Effect of Etanercept on Anti-Carbamylated Protein Antibodies in Patients with Rheumatoid Arthritis

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Abstract
Rheumatoid Arthritis (RA) is a systemic, chronic inflammatory disease characterized by inflammation of synovial joints and production of autoantibodies such as Rheumatoid Factor and antibodies directed against modified proteins - i.e. anti-citrullinated peptides antibody (ACPA). Carbamylation, as a post translational modification, has been recently associated to RA since anti-carbamylated protein antibodies (anti-CarP) have been detected in the sera of RA patients. The effect of treatment on anti-CarP level has been never addressed before.

Through this study we aimed to investigate the short term effect of anti-TNF treatment with Etanercept on anti-CarP. We enrolled consecutive RA patients before starting treatment with Etanercept. Clinical data and serum samples were gathered from each patient at baseline and after 3 months of treatment. Disease activity was assessed at baseline and after 3 months by using the C-reactive protein - Disease Activity Score (DAS) 28. Sixty-three age and sex matched healthy donors served as controls. Anti-Car-P antibodies were investigated by immune-enzymatic assay.

We enrolled 17 RA patients (F:M 15:2, mean age 44.1 ± 10.7 years, mean disease duration 7.9 ± 5.8 years). Six patients (35.3%) were positive for anti-CarP antibodies at baseline while three months after only 4 patients (23.5%) remained positive. Mean serum level of anti-CarP antibodies at baseline and after 3 months were: 253.0 ± 139.8AU/ml and 271.0 ± 132.4AU/ml respectively. Considering the persistently anti-CarP positive patients, the mean antibody titre increases from 386.2 ± 49.3AU/ml at baseline to 421.8 ± 144.0AU/ml at follow up.

The effect of anti-TNF treatment on autoantibody status is still controversial; in particular, data on ACPA variation during treatment are discordant. In our cohort of long standing RA patients, a short term course of Etanercept did not affect the anti-CarP status.

In conclusion, this pilot study demonstrated a slight reduction in the percentage of anti-CarP positive patients but an overall increase of antibody titre unrelated to the clinical response to TNF blockade was observed.

Keywords
Rheumatoid arthritis, Anti-TNF, Anti-carbamylated protein antibodies

Introduction
Rheumatoid Arthritis (RA) is a systemic, chronic inflammatory disease mainly affecting the synovial joints, leading to cartilage and bone erosion. A distinctive feature of RA is the production of autoantibodies such as Rheumatoid Factor (RF) and antibodies directed against modified proteins - i.e. anti-citrullinated peptides antibody (ACPA).

Post-translational modifications encompass several enzymatic or chemical reactions that modify and modulate protein functions. Among the others, carbamylation of proteins, leading to the production of homocitrulline, have been recently associated to RA. Anti-carbamylated proteins (anti-CarP) antibodies have been proposed as an additional biomarker of RA, especially in seronegative subjects and even in pre-clinical stage. As previously demonstrated for ACPA [1], anti-CarP antibodies seems to have a prognostic role since their presence is associated to a more severe and aggressive disease course [2].

The effect of treatment of anti-CarP in RA patients has never been addressed up to now. In the present study our objective was to investigate the short term effect of anti-TNF treatment on anti-CarP serum levels.

Patients and Methods
Consecutive RA patients who were due to start treatment with Etanercept were enrolled. Patients with known cardiovascular disease (CVD) or kidney failure were excluded. Serum samples were collected at baseline and after 3 months of therapy, aliquoted and stored at -20°C until tested.

Disease activity was assessed at baseline and after 3 months by C-reactive protein (CRP) - Disease Activity Score (DAS)28.

Anti-CarP antibodies were investigated by using commercial ELISA as previously reported [2] using carbamylated Fetal Calf Serum (FCS) (Car-FCS) and unmodified FCS as antigens. A titration curve of two positive reference sera with medium-high ELISA immunoreactivity for anti-Car-FCS was carried out in order to show the performance of the test and to transform the absorbance of Car-FCS to arbitrary units per milliliter (aU/mL). The cut-off was established as the mean...
The objective of this study was exploratory and no appropriate information to base a sample size was available. Data presented a normal distribution and were expressed as mean ± standard deviation. Differences in quantitative variables were analysed by the Student’s t test while Pearson’s correlation coefficient was used to assess correlation between other variables. P values <0.05 were considered statistically significant.

Results

In this pilot study we enrolled 17 patients with long standing RA fulfilling the 2010 ACR criteria [3]. Concomitant therapy remained stable throughout the 3 months of follow-up in all the patients. Table 1 reports demographic and clinical data of the cohort.

At baseline, 6 patients (35.3%) were positive for ant-CarP antibodies; at follow-up, 4 patients were still positive (23.5%) while 2 patients turned negative. Mean serum level of anti-CarP antibodies in the whole cohort was 253.0 ± 139.8 AU/ml at baseline and 271.0 ± 132.4 AU/ml at the third month of Etanercept treatment. (Figure 1). Considering the sub-population of anti-CarP positive patients, the mean antibody titre was 386.2 ± 49.3 AU/ml at baseline and increased to 421.8 ± 144.0 AU/ml after 3 months (Figure 1). After 3 months of anti-TNF treatment with Etanercept we detected a significant decrease of DAS28 (5.14 ± 1.3 to 3.73 ± 1.3, p=0.003).

At baseline, anti-CarP positive patients presented with a high disease activity (DAS28=5.9 ± 1.2). All the 4 patients persistently positive for anti-CarP showed a clinically meaningful reduction of DAS28 (-2.8 ± 2.3) and were considered EULAR moderate responders; one of them was considered in remission, another one showed a low disease activity and the remaining two a moderate disease activity. We did not find any correlation between anti-CarP serum level and DAS28 at baseline nor at follow-up.

Discussion

This pilot study is the first attempt to investigate a possible effect of anti-TNF treatment on anti-CarP status. A short term course of Etanercept seems not to affect antiCarP serum levels in a small cohort of RA patients.

Post-translational modifications of self-antigens are responsible for the formation of neo-epitopes that give rise to (auto-)antibodies formation. Besides RF and ACPA, anti-CarP antibodies have recently gained interest as additional diagnostic and prognostic marker of RA. Carbamylation is a post-translational modification in which cyanate binds to amino or thiol groups; this modification is determined by increase of blood urea nitrogen levels (i.e. kidney failure), inflammation [4] or enzymatic conversion of lysine residues into homocitrulline [5].

Recently, Shi et al. [2] started to investigate the immunogenicity to homocitrullinated peptides in RA. Antibodies to carbamylated proteins were detected both in ACPA positive and ACPA negative patients suggesting that anti-citrullinated peptide and anti-homocitrullinated peptides are two separate families of autoantibodies; however, the authors did not definitively exclude

| Table 1: Demographic and clinical data of the 17 RA patients |
|-------------|-----------|
| F:M         | 15:2      |
| Age         | 44.1 ± 10.7 (28-63) |
| Disease duration | 7.9 ± 5.8 (2.5-23) |
| BMI mean ± SD | 27.3 ± 12.3 |
| Smoking habit N(%) | 3/17 (17.6) |
| Glucocorticoids N(%) | 17/17 (100) |
| Methotrexate N(%) | 12/17 (70.6) |
| Hydroxychloroquine N(%) | 2/17 (11.8) |
| Leflunomide N(%) | 1/17 (5.9) |

RA = Rheumatoid Arthritis. The line indicate the cut-off value for anti-CarP positivity (340 AU/ml).

Figure 1: Variation of anti-CarP serum levels during Etanercept treatment in the whole cohort of patients with Rheumatoid Arthritis (left) and in the sub-group of positive patients (right).

value + 3 standard deviation of 63 healthy donors.

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obtained from all the patients for being included in the study. Informed consent was obtained according to the standards on human experimentation.

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**Ethical Statement**

All procedures of the study were conducted following the ethical standards on human experimentation. Informed consent was obtained from all the patients for being included in the study.