CORRELATION OF NEW GENERATION OF ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES IN COMBINATION WITH RHEUMATOID FACTOR WITH DIAGNOSIS, SEVERITY, FUNCTIONAL STATUS AND PROGNOSIS

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by

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LIST OF SYMBOLS

Adj. b/ b	Unstandardized coefficient
n	Sample size
Z	Confidence interval
σ	Standard deviation
Δ	Precision
α	Type I error
m	Ratio of case to control group
δ	Detectable difference
mL	milliliter
IU/mL	International unit per milliliter
µl	Microliter
mm	Millimeter
mm/h	Millimeter per hour
t-stat	t-statistic
CI	Confidence interval

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated peptide/protein antibodies
ACR	American College of Rheumatism
AKA	Anti-keratin antibody
Anti-CCP	Anti-Cyclic Citrullinated Peptide
Anti-CCP1	First generation of anti-cyclic citrullinated peptide
Anti-CCP2	Second generation of anti-cyclic citrullinated peptide
Anti-CCP3	Third generation of anti-cyclic citrullinated peptide
APF	Anti-perinuclear factor
CDAI	Clinical disease activity index
DAS28	Disease activity score of 28 joints
DMARD	Disease-modifying antirheumatic drugs
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte Sedimentation rate
EULAR	European League against Rheumatism
Fc	Fragment crystallizable
HAQ	Health assessment questionnaire
HCQ	Hydroxychloroquine
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
LR	Likelihood ratio
mHAQ	Modified health assessment questionnaire
MLR	Multiple Linear Regression
MTX	Methotrexate
NIAR	National Inflammatory Arthritis Registry
NPV	Negative Predictive Value
PAD	Peptidyl-arginine deiminase
PBS	phosphate-buffered saline
PPV	Positive Predictive Value

PTM	Post-translational modification
PT3/PMR	Peperiksaan Tingkatan 3/ Peperiksaan Menengah Rendah
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
ROS	Reactive oxidative species
SD	Standard deviation
SE	Shared epitope
SLR	Simple Linear Regression
SPM/SVM	Sijil Pelajaran Malaysia/ Sijil Vokasional Malaysia
SSZ	Sulfasalazine
STPM	Sijil Tinggi Pelajaran Malaysia
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumor necrosis factor
UPSR	Ujian Peperiksaan Sekolah Rendah
USM	Universiti Sains Malaysia
QoL	Quality of Life
VAS	Visual Analog Scale

LIST OF APPENDICES

PERKAITAN ANTARA GENERASI BARU ANTIBODI ANTI-CYCLIC CITRULLINATED PEPTID DAN FAKTOR REUMATOID DENGAN DIAGNOSA, KETERUKAN, KUALITI HIDUP DAN PROGNOSIS

ABSTRAK

Artritis reumatoid (RA) merupakan penyakit autoimun yang menyebabkan kerosakan sendi dan mempengaruhi kualiti hidup pesakit. Disebabkan faktor reumatoid (RF) mempunyai kemampuan diagnostik yang terhad, generasi baru antibodi peptida citrullinated anti-siklik (anti-CCP) telah diperkenalkan. Gabungan antibodi RF dan anti-CCP sangat penting untuk diagnosis, prognosis, dan rawatan klinikal RA. Kajian ini bertujuan membandingkan status serum antibodi RF, anti-CCP2 IgG, anti-CCP2 IgA dan anti-CCP3.1 IgG/IgA dalam kalangan pesakit RA dan golongan kontrol. Perkaitan antara aras penanda-penanda serologi dengan keterukan penyakit RA dan faktor-faktor yang berkaitan dengan kualiti hidup pesakit RA juga dikaji. Kajian rentas ini telah dijalankan dalam kalangan 46 pesakit RA dan 40 golongan kontrol di Klinik Reumatologi, Hospital USM. Borang soal selidik kendiri mengandungi tiga bahagian; sosio-demografik, karakteristik klinikal, dan borang soal selidik ringkas penilaian kesihatan (mHAQ) telah digunakan. Kemudian, skor aktiviti penyakit (DAS28) pesakit RA dinilai dan lima milimeter darah diambil dari semua peserta kajian. RF dianalisa menggunakan ujian Direct Latex manakala antibodi anti-CCP2 IgG, anti-CCP2 IgA dan anti-CCP3.1 IgG/IgA dianalisa dengan ujian enzim berkait imunosorben (ELISA). Data dianalisis menggunakan perisian SPSS versi 26.0 dengan nilai p <0.05 dianggap signifikan. Perkaitan antara DAS28 dan penanda-penanda serologi diuji menggunakan ujian-t sampel tidak bersandar manakala, faktor-faktor berkait dengan mHAQ diuji dengan analisis regresi berganda (MLR). Majoriti

pesakit RA mempunyai status positif untuk serum RF (78.3%), anti-CCP2 IgG (63.0%) dan anti-CCP3.1 IgG/IgA (63.0%). Terdapat perbezaan yang signifikan dalam status antibodi serum RF (p<0.0001), anti-CCP2 IgG (p<0.0001), anti-CCP2 IgA (p<0.0001) dan anti-CCP3.1 IgG/IgA (p<0.0001) antara pesakit RA dan golongan kontrol. Pesakit RA mempunyai tahap keterukan penyakit yang sederhana berdasarkan skor DAS28 dengan purata skor 3.52 (SD 1.13) manakala skor mHAQ adalah pada tahap ringan dengan purata skor 0.47 (SD 0.61). Tiada perkaitan yang signifikan antara keterukan penyakit dengan semua penanda serologi. Namun begitu, terdapat perkaitan signifikan antara skor kesakitan (p <0.0001) dan status antibodi anti-CCP2 IgG (p =0.049) dengan kualiti hidup pesakit RA. Majoriti pesakit RA yang mempunyai status positif untuk serum RF, anti-CCP2 IgG, dan anti-CCP3.1 IgG/IgA. Ini menunjukkan kepentingan penanda serologi ini dalam diagnosis dan prognosis penyakit RA. Antibodi anti-CCP2 IgG boleh dikatakan sebagai alat diagnostik terbaik berbanding penanda serologi yang lain kerana ia mempunyai perkaitan yang signifikan dengan kualiti hidup. Kajian lanjut pada masa hadapan sangat penting untuk mendapat lebih pemahaman tentang peranan antibodi anti-CCP generasi baru dalam diagnosis dan patogenesis penyakit RA.

CORRELATION OF NEW GENERATION OF ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES IN COMBINATION WITH RHEUMATOID FACTOR WITH DIAGNOSIS, SEVERITY, FUNCTIONAL STATUS AND PROGNOSIS

ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease which lead to progressive joint damage and affects patient's quality of life. Since rheumatoid factor (RF) has limited diagnostic performance, new generation of anti-cyclic citrullinated peptide (anti-CCP) antibodies were then introduced. The combination of RF and anti-CCP antibodies is ultimately important for diagnosis, prognosis, and clinical management of RA disease. This study aims to compare serum RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status in RA patients and healthy controls. The association between these serological markers and disease severity, as well as factors associated with functional status of RA patients were also evaluated. This cross-sectional study was conducted among 46 RA patients and 40 healthy controls in Rheumatology Clinic, Hospital USM. Self-administered questionnaires containing three parts; sociodemographic, clinical characteristics, and modified Health Assessment Questionnaire (mHAQ) form were used. Then, disease activity score (DAS28) of RA patients were assessed and five millilitres of blood was withdrawn of all subjects. RF was analysed using Direct Latex, whereas anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies were assayed using enzyme linked immunosorbent assay (ELISA). The data were analysed using SPSS version 26.0 with the p value < 0.05 was considered significant. The association of DAS28 with serological markers were analysed using independent sample t-test while factors

associated with mHAQ were analysed using multiple linear regression (MLR). Majority of RA patients had positive status of serum RF (78.3%), anti-CCP2 IgG (63.0%) and anti-CCP3.1 IgG/IgA (63.0%). Significant differences were found in RF (p<0.0001), anti-CCP2 IgG (p<0.0001), anti-CCP2 IgA (p<0.0001) and anti-CCP3.1 IgG/IgA (p<0.0001) antibodies between RA and control groups. RA patients had moderate disease severity based on the DAS28 score, while their mHAQ score showed mild functional status with mean score of 3.52 (SD 1.13) and 0.47 (SD 0.61) respectively. No significant association was found between disease severity with all serological markers. Significant association were found between pain score (p <0.0001) and anti-CCP2 IgG antibody status (p =0.049) with functional status of RA. No significant association between RF with DAS28 and mHAQ score. Positive status of RF, anti-CCP2 IgG, and anti-CCP3.1 IgG/IgA antibodies in majority of RA patients indicates the significance of these serological markers in diagnosis and prognosis of RA. Anti-CCP2 IgG antibody can be considered as the best diagnostic tool compared to other serological markers since it has significant association with the functional status. Future studies were important to obtain more understanding regarding the role of new generation anti-CCP antibodies in diagnosis and pathogenesis of RA.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Rheumatoid arthritis (RA) is an autoimmune disease that progressively promote joints soreness, destruction and articular cartilage erosion involving small and large joints which commonly justified as the prototype seropositive arthritis. Although the exact pathogenesis of RA remain unclear, it manifests as symmetrical polyarthritis which may concurrent with non-specific symptoms such as fatigue, malaise, and mild fever along with a wide spectrum of extra-articular manifestations (Samar *et al*., 2020). It was very common that together with the symptoms, RA can be identified by the presents of certain antibodies in the blood such as rheumatoid factors (RF) or anti-cyclic citrullinated peptide (anti-CCP) antibodies. Previous studies reported that presence of these autoantibodies were associated with the joint inflammation occurrence where it was hypothesise that seropositive RA expressed rapid deformities as well as more severe symptoms (Peter *et al*., 2019).

Even though main aetiology of rheumatoid arthritis remained indistinct, there were several presumptions in favour with the predisposing factors of RA including multiple hereditary and environmental influences. The strongest association that have been seen among RA population was gender where women are more at risk of having RA compared to men as many large number of epidemiologic studies showed ratio 2:1 of women to men from those who develop RA (Deane *et al*., 2017). There was also deduction explored from previous study including early menopause, the presence of polycystic ovarian syndrome, pregnancy and post-partum period, usage of contraceptive pills and hormone replacement therapy increased the risk of getting RA

(Fillingim *et al*., 2009). Furthermore, the decrease in estrogen level during reduction of ovarian follicles reservoir had suppress the humoral immune system either caused by oral contraceptives, pre- or post-menopause had association with high incidence of mild RA in women (Pikwer *et al*., 2012). Existing study also demonstrated that longer breastfeeding duration increase risk and severity of RA compared to non-breastfeeding mother because they found a relationship between HLA-DRB1 alleles and the prolactin gene on the chromosome nonetheless, there was reduced occurrence of RA during pregnancy and increased rate of clinical remission among women with RA who become pregnant (Peschken *et al*., 2012; Pikwer *et al*., 2012; Talsania & Scofield, 2017).

Furthermore, smoking and presence of shared epitope (SE) alleles of human leukocyte antigen namely HLA-DRB1 were associated with increased risk of having positive anti-citrullinated peptide antibodies (ACPA) among RA population in Malaysia (Too *et al.*, 2012). A gene-environment interaction of smoking and genetic was not only associated with positive rheumatoid factors and anti-cyclic citrullinated peptide (anti-CCP) antibodies but also with other RA-related autoantibodies because smoking exerts its influence many years before disease onset by causing citrullination of peptides in the lungs (Terao *et al*., 2014). A multicentre cohorts study in three different country; United Kingdom, Netherlands and Sweden found that smoking appears to be the most important factor for the formation of an autoimmune response against several RA-associated autoantigens whereas HLA-DRB1 gene contribution found later in the onset of RA diseases (Wesemael *et al*., 2016). It was also speculated that the metabolism of smoke substances could generate reactive oxidative species (ROS) that might activate the modification of autoantigens or DNA adduct formation

that explained association of smoking with RF and ACPA antibodies (Lu *et al*., 2014; Másdóttir *et al*., 2000; Terao *et al*., 2014).

Previous studies hypothesized that T cells and B cells produce antibodies to protect from infection or diseases that abnormally attack healthy cells which later on causing swelling, tenderness, and restriction of joint movements (Sivalingam *et al*., 2007). Activation of the innate immune response in RA including dendritic cells, macrophages, activated B cells, and T cells contribute to RA pathogenesis through antigen presentation, production of antibodies, autoantibodies, and cytokines. Besides, the reaction of RF and anti-CCP autoantibodies with antigens can lead to formation immune complexes which will eventually triggers the production of pro-inflammatory cytokines in RA such as Interleukin 1 (IL-1) and Tumour necrosis factor alpha (TNFα) (Josef & Günter, 2003; Ernest, 2012). Presence of cytokines stimulates the synovial fibroblast and chondrocytes in articular cartilage near the joints to secrete enzymes that degrade collagen and proteoglycans in which eventually leads to tissue destruction (Günter, 2007).

1.1.1 Rheumatoid factor and anti-cyclic citrullinated peptide antibodies as diagnostic tools in rheumatoid arthritis

In 1940, Erik Waaler discovered the first autoantibody in RA which was RF where it was then found to be reacted against the fragment crystallizable (Fc) region of IgG. RF is an antibody recognizing the conserved portion of human antibodies (Solbritt, 2005). It presents in 60% to 90% of RA patients with established RA but in less than 50% of patients with early RA (Günter, 2007). IgA, IgG, and IgM forms of RF have been identified in RA patients as well as pan-specific and Ga-specific forms of IgM-RF (Song *et al*., 2007). Even though RF is more established as American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) RA classification criteria adopted RF as an important biomarker, RF is likely to have limited specificity because it is also commonly detected in other autoimmune diseases, infectious diseases, and malignancies (Mierau & Genth, 2006). In addition, anti-CCP antibodies that presents at all stages of RA are also used in diagnosis and prognosis of RA whether in preclinical, early, and established disease and have gained wide acceptance because it is more specific and sensitive compared to RF (Kwok *et al.*, 2005).

Although anti-CCP antibodies tests are commercially available and clinically established, there are differences in cut-off values in terms of sensitivities and specificities between first, second, and third generation of anti-CCP antibodies (Rycke *et al.*, 2004). Conflicting evidences found that anti-CCP2 antibody appears to provide the best performance in sensitivity and specificity of test whereas certain studies also showed a higher sensitivity of anti-CCP3 antibody compared to anti-CCP2 antibody (Ine *et al*., 2017). However, in diagnosis of RA, anti-CCP tests are more sensitive,

specific, and higher likelihood ratios compared to RF. Since RA is a deleterious problem to older people in Malaysia with high medication costing, poor diagnosis, and prognosis monitoring in RA patients lead us to further confirmed and proved that newer generation of anti-CCP antibodies are better diagnostic tool in management of RA patients in future.

Additionally, Szekanecz *et al*. (2013) reported that the sensitivity and specificity of anti-CCP3.1 IgG/IgA antibodies test were significantly better in sensitivity and specificity than anti-CCP2 and anti-CCP3 especially in negative status of RF in RA patients. Innala *et al*. (2008) also found that high positive level of anti-CCP3.1 IgG/IgA antibodies together with RF-positive RA patients also had more aggressive joints erosion which fairly predicts the disease activity of early RA patients. Demoruelle *et al*. (2013) reported that although anti-CCP3.1 antibody are more sensitive, but it is less specific compared to anti-CCP2 IgG antibody. Since anti-CCP2 antibody are more specific, it is more preferable for predicting future diagnosis of RA and had better forecast the development of RA within two years of diagnosis (Ger *et al*., 2010).

1.1.2 Disease severity and functional disability in rheumatoid arthritis

Disease activity score (DAS28) was used as part of RA classification criteria by ACR/EULAR involving joints assessment, blood test and patients pain score. Disease activity measure help to investigate the treatment efficacy at the same time facilitate the clinical decision making to achieve disease remission seems to be a realistic goal for many RA patients (Jaclyn *et al*., 2012). DAS28 is commonly used by rheumatologist as an assessment to measure disease activity in RA patients and has been used as the gold standard for rheumatologist in decision making either to start or to stop disease-modifying antirheumatic drugs (DMARD) for RA patients (Fransen & van Riel, 2005; Piet & Lisanne, 2016). Previous studies found that DAS28 was significantly associated with the status of anti-CCP antibodies in RA patients where increase in the level of serum anti-CCP antibodies linked with aggressive RA, severe disease activity, as well as greater risk of erosive joints (c; Imad *et al*., 2018; Natividad *et al*., 2006; Praveen *et al*., 2019; Safi *et al*., 2011).

On another note, among various types of instruments used in monitoring the functional ability in RA, Health Assessment Questionnaire (HAQ) has been chosen since it was simple, short, easy to score, and had been extensively investigated, thus it showed more sensitive when patients treatment modification was done (Graell *et al*., 2009). Modified Health Assessment Questionnaire (mHAQ) was then developed by (Theodore *et al*., 1983) by reducing 20 items from HAQ into only eight items where each one represents each category. A study conducted by Evo *et al*. (2018) found that application of mHAQ able to compare favourably with the calculation of change scores which reflected the important clinical changes in RA patients over time. Assessment levels of comfort and ability of patients to pursue daily activities are very cost-effective

rather than laboratory test or radiographs. Besides, previous study found that anti-CCP antibodies level in RA patients affects the HAQ score before and after the clinical treatment but not RF status (Safi *et al*., 2011).

1.2 Problem statement

RA is characterized by inflammation and damage of the joints together with positive serology where the patients must fulfil the criteria required by ACR/EULAR. Continuous monitoring of anti-CCP antibodies concentration and disease activity may facilitate rheumatologist to understand whether the treatment work well for the patients and minimise the disease progression, thus enable patients to maintain active lifestyle and continue to lengthen their employability period. Unfortunately, 45% of RA cases were diagnosed a year after the onset of RA symptoms and 20% of RA patients diagnosed only after three years of symptoms onset. According to the Malaysian National Inflammatory Arthritis Registry (NIAR) in 2019, this is might be due to lack of awareness and understanding of the disease among public and healthcare providers as well as socio-economic constraints to access early specialised rheumatology care (Azmillah *et al*., 2019). Overcoming the problem of delayed RA diagnosis was very crucial because RA patients are at risk of developing comorbidities that may shorten their life span from the consequences of increased prevalence of cardiovascular diseases, greater risk of infections as well as stimulating the development of certain malignancies (Dougados *et al*., 2014). Even though RF is widely used in diagnosis of RA, it is known to be less specific compared to anti-CCP. Therefore, newer generations of anti-CCP are predicted to be more important for future diagnostic and prognostic tool for RA in terms of specificity and sensitivity.

1.3 Justification of the study

New generation anti-CCP antibodies are important for diagnosis and predict the prognosis for RA. Early detection of these autoantibodies in RA patients may help in early treatment to minimise the complication of the disease. Detection of more than one autoantibody in RA taken at multiple points of time could better predict symptoms and disease activity improvement as well as medication efficacy. Besides, quality of life of RA patients also will be improved if early diagnosis and treatment was given. This research findings were able to fill in the knowledge gap in terms of diagnostic tools with better sensitivity and specificity used to rule out RA more effectively so that the patients can get early treatment as possible leading to improve disease activity, lower functional disability, minimising joints erosions and eventually lower the mortality risk.

1.4 Research objectives

1.4.1 General objectives

This study aims to determine serum RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status in RA patients and the association with disease severity and functional status of the patients.

1.4.2 Specific objectives

1. To determine serum RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status in RA patients.
2. To compare serum RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status between RA patients and healthy controls.
3. To determine disease severity of RA patients.
4. To determine the association between serum RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status and disease severity of RA patients.
5. To determine the functional status of RA patients.
6. To determine the factors associated with functional status of RA patients.

1.5 Research questions

The research questions in this study were:

1. What is the status of serum RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies in RA patients?
2. Is there any significant difference in the RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status between RA patients and healthy controls?
3. What is the level of disease severity in RA patients?
4. Is there any significant association between the RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status and disease severity of RA patients?
5. What is the level of functional status of RA patients?
6. What are the factors associated with the level of functional status of RA patients?

1.6 Research hypothesis

1. There is a significant difference in the status of RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies between RA patients and healthy controls.
2. There is a significant association between the RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status with disease severity of RA patients.
3. Functional status of RA patients is significantly associated with the RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA antibodies status and disease severity.

1.7 Conceptual framework

Several clinical investigations are required by rheumatologist to confirm RA disease after series of joints inflammation. These include physical examination and laboratory analysis as there is no single measure to confirm RA. Figure 1.1 presented is the conceptual framework of RA clinical factors and its association with disease severity and functional status.

1.7.1 Sociodemographic characteristics that associated with the presence of autoantibodies and functional status in RA

The positive status of RA serological markers such as RF, anti-CCP antibodies were found to be directly associated with age because patients with positive IgM-RF, IgA-RF, IgG-RF and anti-CCP2 antibodies were found to be significantly younger at the onset of RA disease compared to seronegative RA patients (Elshafie *et al*., 2019; Münevver *et al*., 2008). In addition, older age was associated with increasing ACPA positivity (Terao *et al*., 2014). Although women were more susceptible with RA compared to men, men are more likely to have positive RF and anti-CCP antibodies with higher concentration and showed more signs of erosive joints than female patients (Hafström *et al*., 2019; Mackey *et al*., 2016; Miriovsky *et al*., 2011). Furthermore, Malay ethnic was more at risk of RA compared to other ethnics in Malaysia (Too *et al*., 2014). The sociodemographic factors (education, marital status, age, and economic circumstances) were also significantly influencing the quality of life (QoL) of RA patients (Jankowska *et al*., 2010).

1.7.2 Clinical characteristics that associated with the presence of autoantibodies in RAs

A multicentre cohort study found that smoking was significantly associated with the positive status of anti-CCP2 IgG antibody and IgM-RF but not in one autoantibody (RF or anti-CCP2 antibody) (Masdottir *et al*., 2000; Terao *et al*., 2014; Wesemael *et al*., 2016). Exposure of tobacco smoke also was purported to be associated with increase in disease activity (Deane *et al*., 2017). Furthermore, changes in hormone levels, cytokine profiles and immune cell function during pregnancy was associated with increased risk of ACPA-negative RA (Fillingim *et al*., 2009; Peschken *et al*., 2012). Early menopause and breastfeeding were also identified as risk factors for RA (Pikwer *et al*., 2012). Apart from that, the presence of comorbidities such as non-cardiac vascular diseases, endocrine diseases, cardiovascular diseases, respiratory diseases, renal diseases, and hematologic malignancies have also increased the risk of RA (Nikiphorou *et al*., 2017). Positive status of RF and ACPA were also reported in other autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Sjögren's syndrome, juvenile idiopathic arthritis and Epstein-Barr virus (EBV) (Gottenberg *et al*., 2005; Mediwake *et al*., 2001; Rossum *et al*., 2003; Too *et al*., 2012).

Erythrocyte sedimentation rate (ESR) was significantly correlated with disease severity and functional status in RA patients (Magaly *et al*., 2014; Pakchotanon *et al*., 2016). Furthermore, severity of pain was also associated with functional status (HAQ) and disease activity score of 28 joints (DAS28) (Garip *et al*., 2011). Functional disability and DAS28 worsen with increasing levels of comorbidity and longer disease duration (Ajeganova *et al*., 2013; Grøn *et al*., 2014; Radner *et al*., 2010).

1.7.3 RA serological markers associated with disease severity and functional status

High disease severity was associated with positive status and higher concentration of RF, anti-CCP2 IgG, anti-CCP2 IgA and anti-CCP3.1 IgG/IgA in RA patients (Carpenter *et al*., 2017; Miriovsky *et al*., 2011; Wanruchada *et al*., 2015). Positive serological markers in RA was also correlated with higher disability score thus poor QoL (Boyd *et al*., 2013; Evo *et al*., 2018; Hamad *et al*., 2014). Moreover, there was correlation between functional status and DAS28 where higher disease severity leads to poorer QoL of RA patients especially in seropositive RA patients (Boyd *et al*., 2013; Hamad *et al*., 2014; Miriovsky *et al*., 2011).

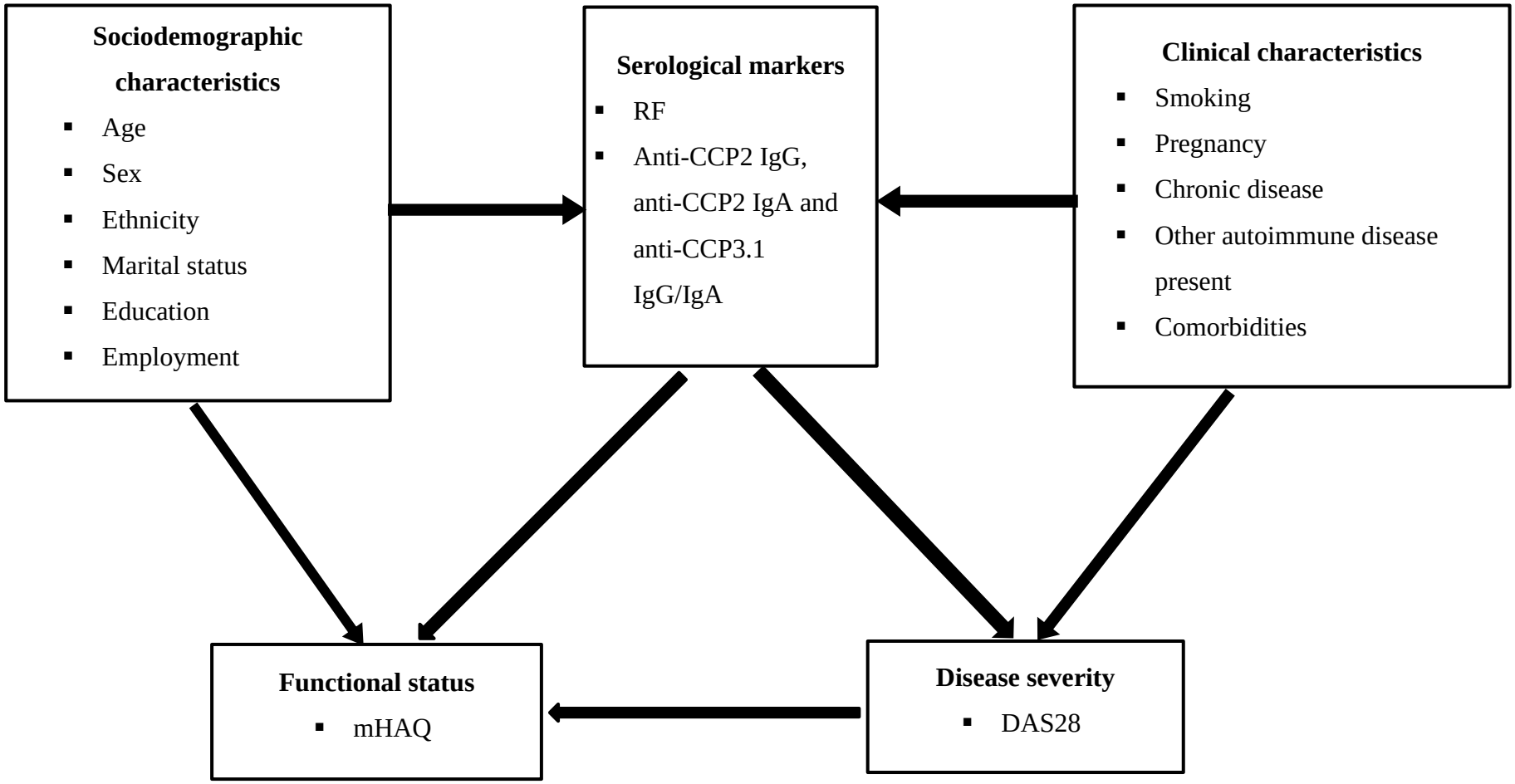

Figure 1.1: Conceptual framework of diagnostic tools and the associated factors in RA

CHAPTER 2

LITERATURE REVIEW

Globally, RA is one of the most common autoimmune disease which affect 1.0% of the world population (Aletaha *et al*., 2010). Malaysian Ministry of Health Consensus reported that the prevalence of RA in Malaysia is 0.5% among adults ranging from 25 to 50 years old and women are at risk of getting RA compared to men and also ranked as the third disease most commonly affect woman in Malaysia (Too *et al*., 2011). RA is an autoimmune disorder characterized by symmetrical chronic inflammation which induces the formation of pannus tissues which eventually lead to erosive synovitis, joint destruction, and premature disability. Since the exact aetiology of RA was still uncertain, serological marker are very important in diagnosis of RA and help in further understanding the pathogenesis of RA. The American College of Rheumatology and European League against Rheumatism (ACR/EULAR) in 2010 (Table 2.1) indicates two important biomarkers in diagnosis of RA which are RF and anti-CCP antibodies. Although RA disease have no exact cure the main aim of treatment was to minimize pain, achieve remission and downturn functional disability which will results in patient's loss of employment. Researchers postulated that the prevalence of RF and anti-CCP antibodies in RA patients greatly associated with increase joints inflammation, destruction and reduce patient's functional health (Carpenter *et al*., 2017; Graell *et al*., 2009; Wanruchada *et al*., 2015).

Table 2.1: The 2010 ACR/EULAR classification criteria for rheumatoid arthritis (Aletaha *et al*., 2010)

	Score
Target population. Who should be tested: Patients who:	
1) have at least 1 joint with definite clinical synovitis (swelling)	
2) with the synovitis not better explained by another disease	
Classification criteria for RA (score-based algorithm: add score of categories A-D; A score of ≥6/10 is needed for classification of a patient as having definite RA)	
A. Joint involvements	
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 (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF and high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

2.1 Rheumatoid factor in diagnosis and pathogenesis of RA

RF were the first autoantibodies that had been discovered in RA as serological evidence presence in serum and synovial fluid that have capability to enhance immune complexes as it were directed against the Fc (fragment crystallizable) region of normal Immunoglobulin G (IgG) where different types of RF can recognize different parts of IgG-Fc sites. RF have germinal center-like and lymphoid follicles structures and was produced by B cells in inflamed synovium (Song & Kang, 2010). The production of RF antibodies was not properly understood because there was no clear evidence that RF are involved either it was the trigger of the disease process or RF triggered by RA disease. The capacity of RF to form immune complexes (containing the normal IgG and RF) not only by self-association but also with other autoantibodies will grant arthritogenicity that eventually associate with poor disease outcome (Volkov *et al*., 2020).

Erik Waaler (1940) who discovered RF more than 70 years ago was depends mainly on IgM-RF isotypes antibodies, RF however can also be found in any forms of immunoglobulins such as IgG-RF, IgM-RF and IgA-RF. The most prevalent RF isotype in RA was IgM-RF as most assays detects IgM-RF which shows it high affinity thus had higher ability to induce agglutination in RA serum that indicate inflammation (Vallbracht *et al*., 2004). It was also detected in more than 75% of RA patients. RF not only can be identified in RA but also in other autoimmune disorders, chronic infections or miscellaneous conditions for instance Sjögren's syndrome, Systemic Lupus Erythematosus, Hepatitis, tuberculosis, sarcoidosis, idiopathic pulmonary fibrosis, smokers or adults more than 65 years old (Shidara *et al*., 2011).

RF becomes reactive when immune complexes were formed after binding with IgG where those formation able to bind to antigen and facilitate the immune response in healthy individual. Nevertheless, a standard RF which is IgM antibody reacts with IgG to form IgM-IgG complex which then shows signs of affinity maturation that fluctuate over time and deposits in the joints of RA patients (Rao, 2006). These incidents were not observed in healthy individuals that probably explained high affinity of RF reflected on high titers RF in patient's serum thus perpetuating inflammation and painful joints (Volkov *et al*, 2020). High titers of RF which indicate three times concentration of minimum positive value of the antibody detected in the serum of RA patients can be measured by commercially available immunoassays such as agglutination latex test, nephelometry or enzyme-linked immunosorbent assays (ELISA) (Datta, 2013).

2.1.1 Sensitivity and specificity of Rheumatoid Factor

There was no single laboratory diagnostic able to confirm RA disease as the blood test must have correlation with the patient's history and clinical manifestations. The presence of RF not only can assist in diagnosis of RA but also assessing prognosis or determining the extent of the disease as certain RF subtypes had been found to predict disease severity in RA patients (Ingegnoli *et al*., 2013). However, RF tests have different sensitivity and specificity in different diagnostic tools measuring different RF isotypes with sensitivity ranging from 60 to 90% and specificity from 50 to 85% where it can also be detected in other disease or healthy individuals (Mierau & Genth, 2006). Sieghart *et al*. (2018) conducted a study using two serological marker which were RF and anti-CCP antibodies found that one-third of total 290 RA patients had negative

status of both autoantibodies. However, it was not fully understood whether these patients generate antibodies species other than routine diagnostic tools used.

Previous research by Sieghart and colleagues (2018) investigated three different RF isotypes (IgA-RF, IgG RF and IgM-RF) using EliA^{TM} platform discovered that IgG-RF had the highest specificity with value 98.6% followed by IgA-RF and IgM-RF with specificity value 95.3% and 90% correspondingly. Nonetheless, IgM-RF had the highest sensitivity followed by IgA-RF and the lowest was IgG-RF subtype with percentage 64.8%, 50.7% and 14.4% respectively. Furthermore, a more recent study by Arumugam *et al*. (2019) found that RF latex agglutination test have lower sensitivity (42.9%) and specificity (76.5%) compared to IgM-RF ELISA with sensitivity (50%) and specificity (82.4%). Despite ELISA method in determining different RF subtypes had higher sensitivity and specificity, RF latex test was more commercially available and widely used as diagnostic tools in determining RA because of its economically affordable and the test can be run with single serum thus more fast yet reliable (Syed *et al*., 2008).

2.2 Anti-cyclic citrullinated peptide (anti-CCP) antibodies in diagnosis and pathogenesis of rheumatoid arthritis

Anti-CCP antibodies have been included as a new serologic criterion in the 2010 Rheumatoid Arthritis Classification Criteria by ACR/EULAR. Generally, this chronic inflammation namely RA induced the formation of pannus tissue which lead to joint destruction. The finding in 1998 proved that the underlying antigen in the antiperinuclear factor (APF) and antikeratin antibodies (AKA) tests contained

citrulline, a modified form of protein from arginine that acted against antibodies (Swart *et al*., 2012). Additionally, these citrulline have antigenic sites which enhance the binding of antibodies forming immune complexes that precipitates mostly in symmetrical joints that eventually contribute to the joints pain and damage. It was reported that anti-CCP antibodies are an important serological marker in the diagnosis of RA thus a commercial test was developed using synthetic cyclic citrullinated peptide (CCP) as an artificial antigen (Venrooij *et al*., 2011).

RA cycle hypothesis was first described by Venrooij and Pruijn (2000) where inflammation triggered from enzymatic process of post-translational modification involving peptidyl arginine deiminase (PAD) converting arginine to citrulline by which process called citrullination. Citrullination is defined as the conversion of peptidyl arginine into peptidyl citrulline (the guanidine group of the arginine side chain is converted into an ureido group) catalysed by Ca^{2+}-PAD enzyme that commonly occurs in dying cells. Firstly, an unlimited influx of extracellular Ca^{2+} ions freely enter the cell membrane that undergoing cell death (apoptosis or necrosis) because of perforated walls. Consequently, PAD enzyme becomes activated and converts peptidyl arginine into peptidyl citrulline. Venrooij *et al*. (2011) also hypothesized that active PAD enzymes together with citrullinated cellular proteins like vimentin and histones are released when the apoptotic clearance mechanism fails to remove the dying cells in time. The RA cycle hypothesis was illustrated in Figure 2.1.

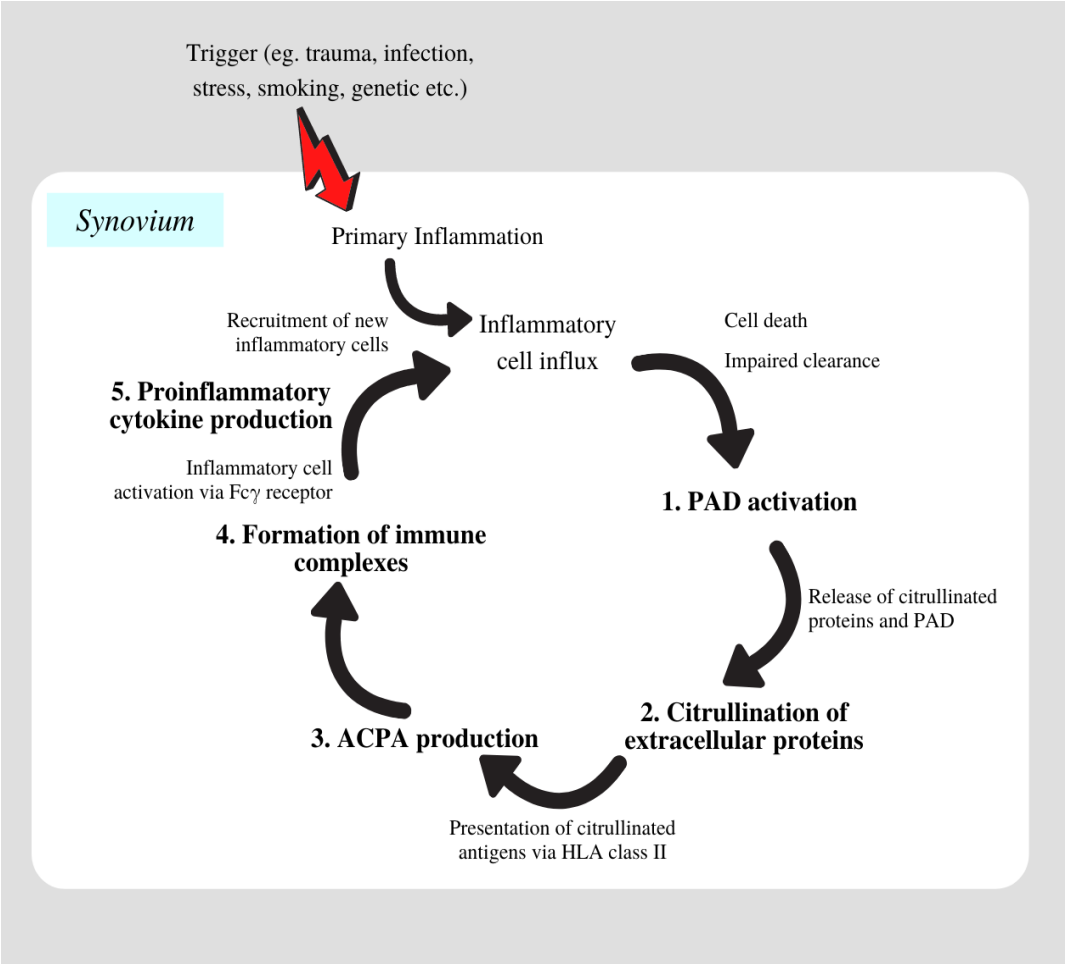

Figure 2.1: Rheumatoid arthritis cycle hypothesis (Venrooij *et al*., 2011)

Furthermore, when the number of dying cells is too high or when there is defect in cell death process the cells can become necrotic and release intracellular proteins into the extracellular space which eventually undergo citrullination. In some individuals citrullinated proteins are exposed to the immune system then trigger an immune response, subsequently leads to the formation of anti-citrullinated peptide/protein antibodies (ACPA) which will trigger the upregulation and precipitation of proinflammatory cytokines mainly find in synovial cytokines (Figure 2.1). Scientist speculate that the released citrullinated proteins function as the first citrullinated antigens to be encountered by the immune system. PAD enzymes will not only activate production of citrullinate vimentin and nuclear histones but also many other arginine-containing proteins in the synovium such as collagen and fibrin/fibrinogen. This will also lead to the exposure of a secondary array of citrullinated peptides to the immune system. Therefore, the diversification of the ACPA pool is an early occurrence in the RA cycle that start way before the disease is clinically recognized.

2.2.1 Evolution of Anti-cyclic citrullinated peptide antibodies as serological markers in rheumatoid arthritis

After RF was identified in 1940, APF antibodies known as keratohyalin granules that contiguous to the granules in human buccal mucosa cells together with AKA antibody that directed against keratinised tissue of animal oesophagus was then explored by Nienhuis *et al*. (1964). However, they have limited diagnostic efficacies due to low specificity, sensitivity, and complicated methodology (Ine *et al*., 2017). Ever since, in 1998 researchers found that the underlying antigen in APF and AKA

antibodies test contained citrulline because during the last stages of terminal differentiation of epithelial cells, filaggrin undergo dephosphorylation and parts of arginine residue was catalysed into citrulline (Schellekens *et al*., 1998). First generation of anti-CCP antibody was then introduced where it relied on a peptide derived from filaggrin protein and able to lead a far better results than RF as serological markers in RA using enzyme-linked immunosorbent assays (ELISA) (Ine *et al*., 2017).

First generation of anti-cyclic citrullinated peptide (anti-CCP1) antibody was found to have higher specificity compared to RF not until 2002 when second generation of anti-CCP (anti-CCP2) antibody was developed with different cyclic peptides and improved performance characteristics that showed a better specificity and sensitivity in RA (Lisiane *et al*., 2009). Subsequently, third generation of anti-CCP (anti-CCP3) antibody containing unique peptide was introduced to increase the sensitivity for the diagnosis of RA disease (Swart *et al*., 2012). It was speculated that higher sensitivity of anti-CCP3 antibody test may be found in cohorts with early RA that helpful in managing early treatment of RA (Ine *et al*., 2017). The latest anti-CCP antibody version established in 2013 was anti-CCP3.1 antibody where it have ability to detect a combination of IgG and IgA isotypes leading to significantly better performance with higher sensitivity of anti-CCP test in RF negative patients compared to other ACPAs laboratory testing that only detect single immunoglobulin (Szekanecz *et al*., 2013). Newer generation of anti-CCP antibodies was no longer derived from filaggrin protein but on peptide precisely designed and optimized (Swart *et al*., 2012).