

## TITLE PAGE

**Title:** Anti-CD26 autoantibodies are involved in rheumatoid arthritis and show potential clinical interest

**Running head:** Anti-CD26 autoantibodies in RA

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63 **ABSTRACT**  
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65 **Objectives:** Rheumatoid arthritis (RA) patients show low serum levels of the Ag dipeptidyl  
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peptidase IV (DPP-IV/CD26), both soluble CD26 (sCD26) concentration and its DPP-IV  
activity. The aim of this study was to test if anti-DPP-IV/CD26 Abs (Anti-CD26) cleared  
sCD26.

**Design & Methods:** Serum Anti-CD26 and Total titers (as comparison) of isotypes IgA, IgM  
and IgG as well as sCD26 concentration and DPP-IV activity were measured in a cohort of  
RA patients undergoing different biological and non-biological therapies (n=105) and  
controls (n= 50).

**Results:** Anti-CD26 levels were increased approximately two-fold for each isotype in RA,  
were not related to the sCD26 clearance, showed several correlations with disease activity  
parameters, were significantly higher in smokers and they were not ACPA. Anti-CD26 Igs  
showed high diagnostic power (82% sensitivity and 96% specificity) and their levels differed  
amongst the different groups of patients stratified by the type of therapy.

**Conclusions:** As DPP-IV/CD26 is an associated to factors triggering RA in the lung and  
periodontal tissue, these results suggest that Anti-CD26 isotypes may participate in  
pathogenesis and may be useful as biomarkers for earlier diagnosis and/or precision medicine.

**Key words:** Autoantibody(ies), Biomarkers, Early diagnosis, Precision medicine, CD26,  
Rheumatoid Arthritis,

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123 **1. Introduction**  
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127           Investigations on anti-citrullinated protein antibodies (ACPA) have revealed  
128 information about the antigen specificity that initiates or perpetuates inflammatory  
129 autoimmune reactions before rheumatoid arthritis (RA) disease onset. For example, aberrant  
130 post-translational modifications of self-proteins such as citrullinated vimentin, type II  
131 collagen, fibrinogen and alpha enolase do play a role in breaking T and B cell tolerance [1,2]  
132 outside the joints. Lungs, lymph nodes and gingival tissues were identified as places where  
133 RA pathogenesis starts [3-7]. Among the environmental factors triggering autoimmunity in  
134 RA, tobacco induces citrullination of proteins in the lung and ACPA production in genetically  
135 predisposed subjects [1-12]. An additional trigger of autoimmunity is the link between  
136 periodontal or gingival disease and RA. In this case, the external agent is the oral microbiome  
137 with *Porphyromonas gingivalis* as the main (but not unique) suspect [29]. *P. gingivalis* is one  
138 of few bacteria that express deiminase, the enzyme that produces citrulline, so may play a  
139 pathogenic role in ACPA-positive RA patients [30].  
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155           However, clinical data of serological biomarkers such as concentration of ESR, CRP,  
156 the presence of ACPA and/or rheumatoid factor or other auto-Abs [11-13], are not enough for  
157 this very early detection. For example, sensitivity of ACPA is about 75% in established RA  
158 compared with 55% in early RA and 40% in very early RA [1,14]. This is neither useful for  
159 disease progression monitoring or responsiveness to therapy. It can be also deduced that if we  
160 attempt to obtain tools for earlier diagnosis or earlier pathogenesis events that might be  
161 targeted with preventive or therapeutic measures, it may be beneficial to look for self-antigens  
162 with higher titers of auto-Abs in serum than in synovial fluid, in which the event occur later  
163 [2,3,5,7-10].  
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174           Dipeptidyl peptidase IV (DPP-IV/CD26) activity is a contributing factor in RA  
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183 [15,16]. DPP-IV as a result of its N-terminal X-Pro cleaving activity, regulates chemotactic  
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185 responses to inflammatory chemokines, including SDF-1, and biologically active peptides  
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187 such as NPY and VIP, recently implicated in RA. DPP-IV is also a neutrophil chemorepellent  
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189 [17,18]. Interestingly, the biofilm formation and production of DPP-IV are correlated in *P*  
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191 *gingivalis*, and both are important factors of virulence in periodontitis in a mouse model [31].  
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193 Also, stimulants such as cytokines or bacterial components from *P gingivalis*, *Prevotella*  
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195 *intermedia*, and *Escherichia coli* augment the CD26 expression by gingival fibroblasts [32].  
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199 Several authors, including ourselves [18,22-24], have shown lower levels of sCD26  
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201 (as well as DPP-IV activity) in RA patients and found important correlations between CD26  
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203 expression on some T cell subsets with disease activity (DAS28) and sCD26 serum levels  
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205 [18]. Autoantibodies against DPP-IV (Anti-CD26) were predicted as a possible way to clear  
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207 soluble DPP-IV (sCD26) from the circulation [19] via bile secretion in the form of IgA-Ag  
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209 complexes [20]. Although high titers of Anti-CD26 IgA were shown in patients of some  
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211 autoimmune diseases with lower circulating sCD26 serum levels [21], supporting this role,  
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213 the same result was not detected in RA patients [21]. We evaluated the levels of Anti-CD26  
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215 auto-Abs in our RA cohort with patients under different biological and non-biological  
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## 2. Methods

### 2.1. Study Design

One hundred and ten patients from the Rheumatology Service (Hospital Meixoeiro-CHUVI) were recruited in a cross-sectional case-control study. Patients fulfilled the American College of Rheumatology (ACR)/EULAR criteria of 2010 [3] and were on different therapies, including biological therapies (BT). The 110 RA patients consisted of 82 women and 28 men. Mean age was 57 years (range: 24– 78). The only exclusion criterion was the patient's decision not to be included in the study. Mean disease duration was 12 years (range: 0.5– 41). A group of 50 healthy donors was also recruited (women, n = 21, men, n = 29). Mean age was 44 years (range: 18–62). All the procedures described were performed according to clinical ethical practices of the Spanish and European Administrations and approved by the local ethics committee (CEIC Galicia (Xunta de Galicia), code n° 2010/298 and Agencia Española de Medicamentos y Productos Sanitarios (Ministerio de Sanidad) n° PYC-INM-2011-01). Written informed consent was obtained from all participants.

### 2.2. Assessment of Disease Activity

Disease activity was assessed by the DAS-28 index, which takes into account the number of tender joints, swollen joints, erythrocyte sedimentation rate (ESR) and Patient Global Assessment (PGA) of disease activity, scored by a numeric rating scale (NRS 0–100). Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), haemoglobin and platelet concentration levels were also recorded as RA activity markers. All patients also completed the HAQ (Stanford Health Assessment Questionnaire), which is a self-report functional status (disability) measure.

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303 *2.3. Smoking history*  
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305 Patients' smoking history was evaluated at inclusion. Current smokers were those  
306 reporting active smoking. Past smokers were all patients who had stopped smoking before the  
307 first examination at inclusion. Non-smokers reported no history of smoking at any time.  
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312 These data, together with other such as the measures of disease activity and others,  
313 were included in a specifically designed database. Some of the most relevant data has been  
314 published before [18].  
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320 *2.4. Measurement of DPP-IV Enzyme Activity and Soluble CD26 Protein*  
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322 Serum collection and both techniques have been described previously [18,21,24].  
323 Since previous studies [25-27] have shown not statistically significant differences in both  
324 levels of sCD26 and DPP-IV activity according to gender or age, values from controls and  
325 RA patients were not matched for these two parameters.  
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333 *2.5. Enzyme-linked immunosorbent assays for Total and Anti-CD26 Igs*  
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335 Concentrations of Total and Anti-CD26 IgA, IgG and IgM in sera of patients were  
336 determined using 96-well cultured plates coated with rDPP-IV (rsCD26) (0.5 µg/mL) (RnD  
337 Systems, USA) or with polyclonal rabbit anti-human IgA, anti-IgG and anti-IgM (2 µg/mL)  
338 (DakoCytomation, Denmark) prepared in PBS pH 7.4. Coated wells were blocked overnight  
339 with PBS 0.5% BSA. Plates were incubated with different dilutions of serum for 1 h at 37 °C.  
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341 Very high dilutions were needed to measure Total Ig types; for Anti-CD26 Igs, between 2 and  
342 5 µL of serum were used. Plates were then washed four times with PBS 0.05% Tween20  
343 before goat anti-human IgM (µ-chain), anti IgG (Fab-specific) and IgA (α-specific)-  
344 peroxidase conjugates (all from Sigma-Aldrich) were used to detect captured Abs. Standard  
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OPD substrate (Sigma-Aldrich) was added following manufacturer's instructions after another

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363 four washing cycles. Absorbance at 450 nm was registered using a BioRad Plate reader  
364 (BioRad, Madrid, Spain). Concentrations were calculated from calibration curves (data not  
365 shown) constructed with affinity-purified human IgA, IgG or IgM.  
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## 371 372 *2.6. ACPA Test*

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374 Elia™ CCP Test (Thermo Scientific/Phadia, Sweden) was used to analyse whether  
375 Anti-CD26 Abs were included in ACPA. Briefly, the test was used to bind the ACPA (anti-  
376 CCP antibodies) from patients' sera to the plate containing CPP Ags, according to the  
377 manufacturers' instructions. The remaining serum was recovered as negative-ACPA fraction.  
378 After washing the Elia-CCP plate to avoid unspecific binding, ACPA were eluted from the  
379 plate with Elution buffer (0.1 M Glycine-HCl pH 2.7) and this buffer neutralized with 1 M  
380 Tris-HCl pH 9 to obtain the positive-ACPA fraction. Both fractions were then checked using  
381 the in-house Anti-CD26 ELISA described in the main text. In the same way, this latter ELISA  
382 was used to obtain a negative Anti-CD26 Ab fraction (recovery of patients' serum after Ab  
383 binding to the plate-bound sCD26 Ag) and a positive-AntiCD26 Ab fraction (obtained after  
384 washing and elution of the bound Ab), and both fractions analysed using the Elia™ CCP test.  
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397 Sera from three RA patients showing high levels of Anti-CD26 IgG isotype and three  
398 healthy donors were chosen. In all these ELISAs, only reagents to detect the IgG isotype were  
399 used (see above).  
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## 406 *2.7. Statistical Analysis*

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408 Kolmogorov-Smirnov was applied to continuous variables to verify normal  
409 distribution. All analyses were parametric as most groups showed normal distribution  
410 although some were small. The ANOVA test was carried out to compare variables among the  
411 four groups of patients with or without biological therapies (BT). The post-hoc Scheffé test  
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423 was used for variables with homogeneous variances and the post-hoc Dunnett's C test was  
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425 used for variables without homogeneous variances. Dunnett's T test was performed to  
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427 compare each group with a control group, either the group without BT or the healthy donor  
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429 group. Student's T-test was also used to compare variables between two groups.  
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432 Anti-CD26 ability to discriminate between RA patients and healthy controls was  
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434 evaluated by receiver operating characteristic (ROC) curves. Sensitivity, specificity,  
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436 likelihood ratios (LRs) and their confidence intervals (CI), positive predictive value (PPV),  
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438 negative predictive value (NPV) and diagnostic accuracy were calculated. To estimate  
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440 positive LR the formula  $\text{sensitivity}/(1-\text{specificity})$  was applied and the negative LR was  
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442 calculated as  $(1-\text{sensitivity})/\text{specificity}$ . The cut-off point was chosen as the value that  
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444 rendered the highest accuracy value.  
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447 Statistical analyses were carried out using the SPSS software version 20 (SPSS Inc,  
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### 3. Results

#### *3.1. Anti-CD26/DPP-IV autoantibody and Total Ig levels and their correlations with serum DPP-IV activity and sCD26 concentrations in the healthy donor cohort*

Mean titers (n=50) of serum Total Ig concentrations for the three isotypes under study were found within expected values [28] by the in-house ELISA, 2.37 ±1.24 mg/mL for IgM, 2.28 ±0.27 mg/mL for IgA and 12.28 ±2.79 mg/mL for IgG. Mean serum Anti-CD26 autoantibody titers are 13.51 ±5.62 µg/mL for IgM, 1.28 ±0.27 µg/mL for IgA and 2.14 ±0.64 µg/mL for IgG, being quite similar to those previously published, slightly lower for the IgM and IgA isotypes [21]. Comparing their published ratios of anti-CD26 /Total Ig isotype [21] with ours (7.12 for IgM, 0.57 for IgA and 0.19 for IgG), our results are more consistent with expected values of Total Ig titers [28].

As demography of control donors and RA patients were slightly (although not statistically) different from healthy donors, levels of Igs were compared according to gender and age (50 or less years old, 51-60, and 61 or more years old). ANOVA did not show any significant difference and the trend to higher IgA levels in elders (data not shown) was only significant by the T-test when comparing the younger and the elder groups.

None of the present values, or their ratios, correlated with serum DPP-IV activity or sCD26 concentrations in this cohort of healthy donors (data not shown). DPP-IV and sCD26 levels behaved different according to gender, as previously described [26,27, manuscript in preparation]. Interesting, the scarcer IgA isotype levels was close to a significant negative correlation (R= -0.529, p= 0.063) with sCD26 in men, where DPP-IV activity and sCD26 concentration did not correlate. Anti-CD26 IgM and IgG strongly correlated between them equally in both genders (data not shown).

### 3.2. Serum Anti-CD26 and Total Ig levels in RA patients undergoing different therapies

Analyzing the whole cohort of RA patients (n=106), levels of both Total and Anti-CD26 Igs were statistically higher compared to healthy donors (T-test  $p < 0.001$ , ~~most of them~~). Mean  $\pm$ SD of Total Ig concentrations for each of the three isotypes were  $4.33 \pm 3.79$  mg/mL for IgM,  $5.61 \pm 1.77$  mg/mL for IgA and  $21.81 \pm 15.35$  mg/mL for IgG. Mean  $\pm$ SD Anti-CD26 concentrations were  $27.97 \pm 19.09$   $\mu$ g/mL for IgM,  $2.01 \pm 1.34$   $\mu$ g/mL for IgA and  $5.87 \pm 3.13$   $\mu$ g/mL for IgG. Anti-CD26/Total isotype ratios were 17.10 for IgM, 0.43 for IgA and 0.75 for IgG. Although all values of Total Igs increased two-fold overall, it is worth noting that IgA ratio is lower compared to healthy donors whereas the opposite was found for IgM and particularly IgG ratios, higher (next to 4-fold) and significantly different compared to healthy donors ( $p < 0.001$ ; ANOVA test), a result that point to the accuracy of our in-home ELISA.

Our patients differed according to therapies and data were calculated for each group, noBT (no biological therapy, under DMARDs), anti-TNF, anti-CD20 and anti-IL6R/Ig-CTLA4 BTs (the latter grouped together because of the similar results) (Table 1). In addition, the results for Anti-CD26 were different to that of Total Igs (Supplemental data), which underlies the specificity of the ELISA

### 3.3. Diagnostic power of serum Anti-CD26 levels

We tested whether any of the isotype titers could have diagnostic power, knowing that this cohort cannot be properly used for this purpose, as these patients were already undergoing therapy. Regardless, for the IgG isotype, which does not change amongst the groups, sensitivity of 82% and specificity of 96% was found (Supplemental Figure 2 shows ROC curves for the whole cohort) with cut-off 2.85  $\mu$ g/mL. Testing the group undergoing DMARDs (noBT) alone (perhaps representing an earlier stage of the disease than the BT

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603 groups), a sensitivity of 77% (17/22) was found for IgG with the same cut-off (Figure 1 A). A  
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605 similar result, 73% (16/22) sensitivity was found for IgM (in this case with cut-off 20 µg/mL)  
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607 (Fig. 1 B) and for IgA (with cut-off 1.8 µg/mL) (Fig. 1 C) titers. To note, only 3 out of 22  
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609 patients were negative for both IgG and IgM isotypes and 2/22 for the three.  
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### 612 613 614 *3.4. Correlations of serum Anti-CD26 levels with disease activity parameters in RA patients*

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616 Data of the DAS28 score; the DAS28 components (clinical: TJC, SJC, PGA, and  
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618 laboratory variables: ESR and CRP); or other RA activity markers (HAQ, haemoglobin,  
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620 platelet and haematocrit) were recorded for each patient. Some information was previously  
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622 shown [18].  
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625 Anti-CD26 levels (like Total Igs) did not correlate with the DAS28 score. However,  
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627 the three Ig isotype titers showed negative correlation with TJC ( $r = -0.272$ ,  $p = 0.036$  for IgM;  
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629  $r = -0.210$ ,  $p = 0.032$  for IgG;  $r = -0.273$ ,  $p = 0.005$  for IgA). IgG levels also negatively correlated  
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631 with the haematocrit and IgA with ESR ( $r = -0.360$ ,  $p < 0.001$  for the latter). Finally, the ratios  
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633 of IgG and IgA isotypes correlated with the CRP (data not shown).  
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636 The same analysis was performed in each group of patients separately, as BT are  
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638 confounding factors. In the noBT group, only the IgA ratio showed a very strong correlation  
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640 with TJC ( $r = 0.704$ ,  $p < 0.001$ ). In the anti-TNF BT group, Anti-CD26 IgA strongly correlated  
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642 with TJC and DAS28 (Supplemental Fig 3 A and B, respectively) and IgM levels positively  
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644 correlated with TJC ( $r = 0.295$ ,  $p = 0.024$ ). No correlations were found for the IgG isotype.  
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646 However, in the anti-CD20 BT group, Anti-CD26 IgG correlated strongly with TJC, SJC,  
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648 DAS28 and PGA (Figure 2 A to D) and no correlations were found for the other two isotypes.  
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650 Finally, in the anti-IL6R/Ig-CTLA4 BT group, only the IgG ratio correlated with TJC and  
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652 Total IgG with the hematocrit (data not shown).  
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663 Taken together, the data show: a) a specific relationship between Anti-CD26, the  
664 joints counts and the DAS, b) Anti-CD26 levels are variably affected by each therapy (or,  
665 alternatively, they might mark patients responders to each therapy), and c) they provide  
666 different information to that of most frequently used disease activity parameters (ESR, CRP,  
667 platelet count, Hb levels or haematocrit).  
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### 675 676 *3.5. Higher serum Anti-CD26 titers in smokers*

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678 The whole cohort was divided in three groups according to their smoking status,  
679 current (n=18), past (n=28) or non-smokers (n=59) and means of the three Anti-CD26 isoypes  
680 were compared. Whereas IgA titers were similar among the three groups, both IgM and IgG  
681 showed higher levels in past smokers in particular (26.0 µg/mL in non-smokers compared to  
682 31.8 µg/mL in past smokers for IgM, and 5.5 µg/mL to 6.7 µg/mL for IgG), although these  
683 differences did not reach statistical significance.  
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691 When the analysis was done in each group of patients (Table 2), in the noBT group  
692 IgM titers were significantly higher in past smokers compared to non-smokers. Although  
693 there were few current smoker in this group, they also showed higher titers. The IgG isotype  
694 also showed a trend to higher titers in past smokers, compared to the other groups, although  
695 this trend did not reach statistical significance. To note, whereas Anti-CD26 IgG or IgA levels  
696 did not change among the different therapies, IgM levels were attenuated in past smokers in  
697 the anti-TNF BT group and in all patients in the anti-CD20 BT group. However, in the anti-  
698 IL6R/Ig-CTLA4 BT group non-smokers showed increased levels of IgM (Table 2).  
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### 710 711 *3.6. Serum Anti-CD26 are not ACPA*

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713 Combination of the well-established second-generation Elia™ CCP tool in the clinical  
714 practice together with our in-house ELISA for Anti-CD26 detection (only the IgG isotype was  
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723 tested, see methods) showed that Anti-CD26 are not ACPA. When supernatants of serum  
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725 from both healthy donors and RA patients were recovered from the ACPA test and tested for  
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727 the presence of Anti-CD26 (Fig. 3 A, RA patients showing highest absorbance levels), a  
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729 similar absorbance to that obtained with the same serum sample used only in the Anti-CD26  
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731 ELISA test (not shown) could be detected. The ACPA test showed the expected results, with  
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733 higher levels in the three patients analyzed than in the three healthy donor samples (except  
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735 one which was also positive) (Fig. 3 B, dark gray).  
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739 To verify these results, we also performed the inverse analysis, where supernatants  
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741 recovered from the Anti-CD26 ELISA were then used in the ACPA test. Although  
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743 absorbances were quite similar to those obtained after the direct incubation of the same serum  
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745 in the ACPA test, one healthy donor and one RA patient showed more important differences  
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747 between samples (Fig. 3 B). In these cases, when proteins captured in the Anti-CD26 assay  
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749 were eluted from the plate well and tested in the ACPA assay a minor positive signal was  
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751 observed, pointing to the possibility that a minor part of ACPA autoantibodies may also bind  
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753 to CD26 (data not shown).  
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### 757 *3.7. Serum Anti-CD26 isotypes and their correlations with DPP-IV activity and sCD26* 758 759 *concentrations in RA patients* 760

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762 As in the healthy control group, correlations of the isotypes and their ratios with the  
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764 serum DPP-IV activity and sCD26 concentrations were analysed in our cohort of patients.  
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766 Only next to statistically significant positive correlations were found between Total IgG and  
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768 Anti-CD26 IgG with the sCD26 concentration but not with the enzymatic activity (data not  
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770 shown).  
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773 The same analysis was then performed in each group of patients undergoing different  
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775 therapies: a) In the noBT group, Total IgA and IgM positively correlated with the DPP-IV  
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783 activity but not with the sCD26 concentration (Figure 4 A); b) In the anti-IL6R/Ig-CTLA4 BT  
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785 group, Total IgM and IgG titers negatively correlated with the DPP-IV activity (Figure 4 D);  
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788 c) In the anti-TNF $\alpha$  group (with increased DPP-IV activity levels), in addition to Total IgA  
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790 and IgM titers, also Anti-CD26 IgG positively correlated with DPP-IV activity (Figure 4 B).  
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792 Only in this group Total IgM and IgG and Anti-CD26 IgG and IgM titers correlate positively  
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794 with the sCD26 concentration (Figure 4 B); d) in the anti-CD20 BT group, with decreased  
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796 levels of IgMs and Igs in general, both Anti-CD26 IgM titers and ratio show a strong negative  
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798 correlation with the sCD26 concentration (Figure 4 C).  
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801 It can be concluded that: a) Anti-CD26 isotypes are not related to the impairment of  
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803 sCD26 Ag concentrations in RA patients, since the only correlation (in the anti-TNF BT  
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805 group) was positive; b) different levels of correlation between DPP-IV activity and sCD26  
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807 concentration are found in each group of patients (they do not correlate in the noBT and in the  
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809 anti-IL6R/Ig-CTLA4 BT group and correlate strongest in the anti-CD20 BT group, data not  
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811 shown); c) together with the fact that DPP-IV activity and sCD26 concentration, impaired in  
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813 RA patients and targeted by therapies [18], are related to the same pathway that affected Anti-  
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815 CD26 titers, we conclude that DPP-IV activity and sCD26 concentration do not play parallel  
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817 roles in RA pathogenesis; d) different therapies have a profound impact also in these  
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819 pathways even in opposite ways.  
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#### 4. Discussion

González-Gronow and colleagues observed that myocardial infarction patients who received streptokinase (SK) as fibrinolytic therapy developed anti-CD26 autoantibodies as well as anti-SK and anti-plasminogen (Pg) auto-Abs [40]. Both Pg and SK bind to CD26 perhaps explaining the biochemical mechanism that originates these auto-Abs [40]. The present work shows important differences to theirs [21,40]. First, in RA and other autoimmune diseases they found higher levels of anti-CD26/DPP-IV autoantibodies (Anti-CD26) of the IgA isotype but not IgG and IgM isotypes. We found additional elevated levels of IgM and IgG isotypes, which showed in fact better diagnostic values than IgA. The differences between both studies may be related to the fact that we used a recombinant sCD26 protein as the ELISA Ag instead of sCD26 isolated from patient sera [21], a more complex Ag [21,27]. In our data, in contrast to theirs [21], Total Ig isotypes levels fit with their known values in human serum [28]. Importantly, in additional experiments we have found people with intestinal anomalies where Anti-CD26 levels significantly decreased (manuscript in preparation), whereas we also found higher levels of only IgM, or only IgA, respectively, in autoimmune diseases such as systemic lupus erythematosus (lower than in RA) and Sjögren Syndrome (higher than in RA) (data not shown).

The expected correlation between lower circulating levels of DPP-IV and the higher levels of Anti-CD26 IgA [21,40] was found in healthy men but in RA, only in patients undergoing anti-CD20 BT (Fig 4), probably because the reduction of confounding Anti-CD26 isotype. On the contrary, the correlations found were positive and mostly for the Anti-CD26 IgG and IgM isotypes in the case of TNFi BT, a therapy that is enhancing the CD26 levels in coherence with previous data [41]. It is easy to conclude that Anti-CD26 generally has a much wider role than of serum sCD26 clearance.

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Although the elevated levels of Anti-CD26 found in most RA patients (present also at lower levels in healthy people) may fit in the context of periodontal infection [29-32], the fact that in our cohort, past smokers (but no current smokers, perhaps indicating that the former smoked a higher amount of cigarettes in their lives) showed elevated levels of Anti-CD26, whereas Anti-P gingivalis Ab titers were found to be associated with, on the contrary, non-smoking status in early RA [29], may rise an alternative scenario of immunological tolerance breaking in the lung: CD26/DPPIV expression is enhanced in bronchial epithelial cells (BECs) by IL-13 stimulation and may play a role in asthma [34,35]. Type II collagen and fibrinogen are typically citrullinated antigens in ACPA-positive RA [5,6], and CD26/DPPIV was found bound to proteins of the extracellular matrix as well as fibrinogen (in pleural effusions samples) [36]. Intriguingly, DPP-IV and fibrinogen-like 2 (FGL2) genes are genomic markers (allele and SNPs frequencies) identified for severe periodontitis [37].

That these autoantibodies are not ACPA may be relevant. A recent study in patients with periodontitis showed higher titers of Abs against non-citrullinated antigens, suggesting that the breaking of tolerance to non-citrullinated peptides could precede later events [33]. B cell clones producing mAbs recognizing citrullinated peptides failed to recognize the native non-modified arginine version of the peptide [38]. This observation suggests that several different target antigens may initially drive the autoimmune responses before being refined and focused on one particular epitope. In this sense, it is also remarkable the fact that a 10-aminoacid long peptide epitope (145-154) of the human CD26/DPP-IV has been recently found as an MHC class I-associated peptide (MIPs) by elution from B lymphoblasts (from the HLA-B\*44:03 allele, [www.iedb.org](http://www.iedb.org)) [39]. Therefore, Anti-CD26 may come from one of those early events and provide different diagnostic information than ACPA, however the existence of anti-citrullinated CD26 autoAbs should be investigated (CD26 has many Arg).

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963 Anti-collagen and anti-fibrinogen Ab titers are higher in the synovial fluid [5,6] and  
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965 may be related with the articular damage because mAbs isolated from ACPA-positive B  
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967 lymphocytes stained inflamed synovial tissues from RA patients [38]. Anti-CD26 showed  
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969 intense correlations with the articular damage, even with the DAS28 score, a clear advantage  
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971 with respect to other autoAbs in clinical use (such as ACPA) that do not correlate with  
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973 disease activity [28]. The fact that the isotype IgA, IgM or IgG classes that correlate with TJC  
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975 or DAS28 were different in each group of patients, with each of them showing different  
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977 correlations with parameters of disease activity, supports that they may provide specific  
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979 biological information. In conclusion, autoantibodies against CD26/DPP-IV may participate  
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981 in the pathogenesis of this autoimmune disease, their preliminary clinical value suggesting  
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983 that they could be tested as biomarkers in different studies.  
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1503 **FIGURE LEGENDS**  
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1508 **Figure 1. Cut off values for Anti-CD26 Igs and frequency of anomalous values in RA**  
1509 **patients of the noBT group (patients under DMARDs) and healthy donors.** Titers of the  
1510 **IgG (A), IgM (B) and IgA (C) classes in both groups are shown. Cut-off values (dotted lines)**  
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1512 2.8, 20 and 1.8  $\mu\text{g mL}^{-1}$ , respectively, were defined as described in the Results section. HD:  
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1514 Healthy donors; noBT: RA patients under DMARD.  
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1520 **Figure 2. Correlations between RA disease activity parameters and Anti-CD26 IgG**  
1521 **titers in the anti-CD20 BT group.** Statistically significant correlations between the IgG  
1522 isotype with TJC (A), SJC (B), DAS28 (C) and PGA (D) were detected in the group of RA  
1523 patients treated with anti-CD20 antibodies.  
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1531 **Figure 3. Anti-CD26 Abs are not ACPA.** (A) Supernatants recovered from the ACPA test  
1532 after incubation of serum from 3 different RA patients (filled columns) and 3 different healthy  
1533 donors (clear columns) were subsequently used for the Anti-CD26 test. Similar absorbance  
1534 values were observed when the same serum samples were used directly in the Anti-CD26 test  
1535 (data not shown). (B) Absorbance values for the same subjects tested in (A) in the ACPA test  
1536 (dark grey columns) and for the supernatants from the serum samples tested previously in the  
1537 Anti-CD26 test (clear gray columns). In two cases, one RA patient (rightmost) and one  
1538 healthy donor (leftmost), there were differences between both absorbance values pointing to  
1539 the possibility that some of the ACPA autoantibodies bind to CD26. Background values were  
1540 extracted from the shown absorbance data.  
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1563 **Figure 4. Correlations of Anti-CD26, or Total Ig titres of the three isotypes (A,G or M),**  
1564 **or their ratios (Anti-CD26/Total), with serum sCD26 concentration and DPP-IV**  
1565 **enzymatic activities in the cohort of RA patients under different therapies.** Correlations  
1566 found in patients under DAMARDs (noBT) **(A)**, anti-TNF $\alpha$  BT **(B)**, anti-CD20 BT **(C)** and  
1567 anti-IL6R/Ig-CTLA4 BT **(D)** therapies, for Total Ig titres (tIgG, tIgM or tIgA; black  
1568 columns), Anti-CD26 (aIgG, aIgM or aIgA; grey columns), or their ratios (rIgG, rIgM or  
1569 rIgA; empty columns) against both sCD26 serum levels and DPP-IV enzymatic activity, are  
1570 shown. r: Pearson's correlation coefficient, and \* p<0.05, \*\* p<0.005 are indicated. Only in  
1571 the anti-TNF BT group **(B)** some coincidences for the DPP-IV and sCD26 were observed.  
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## RESULTS

### *3.2. Serum Anti-CD26 and Total Ig levels in RA patients undergoing different therapies*

In the anti-CD20 BT group, impaired levels of Total IgG and particularly IgM compared to the group without BT (noBT) were found, as expected (anti-CD20 deletes Ab-producing cells) whereas no effect in Total IgA was seen (Table 1). Those drops did not reach the Ig levels of healthy donors. On the contrary, increased levels of both IgG and IgM were found in the other groups (Table 1). The anti-IL6R/Ig-CTLA4 BT group was the only one showing slightly lower levels of IgA.

The results for Anti-CD26 were different to that of Total Igs, underlying the specificity of the ELISA: The IgG isotype was not affected by any therapy; anti-CD20 strongly reduced only IgM, and finally IgA was affected by both anti-TNF and anti-IL6R/Ig-CTLA4 therapies but in contrary directions (Table 1). As consequence, ratios for the three isotypes are quite different compared to healthy subjects, particularly IgM for anti-TNF and anti-IL6R/Ig-CTLA4 BT, IgA for anti-CD20 BT and IL6R/Ig-CTLA4 BT, and IgG for noBT and IL6R/Ig-CTLA4 BT. Most of these differences were statistically significant (see Table 1). The same can be concluded when checking the isotype ratios (A/M, G/A, G/M) for Total or for Anti-CD26 titers: statistically significant differences between patients and donors in some ratios (Supplemental Table 1) and important differences among the therapies (Supplemental Figure 1, check the different behaviour of the G/A ratio in Total and Anti-CD26 titers in the anti-CD20 BT group).

### *3.4. Correlations of serum Anti-CD26 levels with disease activity parameters in RA patients*

Total Ig (which include RF and ACPA) titers of the three isotypes did not correlate with the DAS28 score in the whole cohort, however some statistically significant correlations were found with ESR ( $r= 0.272$ ,  $p=0.005$  for IgM;  $r= 0.213$ ,  $p=0.028$  for IgG;  $r= 0.280$ ,  $p=0.004$  for IgA). Moreover, IgG negatively correlated with TJC and IgM with HAQ (data not shown).

Correlations between the Abs in each group of patients undergoing different therapies (which serve as internal controls of the results shown) were (data not shown): In the noBT group, strong correlations between all Total Ig titers are observed (and show correlations with the platelet count) but in Anti-CD26, only IgA and IgM showed a correlation between them. In the anti-TNF BT group, Total IgA did not correlate with the other two isotypes and showed only a positive correlation with the ESR. Total IgM also correlated positively with Hb and haematocrit and negatively with the CRP whereas IgG correlated negatively with the ESR and the platelet count. In the anti-CD20 BT group, Total Igs did not correlate among them and the only correlations detected were IgM ratio with both HAQ and PGA, and IgG ratio with platelets. In the anti-IL6R/Ig-CTLA4 BT group, Total IgA did not correlate with the other isotype (as in the anti-TNF group) and Anti-CD26 IgA and IgM did not correlate between them (in contrast to the noBT group). Anti-CD26 Igs in the other combinations (IgA with IgG and IgM with IgG) did.

## **SUPPLEMENTARY FIGURE LEGENDS**

### **Sup Figure 1. Therapy effect on Total and Anti-CD26 IgG, IgM and IgA ratios.**

Ratios for either Total (upper row) and Anti-CD26 Ig levels (lower rows) in patients grouped according to treatment are shown. Columns 1: NoBT (DAMARDs); columns 2: anti-TNF- $\alpha$  BT; columns 3: anti-CD20 BT; columns 4: anti-IL6R/Ig-CTLA4 BTs (grouped together). The effects on the different ratios were usually the opposite in the case of Anti-CD26 to that observed for Total Ig levels (compare lower and upper rows).

### **Sup Figure 2. COR curves for the Anti-CD26, Total Ig titers and their ratios in the whole cohort with respect to the control donors.**

Anti-CD26 IgG (see text) and IgM show better curves (AUC: 0.897,  $p < 0.001$  and 0.756,  $p < 0.001$ , respectively) than their Total Ig counterparts (AUC: 0.698,  $p = 0.002$  and 0.647,  $p = 0.022$ , respectively). However, Total IgA isotype (AUC: 0.978,  $p < 0.001$ ) showed the best results while their ratio showed the worst.

### **Sup Figure 3. Correlations between RA disease activity parameters and Anti-**

**CD26 IgA titers in the anti-TNF BT group. (A)** Correlation between the IgA isotype with TJC. **(B)** Correlation between the IgA isotype with the DAS28 score.

**Supplementary Table 1. Levels of total and anti-CD26 Ig isotypes in RA patients under different therapies**

	<b>Controls (n=25)</b>	<b>RA patients (n=106)</b>
	Mean $\pm$ SD	Mean $\pm$ SD
<b>Total Ig ratio A/M</b>	1.3 $\pm$ 1.0	3.7 $\pm$ 6.7**
<b>Total Ig ratio G/A</b>	5.4 $\pm$ 1.3	4.8 $\pm$ 7.3
<b>Total Ig ratio G/M</b>	6.7 $\pm$ 5.2	11.3 $\pm$ 18.6*
<b>Auto Ig ratio A/M</b>	0.11 $\pm$ 0.04	0.13 $\pm$ 0.21
<b>Auto Ig ratio G/A</b>	1.7 $\pm$ 0.6	7.5 $\pm$ 27.1
<b>Auto Ig ratio G/M</b>	0.17 $\pm$ 0.06	0.32 $\pm$ 0.35**

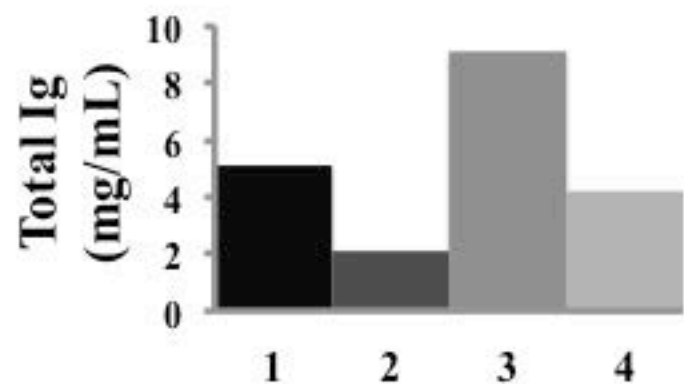
n= number of samples; SD= standard deviation.

\* Values significantly different among groups (not specified) at  $p < 0.05$  with Student's t test

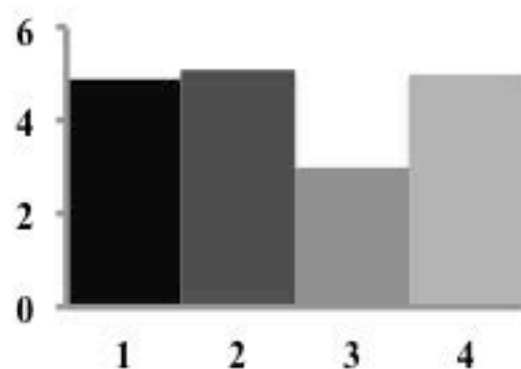
\*\* Values significantly different among groups (not specified) at  $p < 0.001$  with Student's t test

S-Figure 1

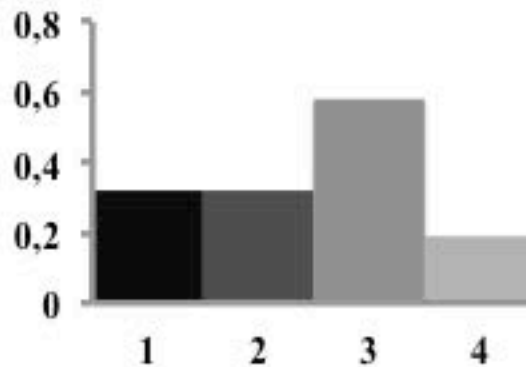
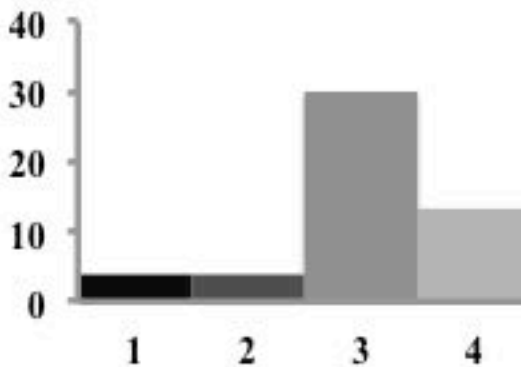
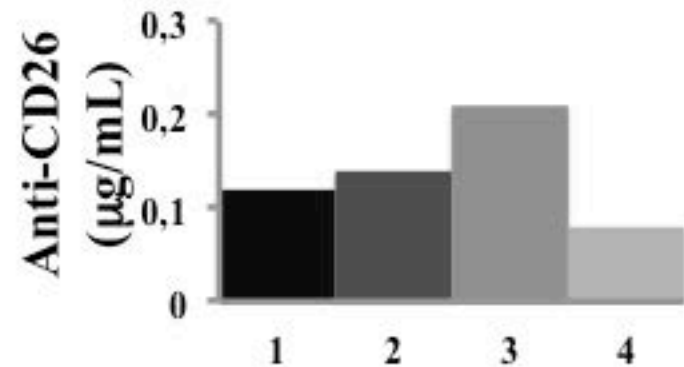
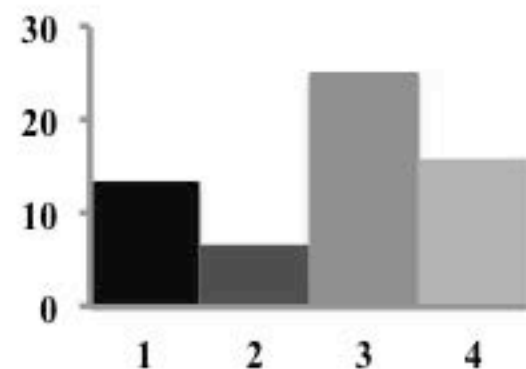
IgA/IgM ratio



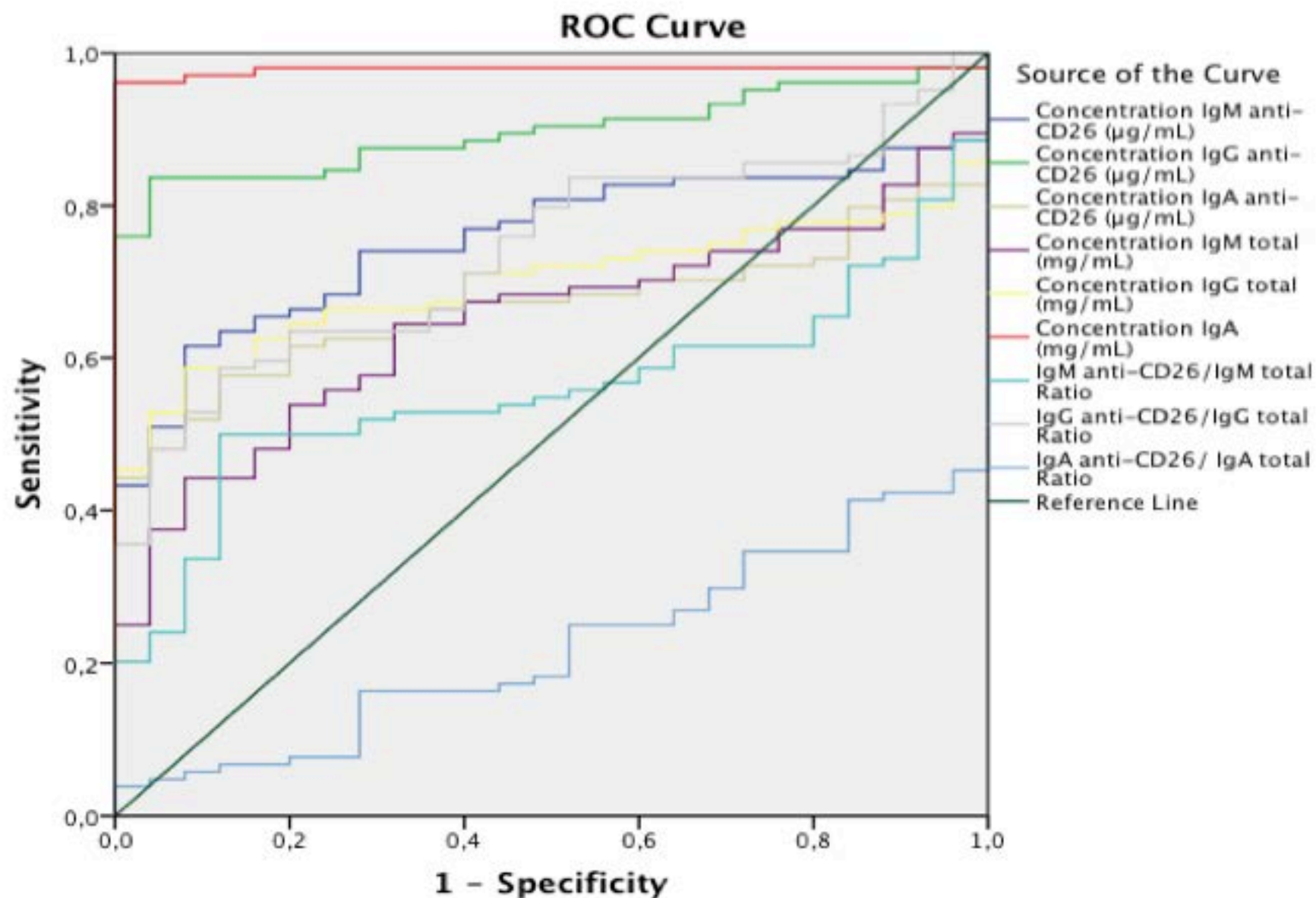
IgG/IgA ratio



IgG/IgM ratio



S-Figure 2



### S-Figure 3

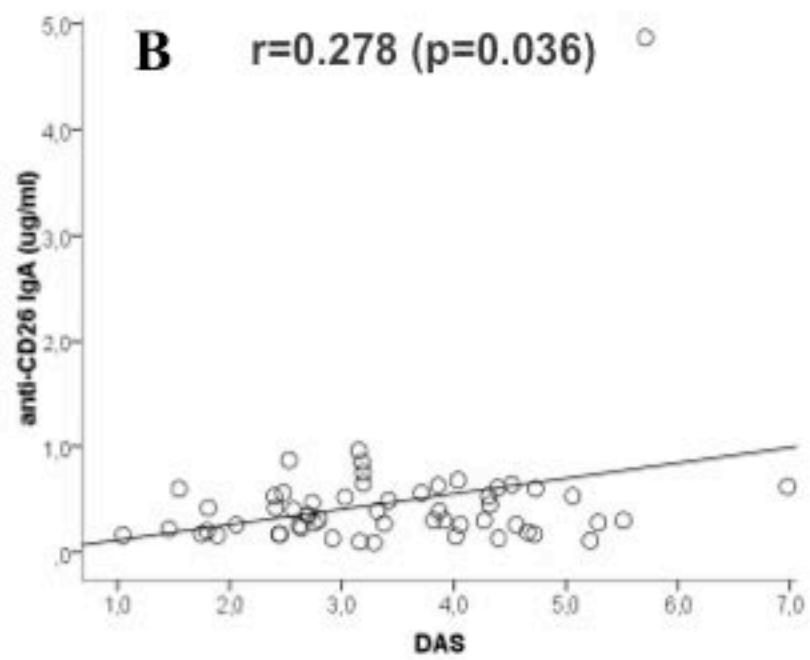
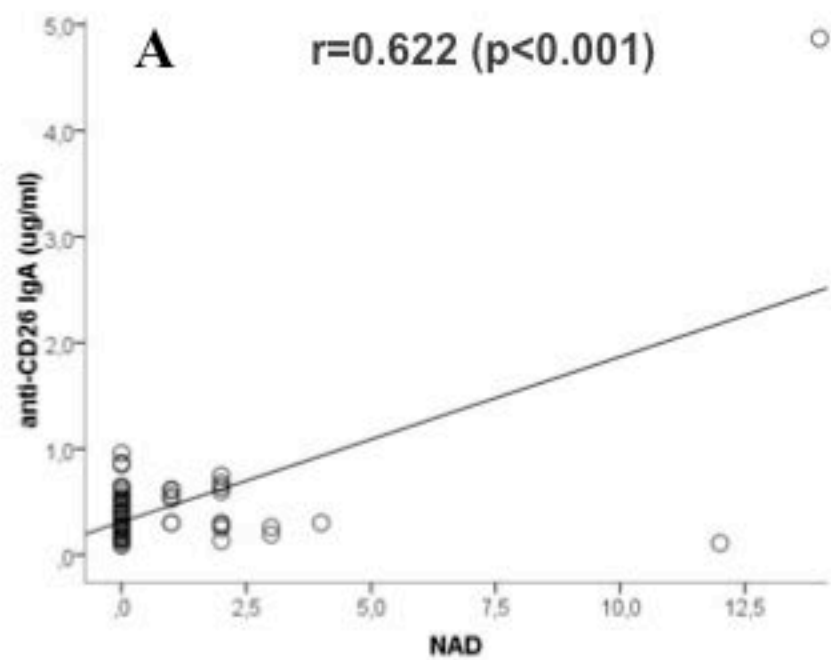


Figure 1

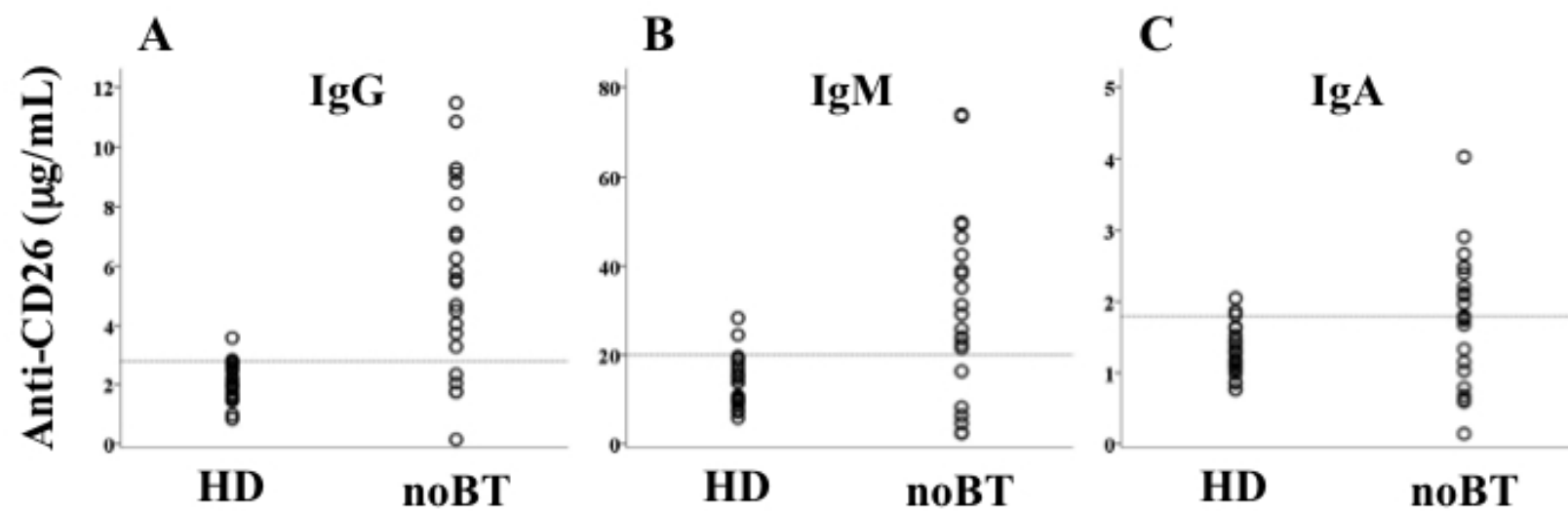
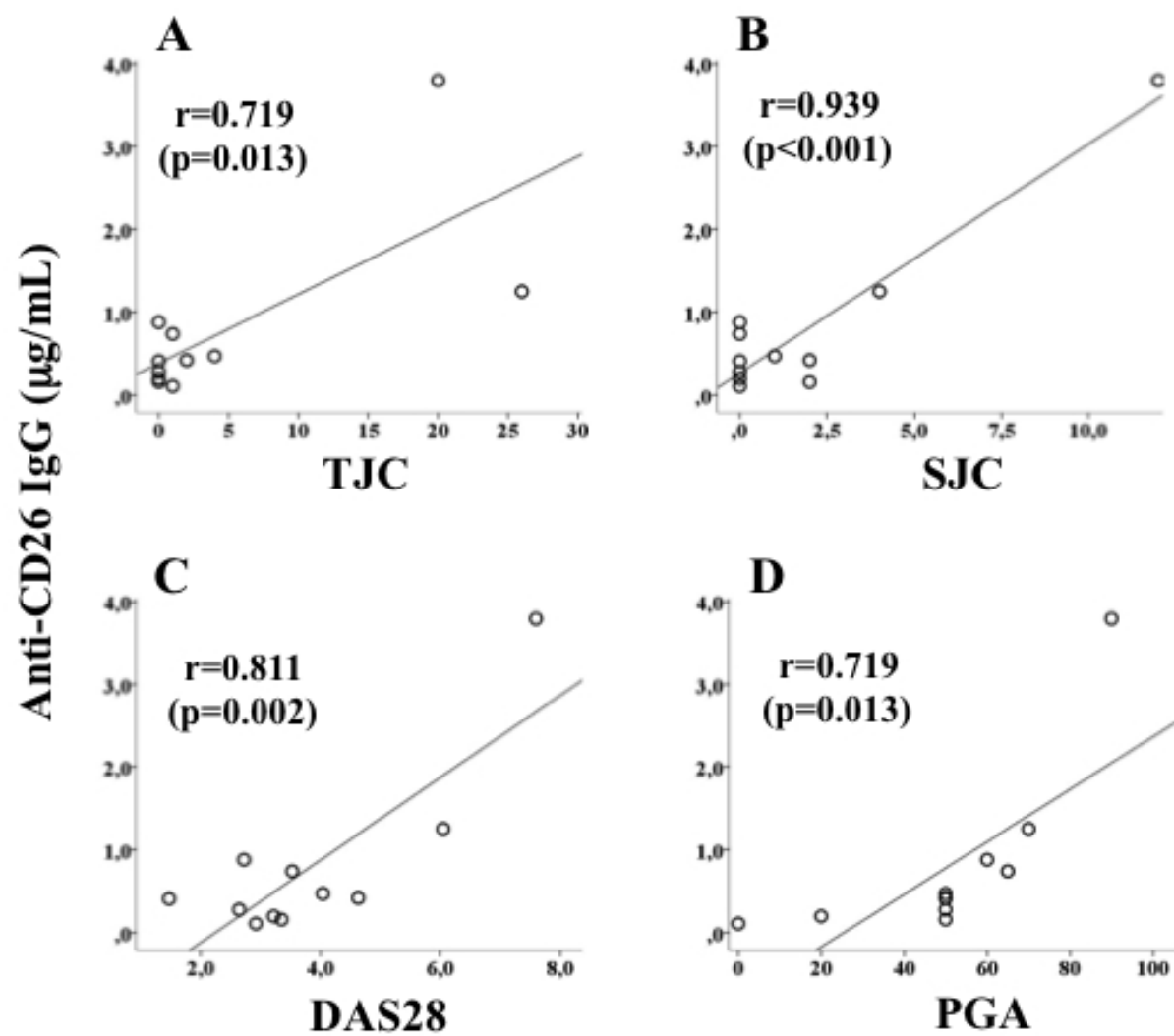
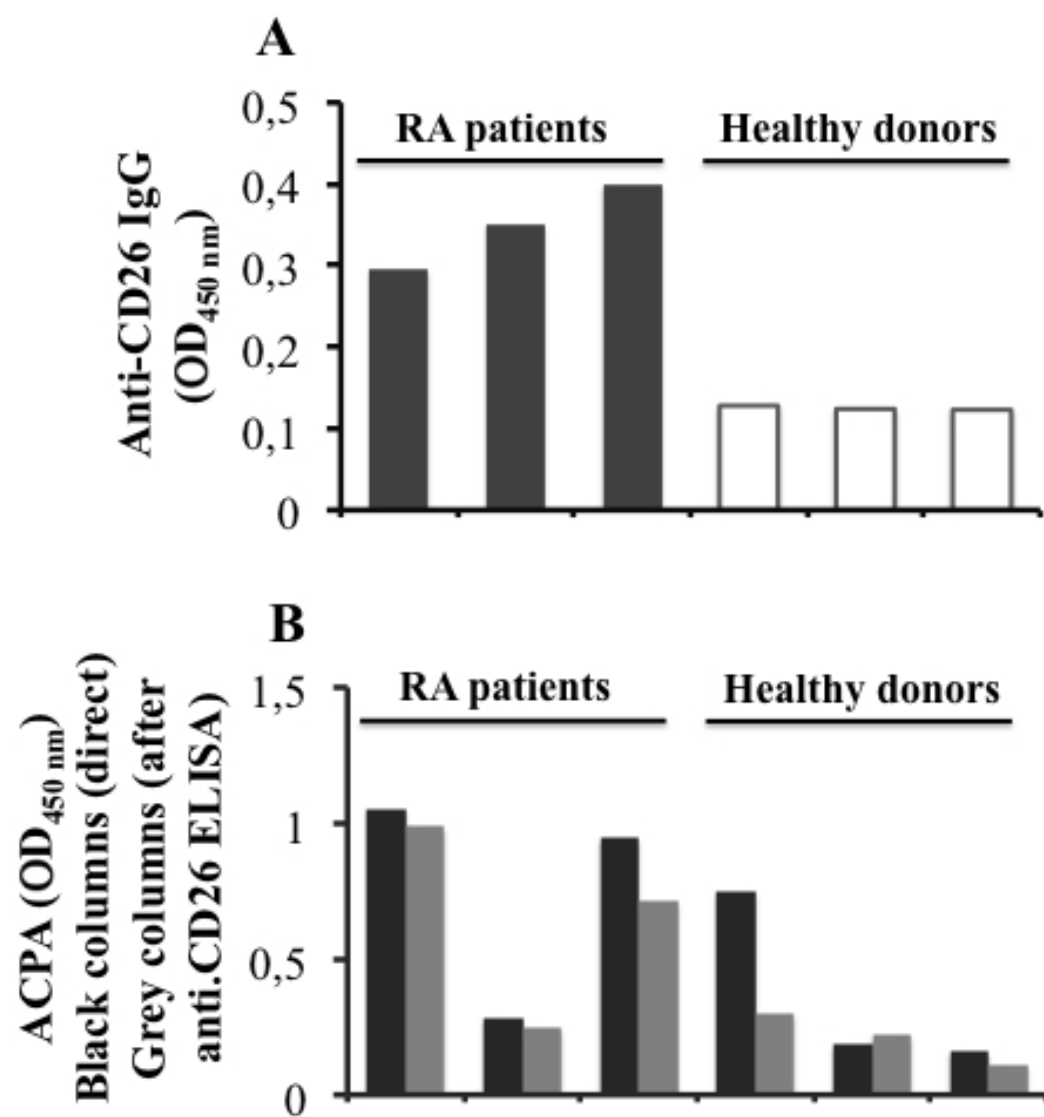


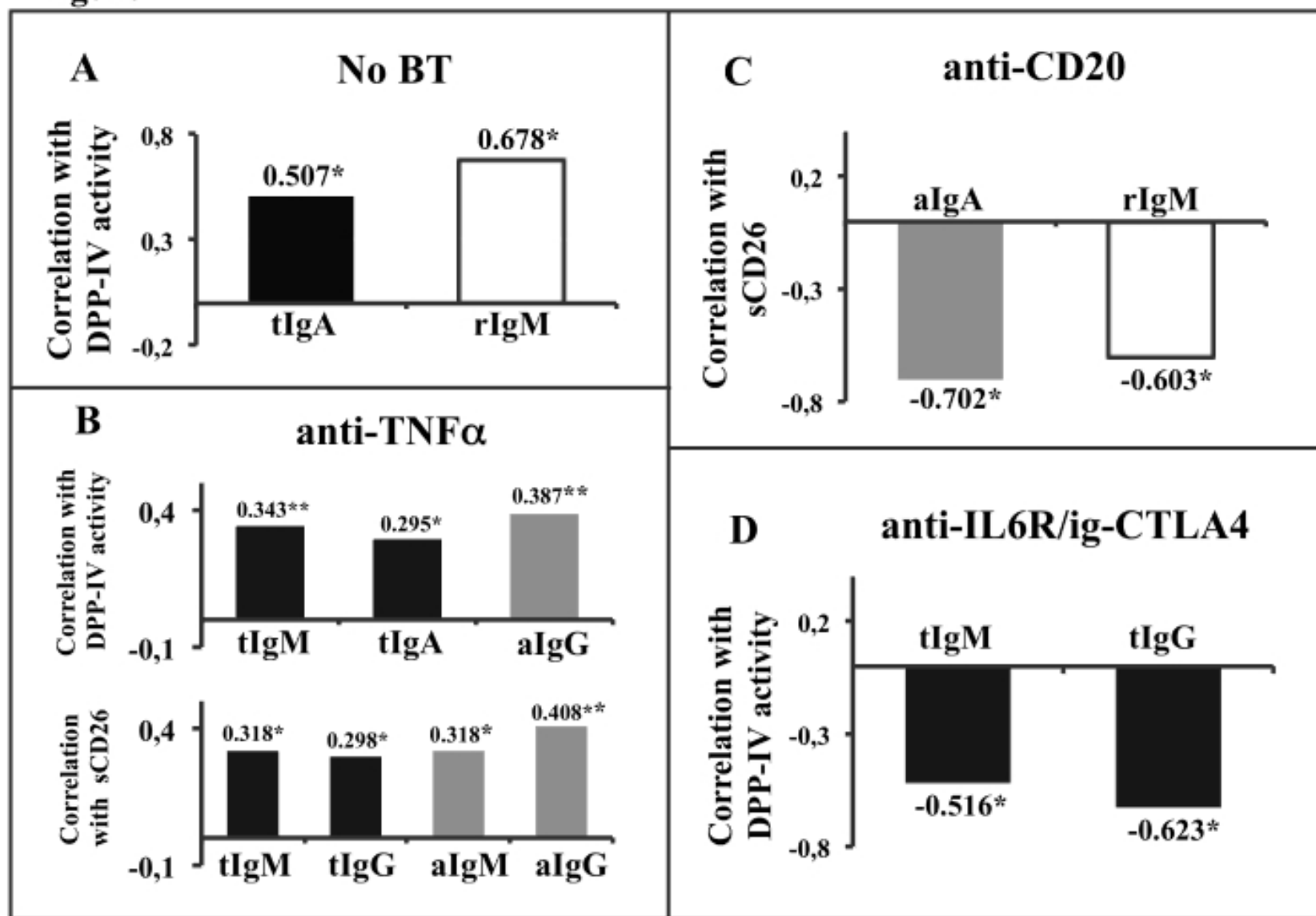
Figure 2



**Figure 3**



**Figure 4**



**Table 1. Levels of Total and Anti-CD26 Ig isotypes in RA patients under different therapies**

	No BT (n=22)		Anti-TNF $\alpha$ BT (n=58)		Anti-CD20 BT (n=11)		Anti-IL6R/Ig-CTLA4 BT (n=15)	
	Mean $\pm$ SD	CI (95%)	Mean $\pm$ SD	CI (95%)	Mean $\pm$ SD	CI (95%)	Mean $\pm$ SD	CI (95%)
<b>Anti-CD26 IgM (<math>\mu</math>g/mL)</b>	30.30 $\pm$ 20.49*	21.21-39.38	27.92 $\pm$ 18.08	23.16-32.67	16.85 $\pm$ 15.15*	6.67-27.03	32.94 $\pm$ 21.82	20.85-45.02
<b>Anti-CD26 IgA (<math>\mu</math>g/mL)</b>	1.76 $\pm$ 0.92*	1.35-2.17	2.32 $\pm$ 1.51*	1.92-2.72	1.81 $\pm$ 1.44*	0.85-2.78	1.34 $\pm$ 0.71*	0.95-1.73
<b>Anti-CD26 IgG (<math>\mu</math>g/mL)</b>	5.60 $\pm$ 3.13	4.21-6.98	6.08 $\pm$ 3.15	5.25-6.91	5.69 $\pm$ 3.68	3.21-8.16	5.62 $\pm$ 2.83	4.05-7.19
<b>Total IgM (mg/mL)</b>	3.26 $\pm$ 3.28*	1.80-4.71	5.22 $\pm$ 3.95*	4.18-6.26	2.23 $\pm$ 2.26*	0.71-3.74	4.02 $\pm$ 3.92	1.85-6.19
<b>Total IgA (mg/mL)</b>	5.56 $\pm$ 2.04	4.65-6.46	5.86 $\pm$ 1.82*	5.38-6.34	5.46 $\pm$ 1.11	4.71-6.21	4.81 $\pm$ 1.38*	4.05-5.58
<b>Total IgG (mg/mL)</b>	17.73 $\pm$ 12.68	12.11-23.35	24.23 $\pm$ 17.1*	19.74-28.73	15.73 $\pm$ 11.35*	8.10-23.35	22.85 $\pm$ 12.76	15.78-29.91
<b>Ratio IgM (<math>\mu</math>g/mg)</b>	20.75 $\pm$ 25.75		10.42 $\pm$ 13.12*		26.36 $\pm$ 33.05*		30.79 $\pm$ 51.75*	
<b>Ratio IgA (<math>\mu</math>g/mg)</b>	0.49 $\pm$ 0.67		0.47 $\pm$ 0.63*		0.32 $\pm$ 0.24*		0.29 $\pm$ 0.16*	
<b>Ratio IgG (<math>\mu</math>g/mg)</b>	0.50 $\pm$ 0.78		0.96 $\pm$ 1.82*		0.79 $\pm$ 1.05		0.29 $\pm$ 0.17*	

n= number of samples; BT= biological therapy; CI= confidence interval; SD= standard deviation.

\* Values significantly different among groups (not specified) at p < 0.05 with Student's t test

+ Values significantly different to those of no biological therapy (No BT) group at p < 0.05 with Dunnett's t test

**Table 2. Titers of anti-CD26 Ig isotypes in RA patients under different therapies according to their smoking status**

		No BT (n=22)		Anti-TNF $\alpha$ BT (n=58)		Anti-CD20 BT (n=11)		Anti-IL6R/Ig-CTLA4 BT (n=15)	
		Mean $\pm$ SD (n)		Mean $\pm$ SD (n)		Mean $\pm$ SD (n)		Mean $\pm$ SD (n)	
<b>Anti-CD26 IgM (<math>\mu</math>g/mL)</b>	Non smokers	22.4 $\pm$ 18.0	(12)	25.4 $\pm$ 19.0	(33)	13.5 $\pm$ 7.7	(3)	37.1 $\pm$ 25.5	(10)
	Smokers	28.6 $\pm$ 3.8	(2)	33.0 $\pm$ 16.5	(13)	9.7 $\pm$ 9.1	(4)	-	-
	Past smokers	42.6 $\pm$ 21.7*	(8)	29.6 $\pm$ 18.0	(11)	25.6 $\pm$ 22.0	(4)	24.6 $\pm$ 8.3	(5)
<b>Anti-CD26 IgG (<math>\mu</math>g/mL)</b>	Non smokers	4.6 $\pm$ 3.2		5.9 $\pm$ 3.4		4.8 $\pm$ 4.0		5.7 $\pm$ 3.1	
	Smokers	8.1 $\pm$ 1.6		6.0 $\pm$ 2.9		4.0 $\pm$ 2.0		-	-
	Past smokers	6.4 $\pm$ 2.9		6.9 $\pm$ 2.7		7.9 $\pm$ 4.0		5.6 $\pm$ 2.7	
<b>Anti-CD26 IgA (<math>\mu</math>g/mL)</b>	Non smokers	1.6 $\pm$ 0.8		2.4 $\pm$ 1.6		1.4 $\pm$ 2.1		1.3 $\pm$ 0.8	
	Smokers	1.7 $\pm$ 1.4		2.1 $\pm$ 1.3		2.1 $\pm$ 0.8		-	-
	Past smokers	2.0 $\pm$ 1.0		2.4 $\pm$ 1.6		2.1 $\pm$ 1.2		1.5 $\pm$ 0.7	

n= number of samples; BT= biological therapy; SD= standard deviation;

\* Value significantly different at  $p < 0.05$  with Student's t test between groups classified according to their smoking status (past smokers *vs* non smokers). Groups were compared only inside each type of therapy.