EVALUATION OF ANTI-CARBAMYLATED PROTEIN ANTIBODIES AS A POTENTIAL BIOMARKER IN ASSISTING THE DIAGNOSIS OF RHEUMATOID ARTHRITIS AND ITS CORRELATION WITH DISEASE ACTIVITY

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ACPA	Anti-citrullinated protein antibodies
AUC	Area under the curve
Anti-CarP	Anti-carbamylated protein
Anti-CCP	Anti-cyclic citrullinated peptide
anti-MCV	Anti-mutated citrullinated vimentin
ACR	American College of Rheumatology
BSA	Bovine serum albumin
CBL	Carbamyl-lysine
CD	Cluster of differentiation
CRP	C-reactive protein
DAS	Disease activity score
DCs	Dendritic cells
DMARDs	Disease modifying anti-rheumatic drugs
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme linked-immunosorbent assay
ESR	Erythrocyte sedimentation rate
ExRA	Extra-articular RA
HAQ	Health assessment questionnaire
HLA	Human leukocyte antigen
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
Ig	Immunoglobulin

IL	Interleukin
NK	Natural killer
MCP	Metacarpophalangeal
MTP	Metatarsophalangeal
MTX	Methotrexate
ml	Milliliters
µl	Microliters
NaCI	Sodium chloride
PAD	Peptidylarginine-deiminase
PIP	Proximal interphalangeal
PTM	Post-translational modification
OD	Optical density
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROC	Receiver operating characteristic
RF	Rheumatoid factor
SD	Standard deviation
SLE	Systemic lupus erythematosus
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
U/ml	Unit per mililiter

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PENILAIAN ANTI-CARBAMYLATED PROTEIN ANTIBODI SEBAGAI PENANDABIO YANG BERPOTENSI MEMBANTU DALAM PENDIAGNOSAN RHEUMATOID ARTHRITIS SERTA KAITANNYA DENGAN AKTIVITI PENYAKIT

ABSTRAK

Rheumatoid arthritis (RA) merupakan penyakit radang reumatik yang dicirikan oleh kehadiran autoantibodi. Kajian ini bertujuan untuk mengukur nilai sensitiviti dan spesifikasi protein anti-carbamylated (anti-CarP) antibodi dalam pesakit RA yang dikaitkan dengan faktor rheumatoid (RF), anti-cyclic citrullinated peptide (anti-CCP) antibodi, C-reactive protein (CRP) dan juga untuk menentukan perkaitan mereka dengan 28-skor aktiviti penyakit (DAS28) dan skor soal selidik penilaian kesihatan (HAQ). Kajian ini melibatkan 105 pesakit RA (57 pesakit RF-negatif dan 48 RF-positif) yang dijalankan di Hospital Universiti Sains Malaysia (HUSM) mulai Januari 2015 sehingga bulan Februari 2016. Lima puluh individu sihat telah terlibat. CRP, RF, antibodi anti-CCP dan antibodi anti-CarP telah diukur. HAQ telah diagihkan kepada setiap peserta kajian. DAS28 bagi 105 pesakit RA telah diukur. Tahap antibodi anti-CarP telah meningkat secara jelas dalam pesakit RA berbanding dalam individu sihat (p=0.042). Sensitiviti dan spesifikasi antibodi anti-CarP bagi pesakit RA masing-masing adalah 42% dan 78%. Kehadiran antibodi anti-CarP dikaitkan secara jelas dengan RF (p=0.019) dan keputusan HAQ (p= 0.010). Walau bagaimanapun, tidak ada perkaitan yang jelas antara antibodi anti-CarP

dengan anti-CCP dan CRP (masing-masing adalah p=0,564 dan p=0.075). Tambahan pula, perkaitan yang jelas antara antibodi anti-CarP dengan DAS28 tidak dijumpai (p=0.165). Kajian ini memberikan bukti lanjut bahawa tahap antibodi anti-CarP adalah tinggi secara jelas pada pesakit RA. Di samping itu, kajian ini menunjukkan bahawa pesakit RA yang mempunyai positif anti-CarP lebih cenderung untuk menunjukkan peningkatan dalam ketidakupayaan berbanding pesakit RA yang mempunyai negatif anti-CarP. Oleh itu, antibodi anti-CarP mempunyai potensi untuk dijadikan sebagai penandabio bagi membantu dalam diagnosis dan prognosis RA.

EVALUATION OF ANTI-CARBAMYLATED PROTEIN ANTIBODIES AS A POTENTIAL BIOMARKER IN ASSISTING THE DIAGNOSIS OF RHEUMATOID ARTHRITIS AND ITS CORRELATION WITH DISEASE ACTIVITY

ABSTRACT

Rheumatoid arthritis (RA) is an inflammatory rheumatic disease characterized by the presence of autoantibodies. This study aims to determine the sensitivity and specificity of anti-carbamylated protein (anti-CarP) antibodies in rheumatoid arthritis (RA) patients in relation to rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) antibodies, C-reactive protein (CRP) as well as to determine their association with disease activity score-28 (DAS28) and health assessment questionnaire (HAQ) score. This study included 105 RA patients (48 RF-positive and 57 RF-negative patients) conducted at Hospital Universiti Sains Malaysia (HUSM) from January 2015 until February 2016. Fifty healthy controls (HCs) were included. CRP, RF, anti-CCP and anti-CarP antibodies were measured. HAQ was administered to each of the study participants. DAS28 of the 105 RA patients was measured. The level of anti-CarP antibodies was significantly increased in the RA patients compared to that in the HCs (p=0.042). The sensitivity and specificity of anti-CarP antibodies in RA patients were 42% and 78%, respectively. The presence of anti-CarP antibodies was significantly associated with RF (p=0.019) and the HAQ results (p=0.010). However, there was no significant association of

anti-CarP antibodies with anti-CCP antibodies and CRP (p=0.564 and p=0.075, respectively). Furthermore, a significant association between anti-CarP antibodies with DAS28 was not found (p=0.165). This study provides further evidence that the level of anti-CarP antibodies is significantly elevated in RA patients. In addition, this study showed that anti-CarP-positive RA patients more likely to demonstrate increased disability compared to anti-CarP-negative RA patients. Therefore, anti-CarP antibodies have a potential to be further develop as a biomarker to aid in the diagnosis and prognosis of RA.

CHAPTER 1

INTRODUCTION

1.1 HUMAN IMMUNE SYSTEM

The immune system is made up of different groups of cells, tissues, as well as organs that work together to protect the body against the invasion by the pathogens. Pathogens can be divided into four kinds; bacteria, viruses, fungi and parasites (Parham, 2014). Natural defence mechanism has the ability to differentiate between the body's own cells and foreign cells. The immune system is classified into innate immunity and adaptive immunity.

1.1.1 Innate immunity

The innate immunity provides the body's early line of defence and aims to prevent the entry of pathogenic substances (Abbas *et al.*, 2014). Innate immunity consisting of various components including skin, mucous membrane, antimicrobial substance, phagocytic cells, dendritic cells (DCs), natural killer (NK) cells and blood proteins (Abbas *et al.*, 2014). Innate immunity is effective to protect the body from infection, however, many pathogens are able to overcome the innate immunity. It is known that the skin immune responses provide effective host defence against many

pathogens, however, it also contributes to inflammatory skin diseases. It has been described that several components including pathogens contribute to complex skin immune reactions (Yazdi *et al.*, 2016). Thus, more effective defence systems of adaptive immunity are required to successfully fight against the pathogenic microbes.

1.1.2 Adaptive immunity

The adaptive immunity develops as a response to infection which improves with each successive exposure to particular pathogens. The typical feature of adaptive immunity is the ability to differentiate between substances, called specificity and the ability to provide a quick and powerful response to repeated exposures of the similar pathogen, known as memory (Abbas *et al.*, 2014).

The adaptive immunity refers to the defence mechanisms characterized by T cell specific for an antigen from the invading pathogens, and by B cells which secreted antibodies that bind to these antigens for their elimination. The successful adaptive immune response in eliminating infection provides long-lasting protective immunity against the particular pathogens.

1.1.3 Autoimmunity and autoimmune diseases

Autoimmunity is a state in which the immune system exhibits immunological responses against its own healthy cells and tissues. Over the year, a significant increase has been observed in the incidence of autoimmune disease worldwide. Immune tolerance is important because it plays a role in preventing the immune system from recognizing self-proteins. The innate and adaptive immunity shown to

be responsible for the inhibition or development of autoimmune disease (Vojdani, 2014).

It has been shown that the loss of the immunological tolerance directed to self-antigens could lead to the development of autoimmunity (Conigliaro *et al.*, 2016). Several genes are known to contribute to the loss of tolerance, in which the gene factors could involve in antigen presentation, lymphocyte differentiation as well as encodes for T and B cells receptors (Gianchecchi and Fierabracci, 2015).

Autoimmunity is characterized by the presence of autoantibodies in susceptible individuals (McInnes and Schett, 2007). A previous study demonstrated that the periodontal pathogen, *Porphyromonas gingivalis* able to citrullinate proteins which provide a potential mechanism for breaking of self-tolerance to citrullinated proteins in rheumatoid arthritis (RA) (Wegner *et al.*, 2010).

1.2 RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) is a chronic disease characterized by the destruction of synovial joint with erosive cartilage and bone damage (Brink *et al.*, 2015; Jeffery, 2014). Increased infiltration in synovial tissue by mononuclear cells, especially T cells and macrophages, and hyperplasia of the synovial sublining are typical features of RA (Firestein *et al.*, 2012). The abnormal regulation of adaptive immunity precedes the onset of clinical symptoms, while the repeated initiation of innate immunity can contribute to the loss of tolerance in RA (Bartok and Firestein, 2010).

1.2.1 Incidence and prevalence

RA affects 0.5% - 1.0% of the adult population worldwide, being women two to four times more likely to be affected than men (Jeffery, 2014). Of 291 conditions, RA was ranked as the 42nd highest contributor to global disability in the Burden of Disease 2010 study (Cross *et al.*, 2014) indicating that RA associated with severe implications for the affected individuals. It is reported that the incidence of RA is 20-45/100,000 in Asians (Alamanos and Drosos, 2005), 10-50/100,000 in Latin American (Gonzalez *et al.*, 2008) and 10-50/100,000 in Middle east populations (Al-Mutairi *et al.*, 2010).

1.2.2 Risk factors

RA remains uncertain in the description of their exact causes and pathogenic mechanisms, but genetic and environmental influences clearly participate (Smolen and Steiner, 2003). Over the decades, a few environmental risk factors such as

smoking, socioeconomic factors, hormonal factors, ethnicity and infectious agents are known to contribute to the development of RA (Alamanos and Drosos, 2005).

A previous study demonstrates a disease concordance rates of 15% - 30% among monozygotic twins and concordance rates of 5% among dizygotic twins (MacGregor *et al.,* 2000). The HLA region has an impact on the genetic risk and may account for RA susceptibility in multiple ethnic groups (El-Saadany *et al.,* 2016). The HLA-DRB1 alleles demonstrated to be associated with disease severity in RA (Weyand *et al.*, 1992). It is reported that most of the RA patients carry the HLA-DRB1*04 alleles (Smolen *et al.,* 2007). Furthermore, the patients that express two HLA-DRB1*04 alleles have a greater risk for nodular disease, major organ failure and joint surgery (Weyand *et al.*, 1992).

Several infectious agents, including Epstein-Barr virus (EBV), proteus, and mycoplasma are associated with RA (Silman and Pearson, 2002). Over the last decades, EBV has been described as the potential risk factor for RA with the growing evidence supporting this. The present of EBV in the synovial fluid of RA patients indicate that it could contribute to the development of RA (Takeda *et al.*, 2000).

Bronchopulmonary infection in RA patients is associated with severe functional disability and the prevalence is greater compared to non-RA patients (Arshad and Rashid, 2008). It is demonstrated that periodontal pathogen known as *Aggregatibacter actinomycetemcomitans* induces hypercitrullination of proteins observed in the joints of RA patients indicating that periodontal infection is associated with RA pathogenesis (Konig *et al.*, 2016a).

1.2.3 Immunopathogenesis

It is known that the pathogenesis of RA is characterized by the interaction of different inflammatory cells and cells contain in the joint which communicates via a network of proteins termed as cytokines. It is known that under normal condition, there is a balance of pro-inflammatory cytokines and anti-inflammatory cytokines. However, the balance shifts toward the production of more pro-inflammatory cytokines in RA.

The pathogenesis of RA involves the activation of the innate immunity by stimulating dendritic cells (DCs) (Figure 1.1). DCs could contribute to the pathogenesis of RA in several ways. A previous study reported that DCs trigger the initiation and perpetuation of RA by the antigen presentation to T cells (Leung *et al.*, 2002). In addition, these DCs could mediate the activation of the infiltrating T cells which is sufficient for the development of the autoantibody and chronic inflammation (Lutzky *et al.*, 2007).

Moreover, DCs are enriched in both the synovial tissue and synovial fluid of joints affected by RA (Thomas and Quinn, 1996). In addition, DCs together with the synoviocytes and macrophages producing the innate inflammatory mediators, in which these mediators mediate the inflammatory condition in RA (Cavanagh *et al.*, 2005; Leung *et al.*, 2002).

The macrophage is a dominant mediator of synovial inflammation in RA. Clinically effective biologic agents are needed in order to reduce macrophage infiltration in the synovial membrane (Haringman *et al.*, 2005). Macrophages play a role in cytokine

production, including tumour necrosis factor-α (TNF-α), interleukin-1, 6, 12, 15, 18 and 23. It is also involved in phagocytosis and antigen presentation (McInnes and Schett, 2011). TNF-α act through activation of cytokine and chemokine and induction of pain (Feldmann *et al.*, 1996; Hess *et al.*, 2011). Furthermore, interleukin 6 (IL-6) plays a role through activation of leukocyte and production of autoantibody (McInnes and Schett, 2011).

It is known that chemokines responsible for the migration of the inflammatory leukocytes including neutrophils and monocytes into the synovium and play a role in arthritic vessel formation termed as angiogenesis (Szekanecz *et al.*, 2009). This evidence indicate that chemokines and chemokine receptors play a crucial role in the inflammatory process of RA and therefore identification of inhibitors which targeted the chemokines should be vigorously initiated for the development of new therapeutic agents.

Synovial hyperplasia is present due to the increased number of macrophages and FLS in the synovial lining layer. Synoviocytes are responsible for several functions under normal conditions including the formation of synovial fluid components, however, the FLS present in the synovial intimal lining can trigger the production of cytokine, leading to the perpetuation of inflammation and cartilage destruction (Bartok and Firestein, 2010). The altered function of synoviocytes in RA could be due to the abnormal regulation of molecular mechanisms influenced by the presence of somatic mutations in major genes (Firestein, 2003). It has been shown that the mutated genes such as the p53 tumour suppressor are present in synovial tissue and synoviocytes of RA patients (Inazuka *et al.*, 2000).

T-cells upregulation is associated with the production of various lymphokines, in which these lymphokines stimulate the T-cells to induce macrophages, B cells, fibroblasts and osteoclasts activation. B lymphocytes produce many cell-surface molecules, including immunoglobulin (Ig), and differentiation antigens (CD20 and CD22). B lymphocytes differentiate into plasma cells that produce antibodies, including autoantibodies such as rheumatoid factor (RF) and citrullinated proteins (Zwerina *et al.*, 2005).

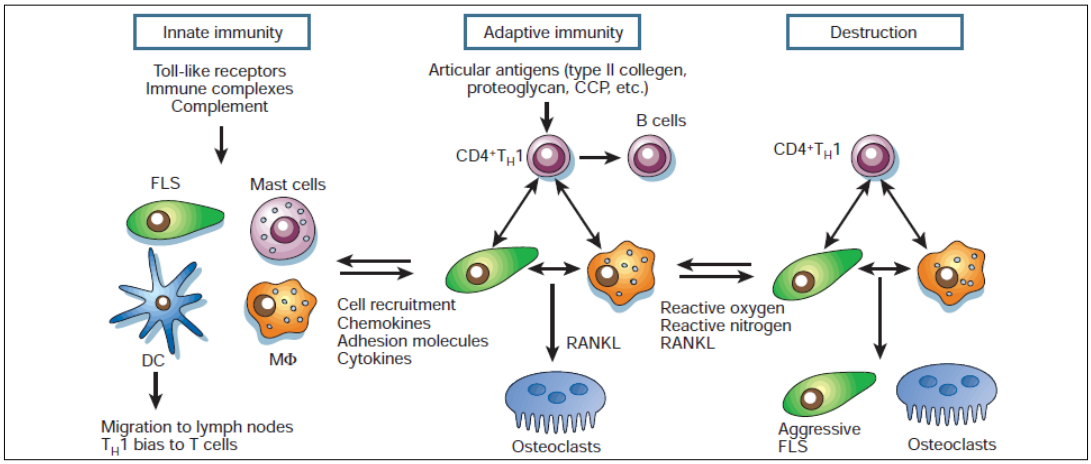

Figure 1.1: Multiple pathogenic mechanisms in RA**.** This model implicating that innate immunity is activated by triggering the immune cells including dendritic cells (DCs), macrophages, fibroblasts and mast cells. After the migration of immune cells into the synovium occurs, the adaptive immune response will present in susceptible individuals. The migration of DCs into lymph nodes bias T cells to a T helper 1 phenotype. RANKL responsible for the activation of osteoclast that mediates bone resorption, while synoviocytes can invade cartilage. Adapted from (Firestein, 2003)

1.2.4 Clinical signs and symptoms

1.2.4 (a) Articular manifestation

RA is characterized by symmetrical polyarthritis that affects the small and large joints (Reid and Miller, 2008) in which hand, feet, and wrists being most commonly affected (Cojocaru *et al.*, 2010). One of the features of RA is the presence of morning stiffness in and around the joints (Grassi *et al.*, 1998). Most of RA patients presented with bilateral pain and stiffness in the joints of the hand (Wahab *et al.*, 2013).

It is found that most of the RA patients presented with more cases of bilateral involvement compared to the unilateral involvement of the shoulder and it is shown that the bilateral involvement is linked to the higher disease activity (Elbinoune *et al.*, 2016). The pathological features of RA are the occurrence of increased thickness of the synovial lining layer comprising of numerous macrophages and fibroblast-like synoviocytes (FLS) (Figure 1.2). However, a healthy joint presented with the small volume of synovium and a thin synovial lining layer containing FLS.

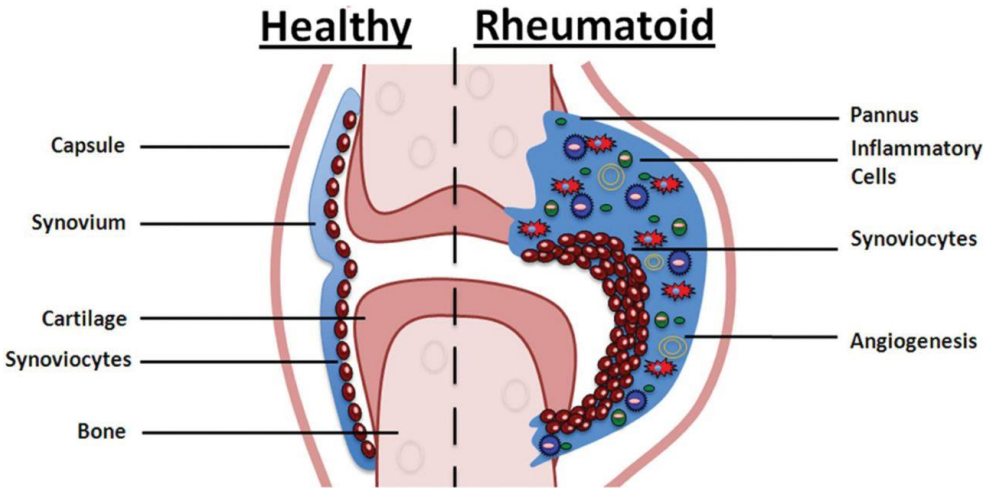

Figure 1.2: Comparison of normal joint and joint affected by RA. Adapted from (Hawtree *et al.*, 2013)

1.2.4 (b) Extra-articular manifestation

In addition to the joint involvement, RA has been shown to be associated with the development of extra-articular manifestations (Turesson *et al.*, 2007). These extra-articular RA (ExRA) manifestations including rheumatoid nodules, rheumatoid vasculitis, anemia, cardiovascular disease, cutaneous and pleuropulmonary (Hochberg *et al.*, 2008; Yildirim *et al.*, 2004). Several non-articular muscular structures including tendons, ligaments, and fascia can be affected by RA (Mielants and Van den Bosch, 2009).

ExRA is associated with premature mortality in RA patients (Bongartz *et al.*, 2007). RA-related mortality mostly occurs at age ≥50 years in 90% of 3955 cases (Pinheiro *et al.*, 2015). Furthermore, the rheumatoid meningoencephalitis that rarely occurs in RA could develop before the onset of clinical signs (Shibahara *et al.*, 2016).

Several predictors of ExRA has been shown including serological, clinical and genetic factors (Turesson and Jacobsson, 2004). The presence of RF shown to be higher in patients diagnosed with ExRA compared to non-ExRA patients, and it is known that patients developed ExRA are associated with higher swollen joint counts (Turesson *et al.*, 2007). RA patients presented with ExRA should be provided with aggressive and early treatment (Young and Koduri, 2007). An improved understanding of the RA pathogenesis and the new treatment strategies are beneficial for RA patients with extra-articular manifestations (Lindqvist *et al.*, 2002).

1.2.5 American College of Rheumatology (ACR) criteria

The 2010 American College of Rheumatology (ACR) classification criteria is useful for the diagnosis of RA in which scores can be obtained from joint involvement, serological markers, acute-phase reactants and duration of symptoms (Aletaha *et al.*, 2010). Classification as having the definite RA is mainly based on the presence of synovitis in at least 1 joint and accumulation of a score of $\geq 6/10$ from the four categories (Table 1.1).

The large joints include the shoulders, knees, elbows, and ankles, while the small joints include the metacarpophalangeal (MCP), metatarsophalangeal (MTP), proximal interphalangeal (PIP), thumb interphalangeal joint and wrists. Duration of symptoms refers to the duration of signs or symptoms of synovitis including pain, swelling and tenderness of joints reported by the patients.

Table 1.1: The 2010 ACR classification criteria for RA. Adapted from (Aletaha *et al.*, 2010)

Target population: patients who (i) have at least 1 joint with clinical synovitis and (ii) with the synovitis not better explained by another disease.	
A.Joint involvement (tender/swollen)	Score
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1
Add score of categories A-D: A cumulative score of ≥6/10 is needed for the classification of a patient as having definite RA	

1.2.6 Serological markers

RA patients are a heterogenous group with well-known differences in severity of disease (Agrawal *et al.,* 2007). This heterogenous group can be subdivided by the occurrence of autoantibodies in the sera of RA patients (Boissier *et al.,* 2012). Autoantibody-positive and negative RA patients were demonstrated to have a different genetic background, disease development processes and responses to treatments (Huizinga and Pincus, 2010).

Combinations of autoantibodies and genetic markers can be highly predictive for the development of RA (Nielen *et al.,* 2004; Rantapää-Dahlqvist *et al.,* 2003). These findings have led to an increased investigation focus on the autoantibodies in order to establish better biomarker in the diagnosis of RA.

There is so far no single diagnostic test to confirm RA. The major basis for the diagnosis is the clinical examination and laboratory tests including rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).

1.2.6 (a) Rheumatoid Factor (RF)

The first autoantibody in RA, rheumatoid factor (RF) was described by Emil Waaler in 1940 (Waaler, 1940). RF is responsible for the initiation of an immune complex, in which the pathogenic role of RF in RA has been subsequently described by Zvaifler in 1972 (Zvaifler, 1972). RF is useful in the diagnosis of RA, which has been included in the ACR classification criteria for RA (Aletaha *et al.*, 2010).

RF can be a prognostic tool for RA, which associated with the presence of more erosive disease, rapid disease progression, and worse outcome (Steiner and Smolen, 2002). RF recognizes the Fc portion of the patient's own immunoglobulin (Ig)G molecule (Sutton *et al.*, 2000). Their presence is determined by agglutination assays, nephelometry, or ELISA tests (Schellekens *et al.*, 1998).

IgM-RF is the most frequent type of RF found in RA which presents in 60–80% of established cases of RA (Nell *et al.*, 2005). Although IgG-RF and IgM-RF are the most abundant in RA, IgE-RF has been demonstrated to be present in RA especially in patients with extra-articular manifestations whereas IgA-RF is also produced in RA including patients who are seronegative as determined by standard clinical tests that primarily detect IgM-RF (Firestein *et al.*, 2012). However, individuals with more than one RF isotypes is associated with the increased incidence of RA (Jónsson *et al.*, 2000).

The RF produced in healthy individuals differ from those produced by RA patients. RF in healthy individuals are polyreactive and are of low affinity (Børretzen *et al.*, 1997) whereas RF in RA patients is monoreactive that are of relatively high affinity

(Bonagura *et al.*, 1998). RF in RA is produced by B cells which present in lymphoid follicles and germinal centers of inflamed synovium (Jones *et al.*, 1984; Wernick *et al.*, 1985) due to the rearrangements and somatic mutations of the germline genes (Firestein *et al.*, 2012). It is shown that the abnormal regulation of spontaneous germinal centers promotes the formation of somatically mutated antibodies which could lead to autoimmunity (Domeier *et al.*, 2017).

However, RF is not unique to RA since it has also been detected in another type of autoimmune diseases, including systemic lupus erythematosus (SLE), primary Sjögren's syndrome and mixed connective tissue disease (Nell *et al.*, 2005; Šenolt *et al.*, 2014). The increased incidence of RF positive reactions has been shown to be associated with aged individuals than in young individuals (Nell *et al.*, 2005). Chronic infection is shown to be associated with a high prevalence of RF and it is reported to be more frequent and severe in aged persons (Gavazzi and Krause, 2002). RF is known to be sensitive but lacks specificity (Wahab *et al.*, 2013). The sensitivity and specificity of RF range between 25-95% and 31-95%, respectively (Avouac *et al.*, 2006).

1.2.6 (b) Anti-citrullinated protein antibodies (ACPA)

Citrullination is a post-translational modification of arginine by deimination (Klareskog *et al.*, 2008; Suzuki *et al.*, 2007). The conversion of arginine to citrulline is catalyzed by a group of calcium dependent, a peptidylarginine-deiminase (PAD) enzyme consisting of five isoforms in mammals, PAD1-4 and PAD6 (Suzuki *et al.*, 2007; Vossenaar *et al.*, 2004a). The functions of PAD1s in normal immune responses is not certain; citrullination of some chemokines can decrease activity, and modification of histones can regulate gene expression in stressed cells (Firestein *et al.*, 2012).

Citrullination activated by calcium influx can occur as a normal part of cell apoptosis (Asaga *et al.*, 1998). It has been shown that increased citrullination was first found in the lining and sublining cells in the joints of RA patients (Baeten *et al.*, 2001). Citrullinated proteins have been detected in inflamed tissues in different types of arthritis (Vossenaar *et al.*, 2004b), including lungs, extra-articular sites in RA, human brain, as well as inflamed muscle and lymphoid organs (Bongartz *et al.*, 2007; Klareskog *et al.*, 2006; Makrygiannakis *et al.*, 2006; Nicholas and Whitaker, 2002).

Citrullination is known to play a role in terminal epithelial differentiation which occurs in tissues such as hair and skin (György *et al.*, 2006). Meanwhile, in the nucleus, citrullination involves in epigenetic regulation (Wang *et al.*, 2004). Citrullination also involved in host defence mechanism and fight against pathogens (Li *et al.*, 2010).

Anti-citrullinated protein antibodies (ACPA) have been identified as sensitive and specific markers in RA (Klareskog *et al.*, 2008), which establish superiority over RF in the diagnosis of RA. ACPA has been added as one of the criteria for the ACR classification for RA diagnosis (Aletaha *et al.*, 2010). It has been hypothesized that induction of ACPA in RA patients occurs in the lungs or gingiva (Klareskog *et al.*, 2006; Nesse *et al.*, 2012). This finding has been confirmed with another study (Janssen *et al.*, 2015) which showed that the presence of IgG anti-CCP associated with bronchiectasis, and demonstrated to exhibit borderline significant trend with periodontis.

ACPA can be detected several years before the onset of RA clinical symptoms (Koivula *et al.*, 2007). They have been demonstrated to be present in 70-90% of established cases of RA with high disease specificity (90-95%) (Schellekens *et al.*, 2000; Suzuki *et al.*, 2009). Furthermore, they are likely rare to be found in other diseases or in healthy individuals (Song and Kang, 2010). ACPA seropositivity is significantly associated with older RA patients (p<0.001) (Alpizar-Rodriguez *et al.*, 2017). Furthermore, a large population-based study consisting of 40136 RA cases in Netherlands demonstrated that the presence of ACPA is associated with several factors including older age, smoking, female gender and joint involvement (van Zanten *et al.*, 2017).

ACPA have been found to mediate the activation of osteoclasts which associate with the systemic bone loss in RA (Llorente *et al.*, 2017). Therefore, it is recommended for RA patients with higher level of ACPA to undergo bone

examinations and be aggressively treated with disease modifying anti-rheumatic drugs (DMARDs) (Orsolini *et al.*, 2017).

ACPA can be detected using the first-generation of the cyclic citrullinated peptides (CCP), second-generation CCP (CCP2) or third-generation CCP (CCP3) (Trouw and Mahler, 2012). CCP test relies on peptide derived from the filaggrin protein (Schellekens *et al.*, 1998), whereas the CCP2 and CCP3 tests rely on artificial peptides (Kessenbrock *et al.*, 2007; Levesque *et al.*, 2009). The formation of filaggrin occurs in the later stages of epithelial cells differentiation during the keratinization (Kuhn *et al.*, 2006). The filaggrin is stored in the keratohyalin granules and cornified epithelium (Hoet *et al.*, 1991; Simon *et al.*, 1993).

Anti-CCP is shown to be an independent marker for radiological damage and disease progression in RA (Forslind *et al.*, 2004). Another study (Ji *et al.*, 2017) demonstrated that the detection of bone erosion is independently associated with the presence of anti-CCP in RA patients indicating that the radiographic progression could provide little-added value in the early diagnosis of RA when anti-CCP antibodies are absent. The high specificity of anti-CCP antibodies in RA (Lee and Schur, 2003) further support the importance of citrullination and the clinical utility of ACPA for the early diagnosis of RA (Schellekens *et al.*, 2000).

1.2.6 (c) Additional autoantibodies

There are many other autoantibodies that have been found in RA, which can be potential biomarkers to improve the diagnosis and prognosis of RA (Verheul *et al.*, 2015a). Other autoantibodies against posttranslational modifications have also been identified, which are the autoantibodies directed against the PAD4. Anti-PAD4 antibodies detected to be present in 22-45% of RA patients and 14% of SLE patients (Halvorsen *et al.*, 2008; Zhao *et al.*, 2008). It is described that anti-PAD4 antibodies are cross-reacts with anti-PAD3 antibodies, however, it is interesting that the cross-reactivity is associated with severe radiological damage (Darrah *et al.*, 2013).

Another type of autoantibody is anti-RA33, directed against an antigen of 33 kDa, which binds to both unmodified and citrullinated heterogeneous nuclear protein (Verheul *et al.*, 2015a). It has been identified that RA33 is citrullinated in the joint of RA patients and shown to be the target of the autoantibodies (anti-RA33) response (Konig *et al.*, 2016b). Anti-Ra33 have been detected in RA patients with the sensitivity and specificity of 32% and 90-96%, respectively (Hassfeld *et al.*, 1989). Interestingly, anti-Ra33 antibodies are associated with mild disease development in patients, which could serve as a prognostic marker (Nell *et al.*, 2005).

Anti-mutated citrullinated vimentin (anti-MCV) antibodies have been detected in early RA with the sensitivity and specificity of 64% and 97%, respectively (Rojanasantikul *et al.*, 2014). A recent meta-analysis consisting of 12 studies among RA patients (n=2003), demonstrated a higher sensitivity of anti-MCV (68.6%) than anti-CCP (61.7%), however, anti-MCV specificity (94.2%) is lower than anti-CCP (97.1%) in the diagnosis of RA (Lee *et al.*, 2015).

1.2.7 Assessment of disease activity

Currently, disease activity in RA patients is monitored using a series of surrogate marker of inflammation consisting of functional disability, visual analog scales, joint involvement, and the acute phase reactants including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (Fransen *et al.*, 2002; Wolfe *et al.*, 2001). To ensure that therapy is effective, frequent clinical assessments are needed (Grigor *et al.*, 2004).

1.2.7 (a) Erythrocyte Sedimentation Rate (ESR) and C-reactive protein (CRP)

The acute-phase reactants, ESR and CRP are the most frequently used biological markers to assess disease activity in RA that appear to be equally useful and reliable as a monitoring test in the management of RA patients (Osei-Bimpong *et al.*, 2007). It is demonstrated that there is an association between the increasing levels of ESR and CRP with the involvement of large joints and/or more joints in RA patients (Shimada *et al.*, 2017).

ESR values associated with disease activity in RA and therefore useful for monitoring therapeutic response (Lane and Gravel Jr, 2002). In addition, ESR is shown to be able to predict prognosis in patients diagnose with multiple myeloma indicating that ESR is an independent prognostic marker in disease (Alexandrakis *et al.*, 2003). Furthermore, elevated values of ESR and CRP are considered a predictor factor in a diagnosis of pneumonia (Hopstaken *et al.*, 2003).

CRP is synthesized by hepatocytes (Okamura *et al.*, 1990) and the increased levels of CRP detectable in sera of patients are associated with various forms of acute and chronic inflammatory conditions (Young *et al.*, 1991). The levels of CRP have been shown to be significantly correlated with disease activity, radiographic progression and response to therapy (Åman *et al.*, 2000; Nakamura, 2000; Plant *et al.*, 2000).

CRP is known to be a specific marker of inflammation for assessment of disease activity since the hepatic synthesis of CRP is associated with the effects of inflammatory cytokines on the liver (Jansen *et al.*, 2000). The severity of the inflammatory response measured by CRP has been demonstrated to be associated with the presence of atherosclerosis in RA patients (Gonzalez-Gay *et al.*, 2005). Furthermore, RA patients with extra-articular manifestations shown to have higher levels of CRP (Turesson *et al.*, 2007). The high levels of CRP associated with the progression of joint destruction in RA patients (Nawata *et al.*, 2016).

1.2.7 (b) Disease activity score 28 (DAS28)

The advancement in therapy has improved the prognosis of RA patients. However, such therapies have high treatment costs and contribute to adverse reactions that pose an economic burden which directly impacts the health care system (Doan *et al.*, 2006). The disease activity score (DAS) incorporates the 44-joint counts, the visual analog score for pain reported by the patient, and the levels of ESR. The DAS has been described to be a sensitive and specific tool to assess disease activity in RA (Van der Heijde *et al.*, 1990).

Disease activity score-28 (DAS28) is the simplified version of the DAS that has been developed because of the greater number of joint counts in the original DAS are time-consuming (Castrejon *et al.*, 2008). It is recommended to perform DAS28 as it is accurately measured disease activity in RA patients, sensitive to change, discriminate between states of remission, low, moderate and high disease activity, and feasible to perform at the healthcare centre (Anderson *et al.*, 2012).

It is demonstrated that high disease activity associated with lower functional capacities (Jansen *et al.*, 2000). Although DAS28-CRP is also available, the DAS28 is frequently used the ESR. However, a previous study demonstrated that DAS28-CRP and DAS28-ESR were significantly correlated in Japanese cohort (Inoue *et al.*, 2007). It has been validated that both measures are necessary and useful for the disease activity assessment in RA patients (Wells *et al.*, 2009). The modified version of DAS28 has been established to assess disease activity when the acute phase reactant values including ESR and CRP are not available, and it is decsribed as a sensitive tool to discriminate between disease states (Bentley *et al.*, 2010).

1.2.7 (c) Health Assessment Questionnaire (HAQ)

The key to successful management of RA is to reduce the impact of the disease on patients' lives and therefore improves the quality of life (Pollard *et al.*, 2005). It is known that RA and other rheumatic diseases associate with functional disability. The health assessment questionnaire (HAQ), established in 1980 is the first instruments to measure health status evaluated with patients' self-reported outcomes (Fries *et al.*, 1980).

This established traditional approach have become one of the main measures for evaluation of health-related quality of life. HAQ has been established as an effective and sensitive tool to measure health status based on the evidence of its use over the past few decades in various settings. A recent study demonstrated that HAQ score is an independent predictor of severe progression in patients with an earlier stage of RA (Joo *et al.*, 2017).

1.2.8 Treatment of RA

Improved understanding of the pathogenesis of RA has led to the development of wide variety of RA treatments. The goal of treatment is to treat RA aggressively in early diagnosis since this could efficiently improve the physical function and quality of life (Schoels *et al.*, 2010). Patients with early RA that have been provided with aggressive treatment shown to have less radiographic progression (Albers *et al.*, 2001). Furthermore, it is shown that irreversible joint damage can be prevented if the patients are treated early in diagnosis especially during the first months of the disease (Bukhari *et al.*, 2003) which suggests that the start of disease-modifying antirheumatic drug (DMARD) therapy is associated with disease severity.

The possible treatment option for RA is either the combination of DMARDs or the biologic DMARDs such as tumor necrosis factor-alpha (TNF-α) inhibitors (Fiehn and Kruger, 2016). The most frequently used drug for treating RA is methotrexate (MTX) which significantly improved the physical function and quality of life (Lopez-Olivo *et al.*, 2014). MTX is shown to be involved in immune activity regulation and could reduce the damage of cartilage and bone (Hawtree *et al.*, 2013).

Furthermore, over the last decades, anti-TNF-α-targeted biologic agents known to have well-established effectiveness (Taylor and Feldmann, 2009). TNF-α is a pro-inflammatory cytokine responsible for the joint destruction in RA (Jenkins *et al.*, 2002). Studies utilizing anti-TNF-α agents demonstrate that they can reduce and control the disease progression in RA patients (Lipsky *et al.*, 2000) which probably involves the suppression of osteoclast in the joint lesions (Redlich *et al.*, 2002), resulting in improves physical function. RA patients achieve reduced disease activity