144.g003
patients with interstitial lung disease showed a nominally increased frequency of anti-CarP antibodies (62.5%). Apart from respiratory diseases, the deaths attributed to neoplasms also showed a trend to increased HR in patients with anti-CarP antibodies (HR = 2.45, 95% CI 0.93 to 6.43, p = 0.07). In contrast, the other two RA autoantibodies did not show association with any of the analyzed causes of death.

**Discussion**

Here, we have found that the increased mortality of patients with RA was associated with the presence of anti-CarP antibodies. This association was the only significant among the autoantibodies and clearly dominant over the trends observed with RF and anti-CCP antibodies. In addition, the anti-CarP antibodies were specifically associated with deaths attributed to respiratory diseases.

Only a previous study has analyzed the relation of the three specific RA antibodies with mortality [9]. It showed dominant associations of the anti-CarP antibodies in one of the three cohorts included, but of RF in other and an additive contribution in the remaining cohort. The authors attributed the different outcomes between cohorts to variation in the antibody assays or to heterogeneity in the cohort inclusion settings. These are plausible explanations, although their influence has never been tested directly. The assay method of anti-CarP antibodies is particularly prone to variation between laboratories as not any standardized antigen or ELISA is yet available [19,23]. Also, genetic and environmental factors could have an influence, as already observed in studies of antibodies against specific citrullinated peptides, where large differences in the frequencies of SE alleles and smoking habit between RA sample collections from Sweden, the Netherlands, the UK and Spain, among other countries, have been reported [28–30]. An additional difference between the patients included in Ajeganova et al. and our patients is that the first were early RA patients, whereas our patients were of established RA. This difference means that our patients were older and, likely, suffered of more
**comorbidities.** This range of factors could also explain the discrepant results observed in the more numerous studies addressing the relative importance of the anti-CCP and RF association with mortality [9–13]. As mentioned in the introduction, a variety of results has been observed, from the dominant association with RF [9,12,13] to the opposite dominant role of anti-CCP [10,11]. The variability in the relative roles of the RA autoantibodies is not exclusive of mortality, it extends to many other patient characteristics [14,15]. A clear example of recent interest is the controversy over the relative association of anti-CCP antibodies and RF with bone erosions [14,31]. A notable factor in all these conflicting results is the high concordance of status between the antibodies [20,21,23], which likely is a consequence of the epitope spreading that is part of the immune dysregulation leading to RA [22,32,33]. This concordance was observed in our study (Fig 1). It means that only a fraction of the patients, only those showing discordant status, are informative for establishing the relative importance of their association with mortality or any other trait. These considerations invite to a circumspect consideration of the relative importance of the autoantibodies until more evidence becomes available. We think this applies also to our results in spite of the clear dominance of anti-CarP antibodies.

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**Fig 5. Conditional analysis of the HR observed with the number of autoantibodies.** The HR for the number of antibodies (# Ab) obtained in Cox proportional analysis was considered either on itself (to the left of the dotted vertical line) or conditional on the presence of each of the three specific autoantibodies (to the right of the dotted vertical line). The asterisks, *, denote significantly increased HR.

https://doi.org/10.1371/journal.pone.0180144.g005
Less abundant are the studies addressing the association of RA autoantibodies with specific causes of death. Only the already mentioned study of Ajeganova et al. has addressed this question with the three antibodies considered here [9]. It showed mortality by respiratory diseases and neoplasms associated with RF and mortality by circulatory system diseases associated with anti-CCP antibodies. Other studies have considered the association of causes of death in sero-positive patients, but not in a comparative way [6,34–38]. The most common finding has been association with deaths by cardiovascular diseases [34–38], although deaths by respiratory diseases were associated with RF in at least four studies [6,9,34,35]. Therefore, the association of RA autoantibodies with death due to respiratory disease has some support in the bibliography, but the association with the anti-CarP antibodies is unprecedented and should be regarded with caution until replicated. In addition, there are particular limitations that affect any analysis of causes of death and that invite to additional prudence. They include the difficulty to establish the cause of death in many patients, and the grouping of the different causes of death by a mixture of pathogenic mechanisms and organ systems as in the ICD coding [39–41].

The association of increased mortality with the RA autoantibodies could be relevant for two aspects of clinical importance: to help elucidate the mechanisms leading to increased mortality, and to act as a useful biomarker identifying patients at increased risk. Unfortunately, there is not yet clear indication of the antibodies value in any of the two aspects. Regarding the possible mechanisms, there are several hypotheses, but none of them is established [3–8]. As mentioned in the introduction, excess mortality is attributed to a wide array of comorbidities, from cardiovascular to gastrointestinal diseases, and from infections to cancer. This wide array of death causes suggests mechanisms involving decreased overall health, as could result from the burden of inflammation, disability, pain and drug adverse events. Most likely, this is not enough. Together with the overall detrimental effect, there are pieces of evidence identifying mechanisms more directly related to specific causes of death. They include the effect of chronic inflammation in reducing the threshold for atherosclerosis and coronary artery disease [42]; the disorganized immune system as the cause of the increased infections, which could be also exacerbated as a consequence of corticosteroid treatments and other immune modulator drugs [43,44]; and the chronic stimulation of lymphocytes and, again, the dysregulated immune system behind the increased frequency of lymphoma and other cancers. The presence of autoantibodies has often been considered in generic terms, as a feature correlating with a more aggressive or severe disease. This participation is applicable to any of the three RA antibodies because the three correlate with severe RA [1,2,14,15,17]. Perhaps, a more direct role of the antibodies in the increased mortality could be behind the association. First evidences in this direction were reported recently for anti-CCP antibodies in the atheroma plaques [18]. However, some of the findings supporting this mechanism have been contradicted in a subsequent study by our group [45]. Therefore, we still do not have a clear indication of what could be the mechanisms linking the RA autoantibodies with mortality. This applies also to the anti-CarP antibodies. No particular mechanism has been yet explored for them, but as suggested in the single study considering this type of antibodies [9], carbamylated proteins indicate a possible relationship. In effect, carbamylated proteins are increased in patients with chronic kidney disease, in whom they are associated with atherosclerosis and with cardiovascular and all-cause mortality [46,47]. However, we did not know yet whether the levels of protein carbamylation are increased in RA patients or in patients with anti-CarP antibodies, and our results did not show an increase of mortality by circulatory system diseases. An alternative mechanism is suggested by the association of anti-CarP antibodies with diminished lung function measured as FEV1 and with *Pseudomonas aeruginosa* infection in patients with cystic fibrosis [48]. This association was attributed to the role of anti-CarP antibodies as marker of neutrophil-driven bronchial inflammation. This interpretation could also pertain to RA because
signs of bronchial inflammation are common in seropositive patients with RA [49,50], and even in subjects at risk of RA that have RA autoantibodies [51]. In addition, plasma cells isolated from inducible bronchus-associated lymphoid tissue (iBALT) produce RA autoantibodies [52].

Regarding the possible value of the RA antibodies as biomarkers of increased mortality risk, there are not yet any studies. However, the anti-CarP association with mortality was in the same order of magnitude than smoking in our patients, making it plausible that they could be used in preventive strategies.

Interpretation of our results should reckon some limitations. One that is shared with other studies on this matter is the partial follow-up of the patients. Ideally, all patients should be followed since disease onset to account for all deaths that could be related with RA [3,7]. However, this approach was unfeasible in our case, because both the sample size and the death rate will be much decreased if only new RA patients were included. In addition, confidence in our results is supported by considering that the increase in RA mortality does not take place until several years, about 10 years, after disease onset [7,53]; and because results from established RA patients are similar to the reported for inception cohorts, except for a higher SMR in the established patients [7].

Conclusions
The anti-CarP antibodies, which are a new type of RA autoantibodies, were associated with increased mortality in Spanish patients with RA, and this association was dominant over the other RA-specific autoantibodies. In addition, it showed specificity for deaths due to respiratory diseases. However, these results require further validation because the relative roles of the different RA autoantibodies are difficult to disentangle due to their high concordance in patients.

Supporting information
S1 Table. Excel table with data from individual patients used in the analysis of survival. (XLS)

Acknowledgments
The authors are indebted to the patients that generously have contributed the samples and time to this work. They thank Carmen Pena for her excellent technical support. Funding was provided by the Instituto de Salud Carlos III (Spain) through grants PI14/01651, PI12/01909 and RD12/0009/0008, which are partially financed by the European Regional Development Fund of the EU (FEDER).

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