The anti-CarP antibody response is of overall low avidity despite extensive isotype switching

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Abstract

Objective: To better understand the contribution of autoantibodies in RA and the biology of their responses, we evaluated the avidity of the anti-CarP antibody response.

Methods: The avidity of anti-CarP antibody, anti-citrullinated protein antibody (ACPA) and anti-Tetanus Toxoid (TT) IgG were determined using elution assays. Anti-CarP IgG avidity was measured in sera of 107 RA-patients, 15 paired synovial fluid and serum samples and 8 serially sampled sera before and after disease onset.

Results: The avidity of anti-CarP IgG is low compared to the avidity of anti-TT IgG present in the same sera. Likewise, although less pronounced, anti-CarP also displayed a lower avidity as compared to the avidity of ACPA IgG. No difference in anti-CarP IgG avidity is observed between ACPA positive or ACPA negative patients. Anti-CarP IgG avidity is higher in anti-CarP IgM-negative compared to IgM-positive individuals. Furthermore, the anti-CarP avidity in serum is higher than in synovial fluid. Using samples of individuals that over time developed RA we observed no anti-CarP avidity maturation in the years before disease onset. In contrast to ACPA avidity, the anti-CarP avidity is not associated with severity of joint destruction.

Conclusions: The anti-CarP response is of overall low avidity, even lower than the ACPA IgG avidity and does not show apparent avidity maturation before or around disease onset. Overall, isotype switch and avidity maturation seem to be uncoupled as isotype switch occurs without avidity maturation, pointing towards a commonality in the regulation of both autoantibody responses as opposed to the pathways governing recall responses.

Clinical trial registration number:

Informed consent was obtained for all individuals participating and all protocols were approved by the local ethic committee of the LUMC (P237-94) or by the Regional Ethics Committee at the University Hospital of Umea.

Keywords: Autoantibodies, anti-CarP antibodies, Rheumatoid Arthritis, antibody avidity, antibody avidity maturation

Key messages:

1. The anti-CarP response has a low IgG avidity, also in synovial fluid
2. No clear anti-CarP avidity maturation despite isotype switching, these processes seem to be uncoupled
3. Anti-CarP avidity is lower than the ACPA avidity, indicating a different regulation of both autoantibody-responses

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease mainly affecting synovial joints [1, 2]. Several autoantibodies have been identified in serum and synovial fluid (SF) of RA patients [3]. These may form immune complexes in the joints, leading to the attraction of immune cells through e.g. complement activation [4, 5] which can contribute to chronic inflammation and bone destruction.

Well-known autoantibodies that are currently used in the clinic for the diagnosis of RA are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)[2]. More recently, anti-carbamylated protein (anti-CarP) antibodies have been detected in RA [6]. These antibodies detect carbamylated proteins which are post-translationally modified proteins wherein lysines are converted to homocitrullines by a chemical reaction with cyanate [7, 8].

Various studies have shown a higher prevalence of anti-CarP antibodies in RA patients compared to controls [6, 9-14]. Several observations implicate a role for anti-CarP directed immunity in the pathogenesis of RA as anti-CarP antibodies; are present years before disease onset with a gradual increase before disease onset [9, 15, 16], are associated with the development of RA in arthralgia patients [17] and associate with increased joint destruction in RA [6, 9, 10, 12, 18]. Sera of RA patients can be positive for both anti-CarP antibody IgG and IgM [19], which is of interest as switching towards IgG is typically associated with a large decline or disappearance of IgM-responses in case of conventional T cell dependent antigen responses [20].

During a B cell response, somatic hypermutation, affinity maturation and isotype switching occurs in the germinal center. For somatic hypermutation, activated B cells will enter the germinal center and start to proliferate and undergo hypermutation upon receiving T-cell help. Studies using model antigens have shown that various B cell clones will compete for the antigen on follicular dendritic cells. Antibody avidity is defined as the overall binding strength of polyclonal (multivalent) antibodies to its multivalent antigens. Those B cells expressing the surface immunoglobulin that will bind with higher avidity, will outcompete other B cells because they attract more signals necessary

for B cell survival and proliferation. Due to this process, the avidity of the immune response increases over time and low avidity B cells will typically disappear from the circulation.

While substantial information is available on the avidity maturation of antibody responses against recall antigens [21-23], less information is present on avidity maturation of autoantibody responses. However, it is described that the avidity of autoantibodies (high, moderate and low) associates with different clinical outcomes in several diseases [24]. Interestingly, in celiac disease the avidity of autoantibodies targeting transglutaminase is reported to be much lower than the avidity of anti-*E coli* antibodies present in the same sera [25]. In addition, previous data of our group showed that the average avidity of ACPAs is much lower than the avidity of antibodies to recall antigens, even when many isotypes are used and levels are high [26]. These data indicate that although these B cells underwent isotype switching, and apparently attract sufficient signals for survival and proliferation, no or little avidity maturation was taking place. In a 'normal' B cell response; somatic hypermutation, isotype switching and avidity maturation are expected to occur side by side. ACPA can be detected many years prior to disease onset [27, 28] and during these years there is (limited) avidity maturation for the ACPA response [29]. Interestingly the patients with the lowest ACPA avidity experienced the most pronounced joint destruction [30]. A low avidity does not mean that these antibodies are non-pathogenic as low avidity antibodies have for example enhanced capacity to penetrate deeper into tissue [31], and an enhanced capacity to activate complement [30]. Citrullination and carbamylation are two rather similar post-translational modifications, and although antibodies against both modifications are often seen together in RA, they represent two different antibody families as also ACPA or anti-CarP single positive patients are present and cross-reactivity towards the two modifications is incomplete [3].

Based on these previous observations and our interest in understanding the biology of the anti-CarP antibody response, we have studied the avidity of the anti-CarP antibody response in detail using baseline serum samples of RA patients with long-term follow-up data in the Leiden Early Arthritis Clinic (EAC) cohort [32] as well as samples from patients in the phase before diagnosis [9, 28, 33].

We show here that the anti-CarP antibody avidity is low, even lower than the ACPA avidity and that anti-CarP antibody IgG and IgM positive patients have a lower anti-CarP IgG avidity compared to anti-CarP antibody IgG positive and IgM negative patients.

Methods

Patients and control sera

Sera of 107 RA patients (average age 55.8 and 64.5% female) were analysed for the anti-CarP antibody IgG avidity. As control, sera of 86 RA patients (average age 47.8 and 59.3% female) and next to this 34 age and sex matched RA patients with healthy controls (HC) (average age 45 and 61.7% female) were analysed for anti-tetanus toxoid (TT) IgG avidity as recall antigen. Anti-CarP antibody [6, 19] and ACPA status were already available [19, 34] and used in these analysis. Furthermore, radiographs taken at yearly intervals were available for almost all tested RA patients. Baseline sera of RA patients participating in the EAC cohort [32] were analysed. RA patients were included between 1993 and 2003 and inclusion required a symptom duration <2 years [32]. Healthy control samples were acquired from persons living in the Leiden region as described before [6]. Informed consent was obtained for all individuals and all protocols were approved by the ethics committee of the LUMC.

Paired serum and synovial fluid (SF) samples of 29 RA patients were analysed for anti-CarP antibody IgG avidity. Samples were kindly provided by RA patients and informed consent was signed.

Sera of 8 anti-CarP antibody IgG positive RA patients, sampled before and after symptom onset, were analysed for anti-CarP antibody IgG avidity maturation. These individuals were participating in the Medical Biobank of Northern Sweden or the Mamography screening project [9, 28, 33]. Informed consent was obtained for all individuals when donating blood and all protocols were approved by the Regional Ethics Committee at the University Hospital of Umea, Sweden. Samples

were also collected from these individuals when they were diagnosed with RA later at the Early Arthritis Clinic.

Measurement of anti-CarP antibody and anti-Tetanus Toxoid IgG avidity

To determine the avidity of anti-CarP antibody IgG, ACPA IgG and anti-TT IgG, elution ELISA assays were used [26, 35]. For anti-CarP antibody the appropriate serum dilution was determined by performing a titration using the anti-CarP IgG ELISA [6]. For ACPA and anti-TT IgG, this was done as previous described [26]. The serum dilution at which the response was in the linear part of the curve with an absorbance value around 1.5 at 415nm was selected as optimal. The minimal dilution we used in the avidity assay was 1:12.5.

Protein carbamylation and citrullination and verification of the modification were done as before [6, 19, 36, 37]. To determine the anti-CarP antibody and ACPA IgG avidity, in house coated CCP2 (1µg/ml) [19], citrullinated foetal calf serum (10µg/ml) (Cit-FCS, Bodinco), carbamylated foetal calf serum (10µg/ml) (Ca-FCS, Bodinco) or Carbamylated alpha-1-antitripsin (10µg/ml) (Ca-A1AT, Lee biosolutions) plates were used and incubated with the appropriate serum dilutions. After washing, the wells were incubated with increasing concentrations of chaotropic agent sodiumthiocyanate (NaSCN;Sigma Aldrich) of 0.25, 0.5, 1, 2, 3, 5M, for 15min at room temperature (RT). After washing the bound antibodies were detected using HRP-labelled rabbit anti human IgG (Dako P0214). The amount of antibodies bound to the plate with and without elution by NaSCN were determined using a standard curve.

The percentage restbinding, defined as the ratio of the amount remaining antibodies to an antigen at a certain molarity NaSCN to the amount of bound antibodies in the absence of NaSCN, was calculated [26, 38]. The relative avidity index (AI) is defined as the ratio of the amount remaining antibodies to an antigen at 1M NaSCN to the amount of bound antibodies in the absence of NaSCN, expressed as percentage [26, 38].

The anti-TT IgG avidity was determined using an in-house ELISA as previously described [26, 35].

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) version 23 (IBM). In order to determine differences in antibody avidity between anti-CarP antibody IgG and anti-TT IgG in RA patients, anti-TT IgG avidity in RA patients and HC or anti-CarP IgM/IgA positive and negative RA patients , Mann-Whitney U tests were carried out. In order to investigate whether there are correlations between anti-CarP levels and AI, between anti-CarP AI and ACPA AI and between anti-CarP AI in SF and serum, Spearman Rank tests were performed. To study whether the presence of more anti-CarP isotypes associates with antibody avidity, Kruskal-Wallis tests were performed. Wilcoxon signed ranks test were performed to investigate differences in paired samples. P values below 0.05 were considered statistically significant.

In 107 RA patients, divided in quartiles of 27 patients based on the AI of anti-CarP IgG, the association between anti-CarP antibody IgG avidity and radiographic progression, as assessed by the Sharp-van der Heijde Score [39], was analysed as described before [6, 19, 32, 40]. As repeated radiographs were taken at yearly intervals we have used a multivariate normal regression analysis for longitudinal data. Adjustments for treatment strategy, age and sex had been made. P values below 0.05 were considered statistically significant.

Results

The avidity of anti-CarP is low compared to the avidity of tetanus toxoid

Anti-CarP antibodies can be detected using several antigens in ELISA [6, 36]. Here we have analysed the avidity of anti-CarP antibodies based on Ca-FCS and Ca-A1AT detection. The anti-CarP IgG avidity was compared to the IgG avidity directed against the recall antigen TT. Using increasing concentrations of chaotropic salt in the elution assays we observed a low avidity for antibodies against carbamylated proteins and as expected a high avidity for antibodies directed against the

188 recall antigen TT (Figure 1A). Within the same patients, similar results were found using Ca-FCS or
189 Ca-A1AT as an antigen (Figure 1A). As there were no major differences in the avidity of anti-CarP
190 antibodies as detected by Ca-A1AT or Ca-FCS, we decided to use Ca-FCS as the antigen to study the
191 anti-CarP avidity in a larger cohort.
192 Analysing 107 RA patients positive for anti-CarP IgG antibodies revealed that the avidity of anti-CarP
193 IgG antibodies is generally low (median AI 21.1%). In contrast the IgG avidity against the recall
194 antigen TT was considerably higher (median AI 99.6%) (Figure 1B). We tested whether patients with
195 higher levels of anti-CarP antibodies, as a sign of a more pronounced anti-CarP response, would also
196 have a higher avidity. However, we observed no correlation between the levels and avidity of anti-
197 CarP antibody IgG (Figure 1C) and patients with a high level do also display a low avidity. We verified
198 whether RA patients were actually capable of mounting a proper avidity response by comparing the
199 anti-TT avidity of patients to the anti-TT avidity of healthy controls and observed no difference (data
200 not shown).
201 To summarize, anti-CarP IgG antibodies present in sera of RA patients are of low avidity as compared
202 to the avidity of anti-TT IgG, irrespective of anti-CarP levels.
203
204 *Lower anti-CarP IgG avidity in anti-CarP IgM positive RA patients*
205 The anti-CarP antibody response comprises several isotypes and a substantial proportion of the
206 patients is double positive for the anti-CarP antibody IgG and IgM [19]. This is interesting as
207 switching towards IgG is typically associated with disappearance of IgM-responses and the
208 appearance of high avidity IgG antibodies in case of T cell dependent antigen responses [20].
209 Therefore we hypothesized that RA patients positive for anti-CarP antibody IgG and IgM have a more
210 actively ongoing, immature, anti-CarP antibody response. In such a scenario, the anti-CarP IgG
211 antibody avidity is conceivably lower in the anti-CarP IgM positive group compared to the anti-CarP
212 IgM negative group. To test this hypothesis, the RA patients analysed were subdivided in an anti-
213 CarP antibody IgM positive and negative group. RA patients positive for anti-CarP IgM showed a

slight but significant lower anti-CarP IgG avidity (median AI 17.7%) compared to the anti-CarP IgM negative patients (median AI 24.2%) (Figure 1D). This effect was not found between anti-CarP antibody IgA positive or negative patients, indicating that it is specific for IgM. Furthermore, there was no difference in disease duration between the anti-CarP antibody IgM positive (median 24.1 weeks) and negative patients (median 23.4 weeks) indicating that this is not a reflection of a shorter disease process.

Importantly, the anti-CarP IgG avidity is similar in IgM depleted or non-depleted serum suggesting that the presence of IgM is not interfering with the IgG affinity measurement (Supplementary figure 1).

Overall, these data indicate that a less differentiated antibody response, still including IgM, associates with lower anti-CarP IgG avidity.

Anti-CarP IgG avidity is lower than the avidity of ACPA

Previous data from our group indicates that also the ACPA IgG avidity is low in RA patients [26]. To determine whether the anti-CarP antibody and ACPA IgG avidity is similar, an initial group of 4 patients double positive for ACPA and anti-CarP antibodies were tested for the avidity of the anti-CarP-, ACPA- and anti-TT IgG- response using several concentrations of chaotropic salt in the elution assay. We observed that in almost all patients analysed the avidity of anti-CarP antibodies was lower as compared to ACPA and anti-TT. Data of one representative patient is depicted in figure 2A. Next an additional 18 patients, double positive for anti-CarP and ACPA IgG, were analysed for the anti-CarP, ACPA and anti-TT IgG avidity using 1M NaSCN. The results of these analyses show that both anti-CarP antibody and ACPA IgG are of low avidity compared to the avidity of anti-TT IgG ($p<0.001$ for anti-CarP and $p=0.0014$ for ACPA) (Figure 2B). However, interestingly, the anti-CarP IgG avidity was even lower than the ACPA IgG avidity. Moreover, no obvious correlation is found between the avidity of anti-CarP IgG and ACPA IgG (Figure 2C) and we could not detect a difference in anti-CarP IgG avidity between patients positive or negative for ACPA (n=107) (Figure 2D).

To summarize, the avidity of anti-CarP is lower compared to ACPA and antibody avidities are not associated with each other.

Anti-CarP avidity is slightly higher in serum compared to synovial fluid

To study whether anti-CarP antibodies present in the SF of inflamed joints may have a different avidity than the anti-CarP antibodies in the circulation, we compared the anti-CarP IgG avidity in SF to the avidity of anti-CarP in paired sera that were collected at the same time point as the SF. These analyses revealed a slightly, but consistently, lower anti-CarP IgG avidity in SF (Figure 3A), with the anti-CarP IgG avidity in SF correlating to the anti-CarP IgG avidity in sera (correlation coefficient 0.6089, $p<0.001$) (Figure 3B). Blocking experiments were performed to investigate whether the difference in avidity could be explained by the presence of carbamylated antigens, to which the high avidity anti-CarP IgG might have bound. Paired serum was pre-incubated with or without Ca-FCS or FCS and the avidity was analysed. However, no difference in avidity was observed between the different conditions (data not shown).

No evidence for anti-CarP avidity maturation before disease onset

RA-associated autoantibodies, including anti-CarP antibodies can be detected many years prior to disease onset [9, 16] and for ACPA we have shown previously that there is (limited) avidity maturation taking place during this 'pre-RA' period [29]. As we observed that RA patients display a certain range of low to moderately high anti-CarP IgG avidity at baseline, this might indicate that a certain degree of avidity maturation has occurred in a proportion of the anti-CarP antibody IgG positive patients. Therefore we next wished to study the avidity maturation in longitudinal samples of early RA patients collected before and after the symptom onset [9, 33]. The samples were available over a period of 15 years. Overall, we observed no anti-CarP IgG avidity increase during the period before symptom onset (Figure 4).

Anti-CarP IgG avidity is not associated with more severe joint destruction

Previous data of our group showed that the presence of anti-CarP IgG at baseline associates with a more severe joint destruction over time [6]. Furthermore, we have shown that low avidity ACPA IgG at baseline associates with more radiological damage over time[30]. Next to these findings in RA, it has also been shown that antibody avidity associates with different clinical outcomes in other autoimmune diseases [41-43]. Therefore we investigated whether the high or low anti-CarP IgG avidity associates with more severe joint destruction over time. However, when we compared the joint destruction and anti-CarP avidity, we observed no difference in severity at baseline or over time between the different anti-CarP avidity quartiles (Figure 5).

Discussion

In this study we observed that the anti-CarP IgG avidity is low, in both serum and SF, compared to the avidity of the recall antibody against TT. Furthermore, the anti-CarP IgG avidity seems to be even lower than the ACPA IgG avidity and no anti-CarP avidity maturation has been observed. These data indicate that the regulation of the anti-CarP immune response is different from the regulation of recall responses and the ACPA response.

Although substantial information is available on the avidity (maturation) of antibody responses against recall antigens [24], less information is present on avidity (maturation) of autoantibody responses. It is known that autoantibodies have different avidities (high-, moderate- or low) in autoimmune diseases and different autoantibody avidities associates with a more or less severe disease course (reviewed in [24]). In a few studies, the autoantibody avidity is compared to the avidity of recall antibodies [25, 26] and these studies revealed a low avidity of autoantibodies compared to recall antibodies. We also found in this study a low avidity for anti-CarP antibodies compared to antibodies against TT. This suggests that autoantibodies are overall of low avidity compared to recall antibodies and within this avidity range the avidity of autoantibodies associates with various clinical outcomes. Importantly, a low avidity of autoantibodies does not indicate that

these antibodies are not relevant, as we know that the presence of both ACPA and anti-CarP is associated with e.g. disease development [17] and with severity of the disease [6, 9, 10, 12, 18]. It does however imply that the mechanisms driving the production of protective vaccine antigens is very different from the mechanisms that lead to the production of autoantibodies. Interestingly in this study is the lower anti-CarP IgG avidity in patients positive for anti-CarP antibody IgM. This cannot easily be explained by competing influences for binding of anti-CarP IgM with IgG, as in IgM depleted serum the anti-CarP IgG avidity is similar to total serum. This suggests limited competition for binding between anti-CarP IgM and IgG. In addition, in the context of ACPA avidity, we have previously compared whether the ACPA IgG avidity as measured in serum would be different from that of purified IgG. In ACPA IgG and IgM double positive patients we observed no differences in ACPA IgG avidity in total serum or purified IgG [26]. This indicates that the presence of IgM does not impact on the measured IgG avidity. As a possible explanation for the concomitant presence of anti-CarP IgM with low avidity anti-CarP IgG we consider it conceivable that these patients display a less mature response, as anti-CarP IgG single positive or anti-CarP IgG and IgA double positive patients have overall higher anti-CarP IgG avidities (data not shown). Importantly, there is no difference in symptom duration between anti-CarP IgM positive and negative patients from the EAC. Indicating that anti-CarP IgG avidity is not related to time but rather to maturation of the anti-CarP response. Whether patients with a less mature response at baseline will undergo this maturation later during the disease progression is unknown.

Furthermore, we found that in baseline serum samples of ACPA and anti-CarP IgG double positive patients, the ACPA IgG avidity is higher than the anti-CarP IgG avidity. This might suggest that the ACPA response is differently regulated than the anti-CarP response.

Moreover, despite isotype switching before disease onset [19], no evidence for anti-CarP IgG avidity maturation was observed before symptom onset; however some patients might have a minimal avidity increase after symptom onset. All together, these data suggest that the isotype switch and avidity maturation in the anti-CarP B cell response are uncoupled. This uncoupling could be

318 explained by various non-excluding mechanisms. For example, it might be due to a difference in
319 additional stimulation of the B cells; such as the degree of innate or T cell help (reviewed in [44])
320 and/or the abundance of its antigen. It could be that low avidity antibodies are a marker for chronic
321 antigen overload and chronic antibody responses. Another option could be that anti-CarP antibodies
322 and ACPA cross react with another antigen, which is currently unknown, to which the antibodies
323 might have a more "normal" response.
324 In this study we also investigated whether the anti-CarP antibody avidity associates with joint
325 damage over time. The presence of anti-CarP antibodies, especially in the ACPA negative stratum, is
326 associated with a more severe disease course [6, 19]. When investigating anti-CarP avidity, we did
327 not observe an association with joint damage. This is different from our previous observations
328 regarding ACPA where low avidity ACPA associates with more radiological damage at baseline and
329 over time [30]. Furthermore, low avidity ACPA was a better complement activator compared to high
330 avidity ACPA [30]. Therefor ACPA avidity might be of clinical relevance for the determination of RA
331 patients at risk for a more severe disease course. Moreover, low avidity antibodies might be better
332 in tissue penetration, as shown for anti-tumour antibodies [31], and possibly resulting in immune
333 activation at a deeper location. For ACPA we observed that especially the patients with very low
334 avidity ACPA presented with severe radiological damage [30]. Also anti-CarP antibodies are
335 associated with radiological damage [6] and since the avidity of the anti-CarP response is as low as
336 the lowest avidities for ACPA it is conceivable that this explains why for anti-CarP we do not observe
337 an association between the avidity and severity of joint destruction.
338 To conclude, the anti-CarP IgG avidity is low compared to the avidity against the recall antigen TT
339 and points to a different regulation of anti-CarP antibody responses as compared to anti-TT
340 responses. This is also indicated by the uncoupling of avidity maturation despite extensive isotype
341 switching.
342

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Authors' contributions

MVD set up the study design and performed the experiments, as well as the analysis, interpretation of the data, drafting the article, and approval of the final manuscript. MV contributed to study design and experiments, interpretation of the data, revising the manuscript and approval of the final manuscript. LB and AVDH contributed to the analysis, interpretation of the data, revising the manuscript and approval of the final manuscript. SRD, RT and TH contributed to the interpretation of the data, revising the manuscript and approval of the final manuscript. LT contributed to study design, interpretation of the data, revising the manuscript and approval of the final manuscript.

Disclosure statement

TWJH, REMT and LAT are listed as inventors in a patent application regarding the detection of anti-CarP antibodies for RA.

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Figure legends

Figure 1. The anti-CarP IgG avidity is low and lower in anti-CarP IgM positive RA. Elution ELISA assays were performed to test the anti-CarP antibody IgG avidity on Ca-FCS and Ca-A1AT and TT IgG avidity in sera of 4 RA patients. The percentage restbinding at various molarities of NaSCN is depicted (A). anti-CarP and recall IgG avidity was tested in RA patients (n=107) using Ca-FCS as antigen for anti-CarP IgG and TT as recall antigen (B). The anti-CarP antibody IgG avidity doesn't correlate with anti-CarP antibody IgG levels (AU/ml) (C). The anti-CarP IgG avidity was investigated between anti-CarP IgM or IgA positive and negative patients. The anti-CarP antibody IgG avidity is lower in anti-CarP antibody IgM positive patients (IgM+ n=30, IgM- n=77, IgA+ n=65, IgA- n=41) (D). The avidity index (AI) is depicted as % restbinding at 1M NaSCN (B-D). anti-CarP antibody; anti-carbamylated protein antibody, Ca-FCS; carbamylated fetal calf serum, Ca-A1AT; carbamylated alpha-1-antitripsin, TT; tetanus toxoid, RA; rheumatoid arthritis, NaSCN; sodiumthiocynate, AU/ml;

arbitrary units per millilitre, AI; avidity index. Mann-Whitney test (B, D) *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001, Spearman correlation (C).

Figure 2. The anti-CarP avidity is lower than the ACPA avidity and similar in ACPA positive/negative RA. An initial group of 4 RA patients was tested in titration for the anti-CarP antibody, ACPA and anti-TT IgG avidity. One representative patient is shown (A). Anti-CarP antibody, ACPA and anti-TT IgG avidity in 18 double positive RA patients (B). The anti-CarP antibody and ACPA IgG avidity do not correlate (C) and there is no difference in anti-CarP antibody IgG avidity between patients positive or negative for ACPA (n=107) (D). The avidity index (AI) is depicted as % restbinding at 1M NaSCN. anti-CarP antibody; anti-carbamylated protein antibody, TT; tetanus toxoid, RA; rheumatoid arthritis, ACPA; anti citrullinated protein antibody, AI; avidity index. Mann-Whitney test (B, D) *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001, Spearman correlation (C).

Figure 3. Anti-CarP IgG avidity is slightly lower in synovial fluid compared to serum. To investigate whether the anti-CarP antibody IgG avidity differs between serum and SF, SF and paired sera samples of 29 RA patients were tested (A). The anti-CarP antibody IgG avidity in SF correlates the avidity in serum (B). The avidity index (AI) is depicted as % restbinding at 1M NaSCN. Anti-CarP antibody; anti-carbamylated protein antibody, SF; synovial fluid, RA; rheumatoid arthritis, AI; avidity index. Wilcoxon signed ranks test (A) *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001, Spearman correlation (B).

Figure 4. No evidence for anti-CarP avidity maturation before symptom onset. To investigate the anti-CarP antibody IgG avidity maturation, 8 anti-CarP antibody IgG positive RA patients, sampled before and after symptom onset, were analysed. No anti-CarP antibody IgG avidity was detected before symptom onset. The avidity index (AI) is depicted as % restbinding at 1M NaSCN. anti-CarP antibody; anti-carbamylated protein antibody, RA; rheumatoid arthritis, AI; avidity index.

Figure 5. Anti-CarP avidity is not associated with more radiological damage. The extent and rate of joint destruction were analysed in all RA patients. RA patients were subdivided in anti-CarP IgG avidity quartiles with equal numbers of patients in each quartile. The severity of joint damage is depicted as median Sharp-van der Heijde score (SHS) on the y-axis and the follow-up years on the x-axis. RA; rheumatoid arthritis, anti-CarP antibody; anti-carbamylated protein antibody, AI; avidity index.

Supplementary material

Supplementary figure 1. Anti-CarP IgG avidity in start and IgM depleted serum. To investigate whether the presence of anti-CarP IgM is influencing the anti-CarP IgG avidity measurement, sera of RA patients (n=11) was IgM depleted (A). The anti-CarP IgG avidity was similar in IgM depleted and start serum (B) and correlate well (C). The anti-CarP IgG avidity in this second measurement correlates with the avidity measured in the big cohort (first measurement) (D). The avidity index (AI) is depicted as % restbinding at 1M NaSCN. Anti-CarP antibody; anti-carbamylated protein antibody, RA; rheumatoid arthritis, AI; avidity index. Spearman correlation (C,D).

Figure 1 M.A.M. van Delft

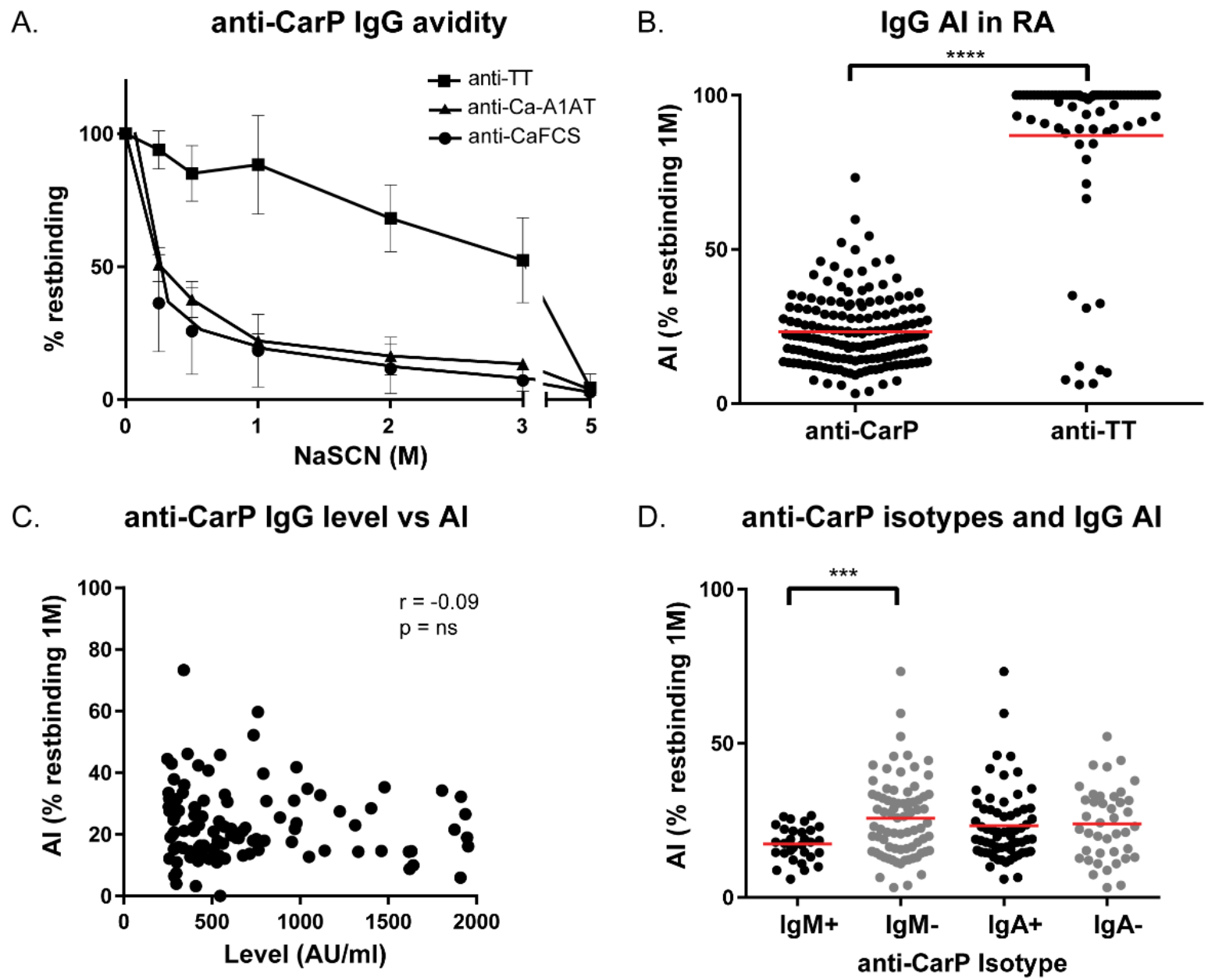
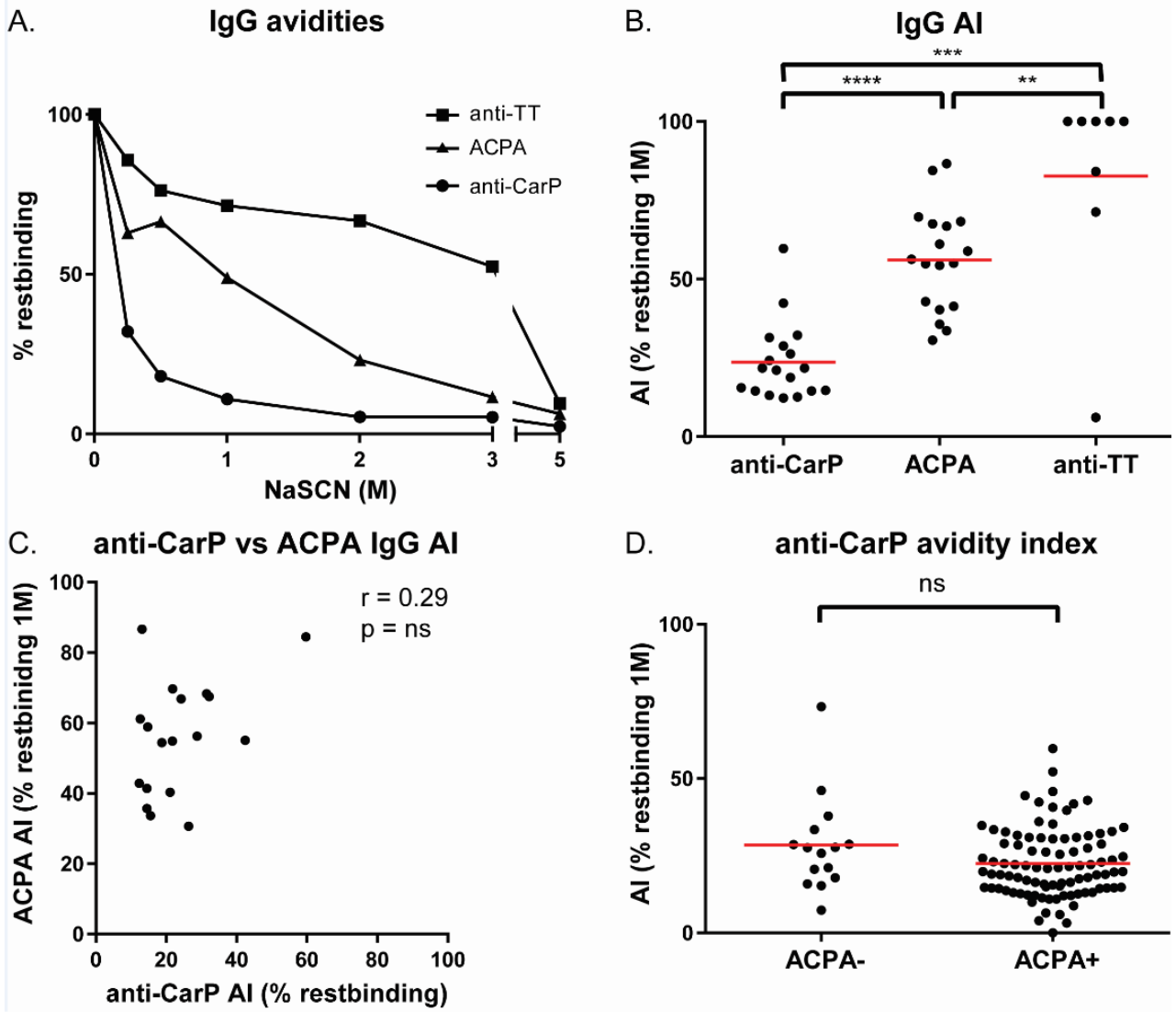


Figure 2 M.A.M. van Delft



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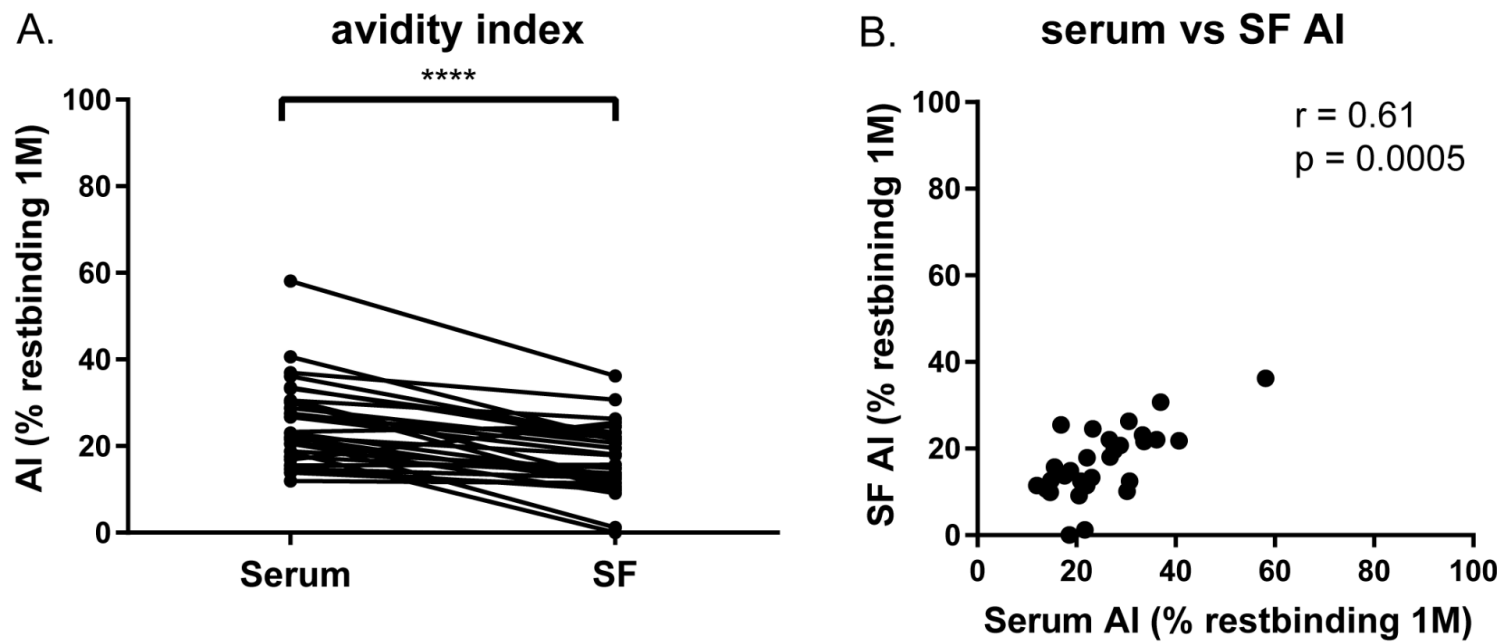

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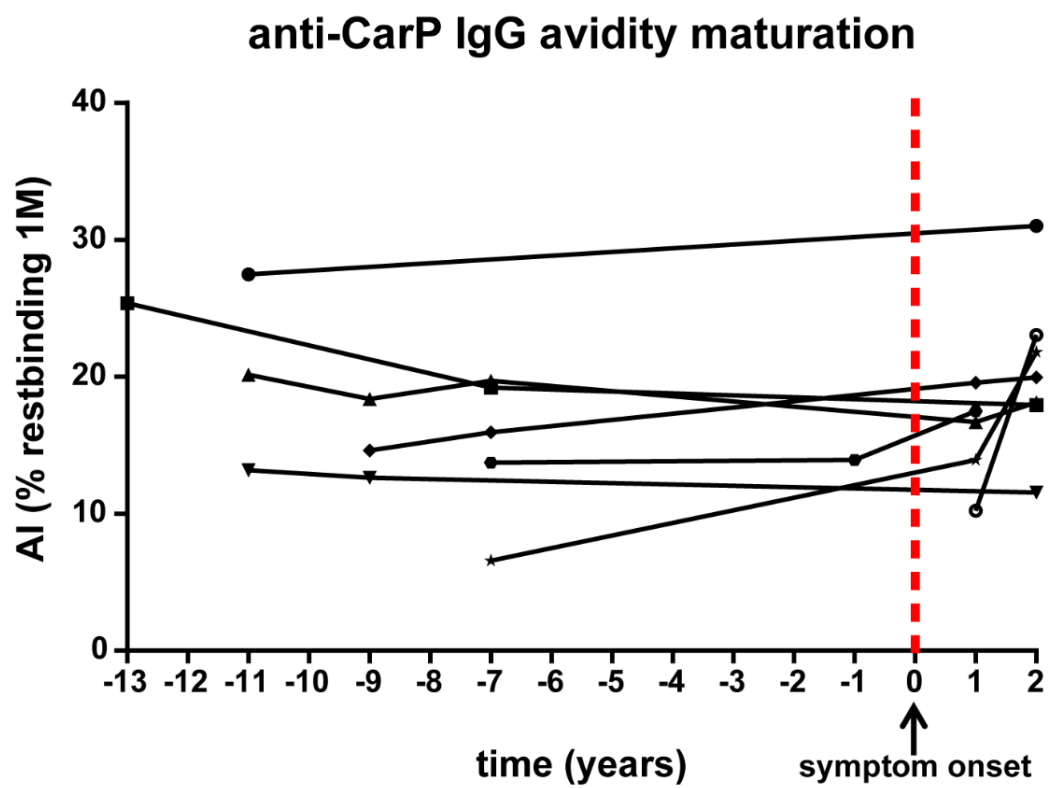
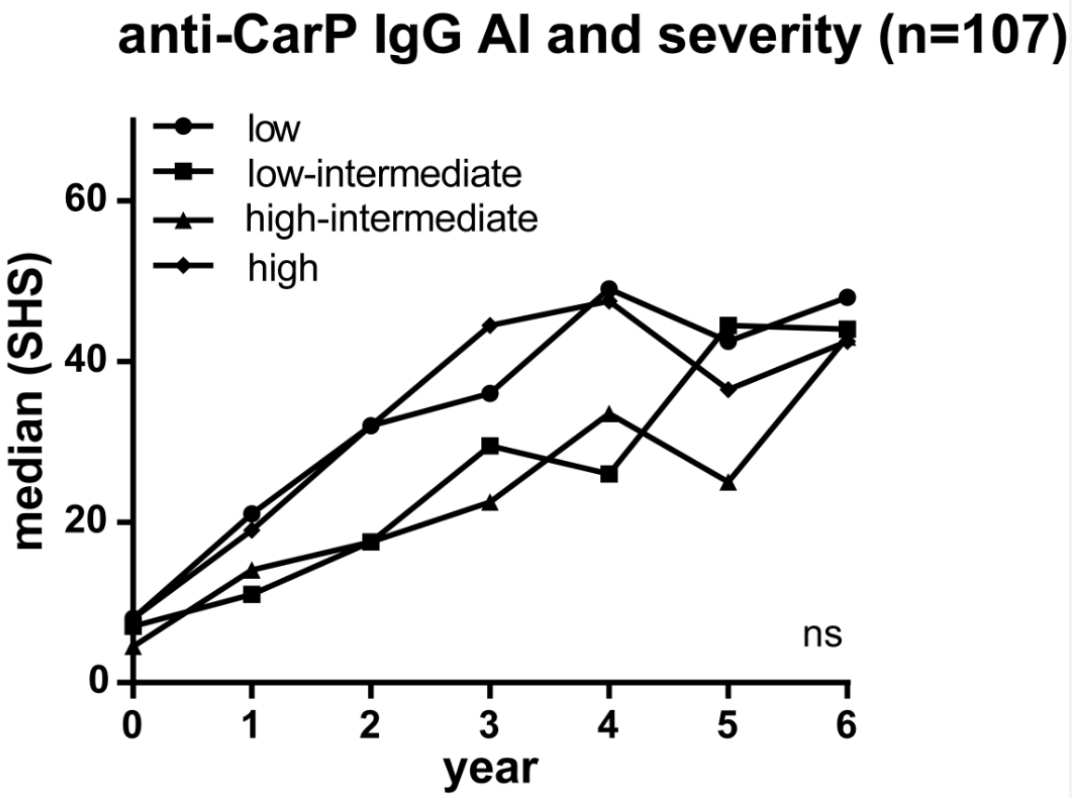
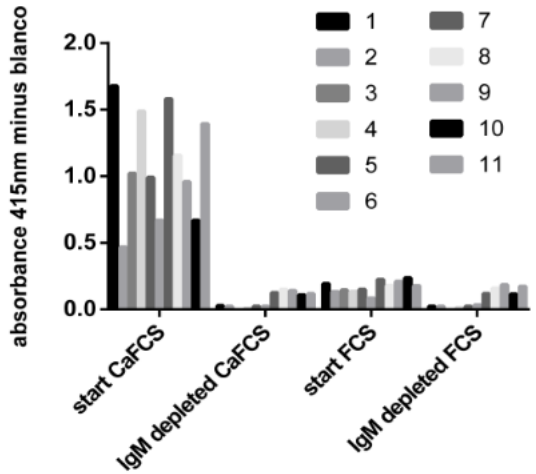


Figure 5. M.A.M. van Delft

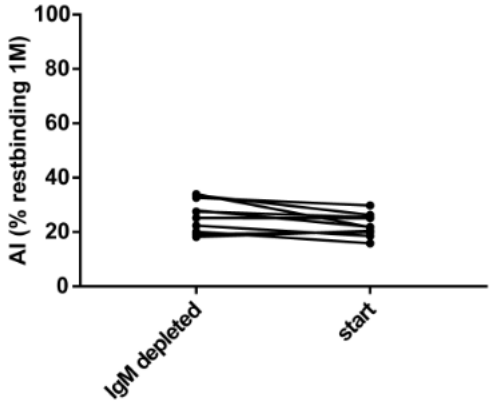


Supplementary figure 1. **MAM van Delft**

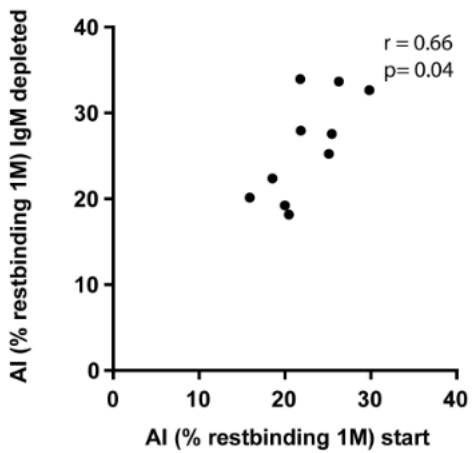
A. **anti-CarP IgM in start and IgM depleted serum**



B. **anti-CarP IgG avidity IgM depleted and start**



C. **correlation AI IgM depleted and start**



D. **correlation start first and second measurement**