Short Communication

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Journal of Molecular Sciences

2018
Vol.2 No.1:5

Low Serum Levels of Immunoglobulin D Recognize Autoantibody Production in Rheumatoid Arthritis

Gravina G[1]*, Erlandsson MC[1,2], Bossios A[3], Ekerljung L[4], Malmhäll C[4], Lundbäck B[4], Silfverswärd ST[1], Pullerits R[1,2] and Bokarewa MI[1,2]

1 Department of Rheumatology and Inflammation Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
2 Rheumatology Clinic, Sahlgrenska University Hospital, Gothenburg, Sweden
3 Department of Medicine, Unit for Heart and Lung disease, Karolinska University, Stockholm, Sweden
4 Department of Internal Medicine and Clinical Nutrition, Krefting Research Centre, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Sweden

***Corresponding authors:**
Giacomo Gravina

giacomo2392@gmail.com

Department of Rheumatology and Inflammation Research, University of Gothenburg, Box 480, SE 405 30 Göteborg, Sweden.

Tel: +46-31-34224021

Citation: Gravina G, Erlandsson MC, Bossios A, Ekerljung L, Malmhäll C, et al. (2018) Low Serum Levels of Immunoglobulin D Recognize Autoantibody Production in Rheumatoid Arthritis. J Mol Sci. Vol.2 No.1:5

Abstract

Background: Immunoglobulin D (IgD) remains an enigmatic molecule due to the limited understanding of its function both in healthy and in patients with autoimmune diseases. In this study, we analyse serum IgD (sIgD) levels in rheumatoid arthritis (RA), paying special attention to clinical and serologic features of RA and treatment.

Methods and finding: Serum levels of IgD, IgM, IgG and IgA were measured in 416 subjects (248 RA patients and 169 healthy controls matched by age and gender), by sandwich ELISA. Here, we show that low IgD, but not IgM, IgG and IgA, is associated with female gender and with the presence of RA-specific autoantibodies. Most prominent reduction of sIgD was found in the patients producing rheumatoid factor, alone (p=0.009) and in combination with antibodies to cyclic citrullinated peptides (p= 0.02). Low sIgD was measured in female RA patients below 50 years (p=0.01), but not in healthy females. Additionally, low sIgD was measured in RA patients treated with a combination of methotrexate and sulfasalazine/hydroxichloroquine compared to those receiving methotrexate and biologics (p=0.01).

Conclusion: In RA patients, sIgD levels present clinical associations which distinct it from IgM, IgG and IgA. Low serum IgD levels appears to be pathological and is associated with autoantibodies and certain anti-rheumatic treatment.

Keywords: Rheumatoid arthritis; Rheumatoid factor; Acpa; Treatment

Received: February 01, 2018; **Accepted:** March 01, 2018; **Published:** March 08, 2018

Introduction

Immunoglobulin D (IgD) was first discovered in 1965 by Rowe and Fahey in myeloma patients [1]. Despite significant efforts, its role in the immune system today remains a mystery. Since the measurement of serum IgA, IgG, IgM and IgE is broadly used for the diagnosis of pathologies in the clinical practice, the measurement of serum IgD (sIgD) is often restricted to research purposes. Serum IgD is detectable in adult human serum although it has lower levels than IgM, IgG and IgA [2]. In childhood, its levels are low or undetectable [3].

On B cells surface, IgD is usually co-expressed with IgM and exerts its function as the B Cell Receptor (BCR) [4]. B cells undergo the initial maturation in the bone marrow, where the membrane-bound IgD expression serves an important marker for the B cells leaving for the spleen or secondary lymphoid organs. After antigenic stimulation, mature B cells shut down membrane-bound IgD expression [5]. Despite the tight regulation of membrane IgD, its function on the B cells surface is not completely understood, opening new frontier of investigations.

The C-terminal part of the membrane-bound IgD is identical to IgM in humans. Findings from genetically modified mice lacking expression of IgD and IgM showed an exchangeable role of these two immunoglobulins. In IgM knock-out mice, IgD replaces the absence of IgM, without any effects on the morphology and the number of cells in lymphatic organs. On the other hand, lack of IgD is largely substituted by an increased amount of IgM [6-8]. Additionally, the analysis of the stability of IgM and IgD reveals that IgD is more stable at the cell surface than IgM. This high stability could explain the high turnover of IgM compared to IgD [9].

Rheumatoid Arthritis is an inflammatory autoimmune disease, present in 0.5-1 % of the population [10] which affects joints and leads to the cartilage and bone destruction. Although the cause of RA is not known, environmental factors, genetic and epigenetic modifications guiding the formation of autoreactive B cells, production of antibodies towards arthritis-specific antigens are found years ahead and predispose patients to develop RA [11]. Therapeutic elimination of the autoantibody producing B cells results in reduction of the RA activity leading often to clinical remission [12,13]. Studies on the bone marrow B cells of RA patients identified the IgD expressing B cells as the major target of successful B cell depleting therapy in RA [14].

Since the pathogenesis of RA is not completely understood and IgD seems to be an enigmatic and neglected molecule, we investigated the serum IgD levels in relation to demographic characteristics and clinical and serologic features of RA patients.

Material and Method

Patients and controls

Two hundred forty eight patients fulfilling the classification criteria of RA [15] were randomly selected from the methotrexate (MTX) treated patient cohorts attending the Rheumatology Clinics at the Sahlgrenska University Hospital in Gothenburg and the Northen Älvborg Hospitals in Uddevalla. The clinical characteristics of RA patients are shown in **Table 1**. At the time of enrolment, all but 12 patients were treated with MTX. In total, 115 patients were treated with MTX monotherapy, additional 73 patients received MTX in combination with biological drugs (infliximab, rituximab, etanercept, adalimumab, golimumab, tocilizumab, abatacept), and 27 patients had MTX in combination with other disease modifying drugs (sulfasalazine, hydrochloroquine and both).

One hundred sixty eight healthy controls (HC) were randomly selected from the participants of the West Sweden Asthma epidemiological study [16] to match the RA patients by age and gender.

The Ethical Evaluation Board at the University of Gothenburg approved the study. All the patients gave the written informed consent to the participation in the study.

Clinical and laboratory assessment

Clinical activity of RA was calculated at the time of blood sampling based on the number of swollen and tender joints, erythrocyte sedimentation rate (ESR) and global health assessment of the patient, and the disease activity score (DAS28) was constructed [17].

Blood samples were obtained from the cubital vein from the RA patients and controls. All blood samples were centrifuged at 800 × g for 15 min, aliquoted, and stored frozen at −70°C until use.

Measurement of serum levels of IgD

Serum levels of IgD were analysed by sandwich enzyme-linked immunosorbent assays (ELISAs) using matched pairs of specific antibodies and standard. For measuring the levels of sIgD, the capture antibodies were mouse anti-human IgD (SouthernBiotech, Birmingham, Alabama) in PBS overnight at 4°C. Serum samples were diluted 1/5000 in 1% BSA and 0.25% tween 20 (Sigma Aldrich, Saint Louis, Missouri, USA) in PBS. Standard, consisting of human serum with known IgD concentration (Siemens, OTRD, Munich, Germany), was serially diluted in the same buffer and incubated for 2 hours at room temperature. Levels of antibodies were detected using biotinylated goat anti-human IgD (SouthernBiotech) and incubate for 2 hours at room temperature. 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Aldrich) was used as the developer and plates were read at 450 nm. Detection limit of the assay was 0.08 µg/ml.

Measurement of serum levels of IgM, IgG, IgA

For the measurement of IgM, IgG and IgA, plates were coated with $F(ab')_2$ goat anti-human IgA, IgG, IgM (109-006-064, Jackson ImmunoResearch, West Baltimore Pike, West Grove, PA, USA) in PBS overnight at 4°C. Serum samples were assayed in serial dilutions in the range from 1/1000 to 1/8000000 in 1% BSA and 0.25% tween 20 in PBS and incubated for 2 hours at room temperature. Levels of antibodies were detected using biotinylated $F(ab')_2$ goat anti-human IgA, IgG and IgM (all from Jackson ImmunoResearch) for 2 hours at room temperature. 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Aldrich) was used as the developer and plates were read at 450 nm. The detection limits of the assays were 0.039, 0.29 and 0.06 optical density 405 nm respectively for IgM, IgA and total IgG. The results were analysed in the absorbance values obtained during simultaneous measurement for all RA patients and the healthy controls in serum dilution of 1/6400 for IgM, 1/32000 for IgA and 1/8000000 for IgG.

Analyses of ACPA and RF

The measurements of ACPA and RF were performed at the accredited laboratories of Clinical Immunology at the Sahlgrenska University Hospital [18]. ACPA was detected using an automatic multiplex method (anti-CCP2, BioRad, Hercules, CA). The cut-off level above 3.0 IU/ml of IgG ACPA was set positive by the manufacturers and verified on healthy individuals. Antibodies against Fc-region of gamma globulin (RF total) were measured by the rate nephelometric technology (Beckman Immage 800, Beckman Coulter AB, Brea, CA). The cut-off for RF positivity was set at 20 U/ml.

Analysis of RF interferences with sIgD

In order to exclude interference of RF in the measurement of sIgD in serum samples, we performed additional control experiments, studying recovery of IgD standard in serum samples with high levels of RF. Three samples RF+ (ranging from 73 to 146 IU/ml) and three samples RF- were spiked with human serum IgD, 75 µg/ml, serial dilutions of the samples were prepared using 1% BSA and 0.25% tween20 in PBS in the range 1:270 to 1:7290. For measuring the levels of sIgD, it was used mouse anti-human IgD (SouthernBiotech), as capture antibodies, and biotinylated goat anti-human IgD (SouthernBiotech) and incubate for 2 hours at room temperature. 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Aldrich) was used as the developer and plates were read at 450 nm.

Measurement of serum levels of IL-6

Serum levels of IL-6 were analyzed in 1:2 dilution with PeliPair reagent set (Sanquin, Amsterdam, the Netherlands) according to the manufacturer's instructions. The minimal detection levels for IL-6 were 1.1 pg/ml.

Statistics

Statistical analyses were performed using Graphpad Prism v.6 and www.open-epi.com for two-by-two tables. In the absence of established cut-off points and in order to retain adequate statistical power, the RA cohort was categorised by sIgD levels in tertiles. sIgD levels below 20.8 µg/ml indicates the 1st tertil, hence the low expression, whereas within the middle and upper tertiles indicated its high expression.

Descriptive data are presented as the median and the interquartile range [IQR] or the number, and the percentage. The differences between groups were assessed by the Mann-Whitney U test. All tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Distribution of serum IgD, IgM, IgA and IgG in healthy controls and in RA patients

sIgD levels displayed a variation in the healthy controls (HC), ranging between 0.16 and 612.6 µg/ml, with the median level of 43 (19-108) µg/ml and in RA between 0.08 and 485.5 µg/ml, with the median levels of 38 (14-97) µg/ml. In the total RA cohort, sIgD levels were similar to HC. Importantly, in 14 of 248 RA patients (5.6%) and none of 168 HC (p=0.0004) had the levels of sIgD <0.08 µg/ml, and were undetectable in the ELISA system used in our study.

The serum levels of total IgM, IgA and IgG were significantly lower in HC compared to the RA patients (**Table 2**).

Effects of age and gender in the serum levels of IgD, IgM, IgG and IgA in RA and controls

To investigate if gender and age affect the sIgD levels, the HC and RA patients were stratified by these parameters. Age above 50 years was chosen to separate in the female group entering or after menopause.

In HC, the levels of sIgD **(Figure 1A**) as well as the levels of total sIgM, sIgG and sIgA (**Figure 1B**) were similar between the genders. Instead, in RA patients, we observed a significantly lower production of sIgD in the female patients compared to the male patients (**Figure 1A**). The median of sIgD levels in RA females were half of that in RA males (34.07 *vs.* 60.7 µg/ml, respectively). In contrast to IgD, the levels of IgM, IgG and IgA were comparable between the female and male RA patients and displayed no consistent association with gender (**Figure 1B**).

In HC, we observed a difference in sIgD levels between the younger females and the females >50 years. These are the young females who had significantly higher sIgD levels compared to females >50 years (p=0.02). The stratification by age identified no difference in the production of sIgD in the male HC. Similarly, no difference was found in the levels of sIgM, sIgG and sIgA in the HC male and female. In RA patients, IgD levels displayed no variation between the groups of patients with different age, suggesting that this steady state of IgD production may have a pathological impact in the disease. Moreover, in RA patients, a decrease of IgM, but not IgG and IgA was found in the females >50 years. In the male RA patients >50 years, a similar tendency towards lower levels of IgM and IgG was observed **(Figure 1C**).

Table 1: Demographic and clinical characteristic of RA patients and healthy controls. HC: healthy controls; AB, antibody; RA: rheumatoid arthritis; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibodies; DAS28: disease activity score-28; ESR: erythrocyte sedimentation rate, MTX: Methotrexate; DMARD: Disease-modifying antirheumatic drugs. Data is reported as median [IQR]

	HC	RA
Number	168	248
Nr. Female	95	184
Nr. male, n	73	64
Age, years	56 [46-64]	55 [55-62]
AB-, n	-	17
ACPA+, n	-	131
RF+, n		165
RF- ACPA+	-	21
RF+ ACPA-	-	55
RF+ ACPA+	-	110
RA patients	**Low* IgD**	**High IgD**
IgD levels µg/ml	8.91 [0.08-20.79]	72.29 [20.91-485.5]
Number	84	164
Nr. Female	68	116
Nr. Male	16	48
Age, years	53 [43-63]	56 [46-63]
DAS28	2.89 [2.04-3.71]	2.99 [1.90-4.16]
Disease duration, years	8 [5-14]	8 [4-17]
ESR (mm/h)	7 [4-13]	10 [5-16]
MTX mono, n	38	77
MTX+DMARD, n	15	12
MTX+Biologics, n	23	50

To understand if IgD is connected to the RA pathology, we compared the levels in patients and controls stratified by age and gender. Interestingly, the low levels of sIgD were found in the young female RA patients (**Figure 1A**), when compared to the young female HC (p=0.018). The level of sIgD in the young RA females resemble the levels of HC females >50 years.

Low serum IgD associated with the presence of RA-specific autoantibodies

To further investigate the potential place of low sIgD in the RA, we asked if the presence of RA-specific autoantibodies, Rheumatoid factor (RF) and antibodies against citrullinated proteins (ACPA) are associated to low sIgD. The comparison of sIgD levels between the RA patients with different antibody pattern revealed the significantly lower sIgD in patients with RF alone (p=0.009) or in those combining RF with ACPA (p=0.028) (**Figure 2**). Importantly, such an antibody-associated difference was not seen in the levels of IgM, IgG and IgA. This latter result suggests that the sIgD production and release, but not IgM, IgG and IgA, could be affected by the autoantibodies.

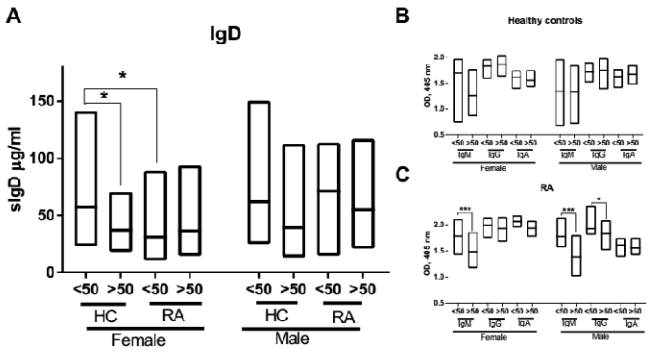

Figure 1 Serum levels of IgD in RA patient and healthy controls stratified by age and gender. **(A)** IgD levels in µg/ml. **(B)** Serum levels of IgM, IgG and IgA levels in healthy controls. The levels are presented as absorbance values of optical density (OD) at 450 nm. **(C)** Serum levels of IgM, IgG and IgA levels in RA patients. The levels are presented as absorbance values of optical density (OD) at 450 nm. The levels of IgM, IgG and IgA are reported in optical density (OD), respectively with the dilutions 1/6400, 1/8000000 and 1/32000. Boxes represent the interquartile range. Statistical comparison between the groups were done in pairs using the non-parametric Mann-Whitney test. P-values <0.05 are indicated with *, p-values <0.001 are indicated with ***.

Due to the fact that RF, through a non-competitive mechanism, may interfere with other antibodies, thereby affecting the analytic measurement in ELISA [19] a control experiment was performed to study recovery of human IgD in serum with high levels of RF.

Serum samples from 3 RF+ and 3 RF- patients were spiked with 75 µg/ml of IgD standard and the mixture was thereafter tested for IgD in 4 dilutions using the established ELISA as described. We found that the dilution curves developed from serum samples RF+ and RF- were increased in a similar manner when spiked with IgD. This let us to conclude that RF does not interfere with the analytic measurement of IgD concentration in RA patients.

Effects of anti-rheumatic treatment on the levels of sIgD in RA patients

Several treatment strategies are available for RA patients with remission as the primary goal. To explore if anti-rheumatic treatment has an effect on the sIgD levels, we categorised the RA patients by the treatment modality at the time of enrolment into the study. Three groups were identified the patients treated with MTX as a monotherapy (n=115), patients treated with a combination of MTX with other DMARD, mostly sulfasalazine (n=27), and the patients treated with a combination of MTX with biologics, mostly TNF-inhibitors (n=73).

Comparing sIgD levels between these groups, we found that low levels of IgD were associated with the combination treatment of MTX with other DMARD. In contrast, the patients treated with MTX monotherapy or the combination between MTX and biological drugs have significantly higher levels of sIgD (**Figure 3A and B**). Consistently, a high proportion of patients treated with MTX and DMARD had low levels of sIgD compared to the patients receiving MTX monotherapy or a combination of MTX with biologics (p=0.03). We found no significant difference in the

Table 2: Differences in the serum levels of IgD, IgM, IgG and IgA in healthy controls (HC) and RA. Levels of IgD are reported in µg/ml whereas the IgM, IgG and IgA levels are reported in optical density, respectively with the dilutions 1/6400, 1/8000000 and 1/32000
††† Statistical difference between the total HC and RA, p<0.001; *statistical difference between genders, p<0.05. Data is reported as median [IQR]

	HC			RA		
	Total	Female	Male	Total	Female	Male
N.	168	95	73	248	95	73
IgD	43	41.7	45.1	38	34.07*	60.7
	[9-108]	[19.5-97.8]	[17.2-121.9]	[14-97]	[11.9-91.1]	[20.8-114]
IgM	1.3	1.3	1.3	1.6†††	1.4	1.6
	[0.7-1.9]	[0.7-1.9]	[0.7-1.9]	[1.7-2.0]	[0.7-1.9]	[1.04-1.9]
IgG	1.6	1.8	1.7	1.9†††	1.9	1.9
	[1.4-2.1]	[1.6-2.0]	[1.4-2.0]	[1.8-2.1]	[1.7-2.1]	[1.6-1.9]
IgA	1.8	1.6	1.7	2.0†††	1.9	2
	[1.5-2.0]	[1.4-1.7]	[1.4-1.8]	[1.8-2.2]	[1.7-2.1]	[1.8-2.1]

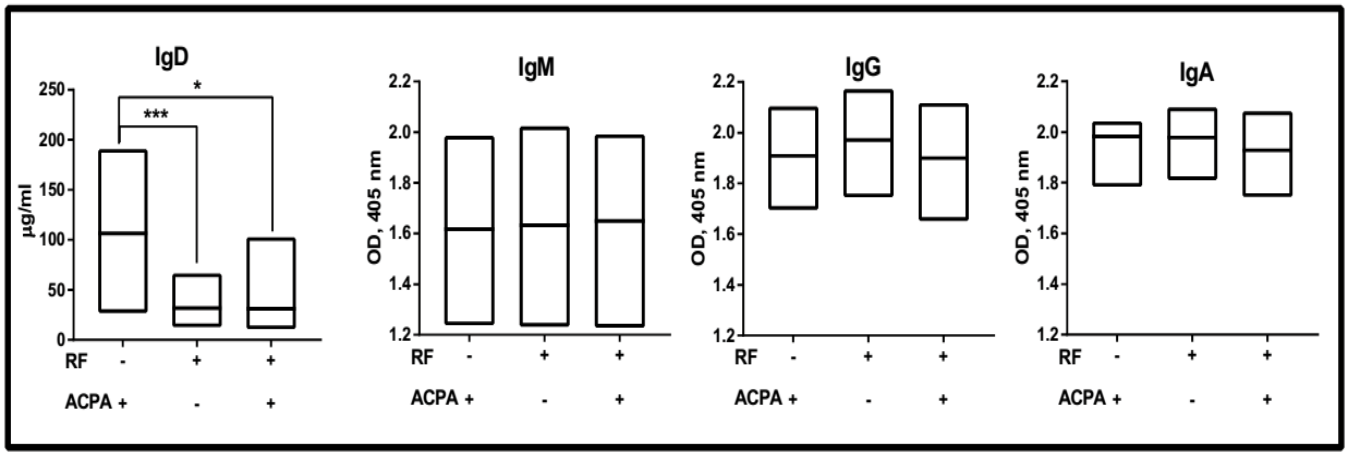

Figure 2 Serum levels of IgD, IgM, IgG and IgA in RA patients with different antibody pattern. RF-ACPA+ (n=18); RF+ACPA- (n=52); RF+ACPA+ (n=88). Boxes represent the interquartile range. RF, rheumatoid factor, ACPA, antibodies against cyclic citrullinated peptides.

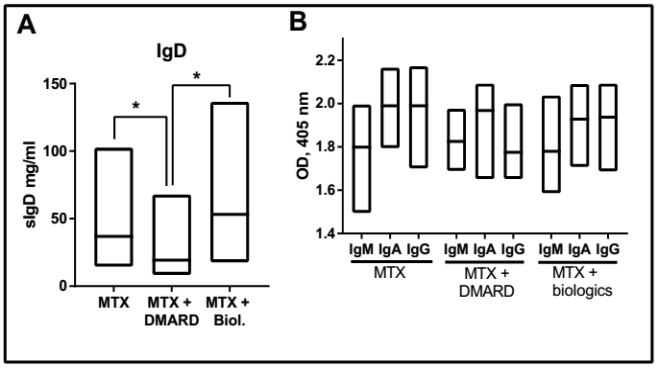

Figure 3 Serum levels of IgD in RA patients with different anti-rheumatic treatment. **(A)** IgD levels in RA patients treated with methotrexate (MTX) monotherapy (n=119), MTX in combination with other disease modifying antirheumatic drugs (DMARDs) (n=27), MTX in combination with biologics (n=73). **(B)** Serum levels of total IgM, IgG and IgA in the patients with different anti-rheumatic treatment. sIgM, sIgG and sIgA levels are reported in optical density (OD), respectively with the dilutions 1/6400, 1/8000000 and 1/32000. Boxes represent the interquartile range. P-value < 0.05 are indicated with *

levels of IgM, IgG and IgA, between the RA patients with different treatment modalities. The group treated with MTX+DMARDs consisted predominantly of females (26 of 27, 97%) compared to the group treated with MTX monotherapy or a combination of MTX with biologics (72% and 67%, respectively).

Association between clinical parameters of RA, serum IgD and treatment

Intrigued by these results, we analysed the clinical parameters of the RA patients in the different treatment groups. We observed that serum levels of IL-6 were significantly higher in the patients treated with MTX+DMARDs, whereas this group was also characterized by having low levels of sIgD, compared to the other treatment groups (**Table 3**). In contrast, the groups treated with MTX monotherapy or MTX+biologics had lower IL-6 and higher levels of sIgD. Regarding disease activity, the proportion of patients with DAS28>2.6 and not in remission, was higher among the patients receiving MTX+DMARD than patients treated with MTX monotherapy (p=0.035) (**Table 3**). Taken together, these findings indicate an association between the low sIgD and higher systemic inflammation. No significant difference was found between the groups with respect to disease duration, Visual Analog Scale for Pain, ESR and the antibody pattern.

Discussion

In this study, we designed and applied the measurement of serum IgD levels and total IgM, IgA and IgG to evaluate their potential place in RA pathogenesis. The results obtained in RA patients and in HC showed that sIgD levels are affected by several disease characteristics important of RA, such as gender and presence of autoantibodies.

IgD is a molecule preserved through the evolution, being detectable in different species, including mammalian species, cartilaginous fishes, bony fishes, frogs and reptiles [20]. Evolutionary, two populations of B cells have been identified IgD+IgM+ B cells and IgD+IgM- B cells [21]. However, Rouaud et al. have found and reported that the class-switch recombination of IgD producing naïve-B cells may involve a different mechanism than plasma cells producing IgM [22]. The IgM-IgD+ B cell population has been associated with more production of IgLδ chain, and higher RNA for secreted IgD [21]. Also, it could be generated from a transcriptional inactivation of IgM locus [20]. Functionally, IgD might be able to recognize bacteria and their products and stimulates basophils to product cytokines such as IL-4, IL-13, BAFF and APRIL [23]. Despite the efforts in understanding its role in both physiological and pathological conditions, it is not completely identified.

We report that RA patients had lower levels of sIgD compared to healthy controls, especially among young women, tempting to speculate on its prognostic potential value in RA patients.

Table 3: Clinical characteristic of RA patients with different treatment modalities.

	MTX mono	MTX+DMARD	MTX+biologics
Total	115	27	73
Nr. of Female	83	26	51
Nr. of Male	32	1	22
Age, years	56 [49-63]	52 [45-63]	55 [42-62]
Dis. Duration	8 [38-53]	8 [5-15]	8 [4-17]
DAS28	2.7 [1.8-3.8]	3.4 [2.6-4.1]	3.1 [1.9-4.2]
DAS<2.6	46 (40%)	*5 (18.5%)	23 (31.5%)
IL-6 pg/ml	1.2 [0-5.1]	7.4 [1.3-17.1]	2.1 [0.2-7.3]
Vas-pain	26 [13-41]	26 [10-63]	32 [12-60]
ESR mm/h	9 [4-14]	7 [5-13]	8 [5-14]
IgD, µg/ml	36.8 [14.4-102.6]*	19.3 [8.3-67.8]	53.2 [17.7-136.6]*
N. IgD low	38	15*	23
ACPA+RF-	27 (23.5%)	6 (22.2%)	17 (23.3)
ACPA-RF+	10 (8.7 %)	4 (14.8%)	4 (5.5%)
ACPA+RF+	42 (36%)	16 (59.2%)	42 (57.5%)

* indicates p<0.05. Data is reported as median [IQR]

Previous findings on this subject gained from an observational study in a Spanish healthy population suggested that IgD levels were negatively associated with age and no association was observed in relation to gender [24]. Further studies are needed to confirm these findings.

Our results suggest that the presence of autoantibodies are associated with low sIgD but not to the serum level of IgM, IgG and IgA. This put sIgD in a unique position among other Ig and aids recognition of patients producing autoantibodies. We speculate that RF could trigger, directly or indirectly, the suppression of IgD production or serum release, which highlights its clinical implication. Low levels of sIgD were also found in patients having high serum levels of IgE and recurrent infection (Job's) syndrome [25]. Variation of sIgD levels has been reported in other diseases, suggesting a clinical relevance of this molecule in the immune-mediated pathologies. Indeed, abnormal production of IgD has been reported in viral infection such as rubella [26], chronic rhinosinusitis [27] and hyper-IgD syndrome [28]. The clinical utility of measuring the serum levels of IgD becomes of remarkable relevance when considering that sIgD are associated with high levels of IL-6, which is a marker of acute phase of inflammation. IL-6, indeed, is able to elicit both the acute phase of inflammation, B cell differentiation and T cell activation [29]. These latter is characteristic for RA. It is still unclear which are the factors triggering the disease but it is widely accepted that environmental factors such as smoking in combination with genetic predisposition may trigger development of RA [11]. Smoking is the established risk factor for cardiovascular diseases [30], which morbidity is often increased in RA patients, rising their mortality [31].

An independent study from our laboratory on the same cohort of patients and controls revealed that smoking, known for its harmful effect, is associated with an high risk to be survivin-positive [32], which maintains persistence of autoreactive cells in autoimmune disease [33] and improves the estimation of RA risk [18]. An increased interest of early diagnosis of RA is catching the attention of several researchers, since an early recognition of RA can improve the efficacy of the treatments. In this context, the measurement of the serum levels of IgD may be clinical useful for the early recognition of RA patients.

In conclusion, the present study shows that sIgD levels are influenced by factors as age, gender and presence of autoantibodies. sIgD seems to have different characteristics than IgM, IgG and IgA, highlighting its unique role in RA pathology. Low serum levels of IgD seem to be pathological due to their association with the presence of RF, higher serum Il-6 levels and not being in remission, which indicates a link between sIgD and disease severity. However, further studies need to investigate the place of sIgD in the pathogenicity of RA, in order to reveal the mechanism behind.

Acknowledgement

The authors are thankful to Miss Caroline Wasén for critical reading of this manuscript.

Funding

This work has been funded by grants from the Swedish Research Council, the Medical Society of Gothenburg, the Swedish Association against Rheumatism, the King Gustaf V:s 80-year Foundation, Torsten Söderberg's Foundation, the Lundberg's Foundation, the University of Göteborg, the Regional agreement on medical training and clinical research between the Västra Götaland county council and the University of Göteborg LUA/ALF.

Declaration of Interest

The authors declare that they have no competing interests

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