Vitamin D Receptor FokI Gene Polymorphism in Rheumatoid Arthritis Patients

Lamiaa Abdel Wahaab Mohammed[1], Ahmed Mohammed Baraka[1], Ghada S. Nageeb[2], Fatma Mohammed Mohammed Alsayed*[1], Ahmed Mokhtar Ahmed[1], Ahmad Sallam Soliman[1], Mahmoud Amer[3]

Departments of [1]Clinical Pathology, [2]Rheumatology, and Rehabilitation, and [3]Internal Medicine,
Faculty of Medicine, Zagazig University, Sharkia, Egypt

*Corresponding author: Fatma Mohammed Mohammed, **Mobile:** (+20) 01151234898, **E-Mail:** dr.mosad@yahoo.com

ABSTRACT

Background: Rheumatoid arthritis (RA) is a chronic, systemic, destructive joint disease. Vitamin D initiates biological responses via binding to the vitamin D receptor (VDR), which is a member of the steroid hormone receptor superfamily located on chromosome 12 (12q12-q14) that regulates gene expression in a ligand-dependent manner. The receptor (VDR) is active in almost all tissues that are necessary for the effects of vitamin D.

Objective: The present work aimed to detect vitamin D receptor FokI gene polymorphism in rheumatoid arthritis patients, to assess its role as a risk factor of the disease.

Patients and Methods: A case-control study was conducted on 44 subjects (22 RA patients and 22 apparently normal volunteers age and sex-matched with patients) at the Clinical Pathology, Rheumatology and Rehabilitation Departments, Faculty of Medicine, Zagazig University.

Results: Serum vitamin D level was found to be significantly lower among RA patients compared to healthy controls. There was a significant difference between the studied groups regarding VDR FOK1 gene polymorphism and its alleles. There was a non-significant difference between the different genotypes regarding age, however, the difference between them was significant regarding sex as it was noticed that FF and ff homozygous genotypes were found only in females compared to their male counterparts while Ff heterozygous genotype was found equally among both males and females.

Conclusion: Vitamin D receptor gene FokI polymorphism could be considered as genetic risk markers of RA susceptibility, associated with vitamin D level deficiency which was found to be significantly lower among FF genotype compared to other genotypes. Having FF and Ff genotypes have almost a higher risk than having ff genotype to be RA patient.

Keywords: Rheumatoid arthritis, vitamin D receptor, FokI gene polymorphism.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, destructive joint disease. It occurs most frequently in middle-aged women, and about 0.5 to 1.0% of the population suffers from the disease in the industrialized world. It is characterized by the presence of autoantibodies including rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA). Rheumatoid patients have an increased risk of morbidity and mortality [1].

Rheumatoid arthritis is the most common autoimmune disease worldwide and is an important public health concern, associating with early death and systemic complications [2].

The characteristic of RA is muscular weakness around the affected joints, and symmetric joint inflammation, stiffness, and pain. It is the most severe form of arthritis [3].

Smoking, diabetes mellitus, citrullination, and genetic variability were shown to be involved in the immune pathogenesis of RA and might contribute to its prevalence [4].

Although major progress has been made in treating RA, many patients still experience premature work disability and co-morbidities. Therefore, there is an urgent need to explore new risk factors in helping early identification and treatment of the disease. About 60% of RA risk is thought to be genetic. [5].

Vitamin D, as an immunoregulatory hormone, is central to the control of bone and calcium homeostasis. The previous meta-analysis showed that low vitamin D intake was associated with an elevated risk of RA development [6]. Greater intake of vitamin D was associated with a lower risk of RA, as well as a significant clinical improvement was strongly correlated with the immunomodulating potential in vitamin D-treated RA patients [7].

Vitamin D initiates biological responses via binding to the vitamin D receptor (VDR) [8], which is a member of the steroid hormone receptor superfamily located on chromosome 12 (12q12-q14) that regulates gene expression in a ligand-dependent manner [9]. VDR is active in almost all tissues that are necessary for the effects of vitamin D. Several genetic variations have been identified in the VDR gene. Among which, TaqI (rs731236 in exon 9), BsmI (rs1544410 in intron 8), ApaI (rs7975232 in intron 8) at the 3'-end, and FokI (rs2228570 in exon 2) at the 5'-end of this gene were the most studied [10].

Although several studies reported the role of VDR polymorphisms in RA risk, the results remain inconclusive. This may due to the non-equilibrium distribution of RA incidences and VDR polymorphisms.

Furthermore, differential VDR expression relates to ethnicity [11] and may affect the genetic associations in RA [12]

The present work aimed to detect vitamin D receptor FokI gene polymorphism in rheumatoid arthritis patients, to assess its role as a risk factor of the disease.

PATIENTS AND METHODS

This case-control study was carried out at the Clinical Pathology, Rheumatology and Rehabilitation Departments, Faculty of Medicine, Zagazig University, after review and approval by the Institutional Review Board (IRB) committee on 44 subjects for ethical consideration.

Ethical consent:
An approval of the study was obtained from Zagazig University academic and ethical committee. Every patient signed an informed written consent for acceptance of the operation. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

The subjects were divided into 2 groups:
Group I (Rheumatoid Arthritis Group): It included 22 previously diagnosed rheumatoid arthritis patients, fulfilled the 2010 American College of Rheumatology/European League against Rheumatism classification criteria for RA patients [13]. They were 18 females and 4 males. Their ages ranged from (35-65) years with a mean value ± SD (55.04± 9.24) years, disease duration ranged from (1-25) years with a mean value ± SD (11.6 ± 5.93) years.

Group II (Control Group): It included 22 apparently normal volunteers age and sex-matched with patients. They were 15 females and 7 males. Their ages ranged from (40 –65) years with a mean value ± SD (50.3 ± 8.70) years. Clinical examination as well as routine laboratory investigations confirmed their healthy state.

Inclusion criteria: All patients fulfilled the 2010 American College of Rheumatology/European League against Rheumatism classification (EULAR) criteria for RA patients**.** For classification purposes, a patient has definite RA if he/she scores at least 6 points in the established classification system [13]**.**

Exclusion criteria: patients with similar clinical features of other rheumatic or autoimmune diseases have been excluded

All studied people were subjected to the following:

A-Full history taking: All patients were subjected to detailed history taking including name, age, sex, disease, and current medication, with special emphasis on Pain, Morning stiffness (MS), Number of swollen joints duration, Number of tender joints, Disease duration, Dryness of the eyes and /or mouth, Fever: character, degree and its diurnal variation, Raynaud's phenomenon, Falling of hair, skin rash, oral or genital ulcers, Subcutaneous nodules, Symptoms of other system affection.

B-General and Systemic examination: General: Pulse, temperature, blood pressure, respiratory rate, L.L. edema, and lymph nodes examination, skin examination for rash, oral ulcers. **Systemic:** chest, heart, abdominal& neurological examination.

C-Local examination of joints: Examination of individual joint by:
Inspection: Swelling: its site, size, and shape. Wasting of the muscles, Deformity. The skin over the joint: (redness, scars, and ulcers).
Palpation: Hotness, tenderness, crepitus, synovial thickening & swelling nature. **Range of motion:** both active and passive.

D- Assessment of disease activity:
We used DAS-28 to assess the patient's disease activity.

The joints included in DAS28 are (bilaterally): proximal interphalangeal joints (10 joints), meta Carpo phalangeal joints (10), wrists (2), elbows (2), shoulders (2), and knees (2) [14]**.**

When looking at these joints, both the number of joints with tenderness upon touching (TEN28) and swelling (SW28) are counted. In addition, the erythrocyte sedimentation rate (ESR) is measured. Also, the affected person makes a subjective assessment (SA) of disease activity during the preceding 7 days on a scale between 0 and 100, where 0 is "no activity" and 100 is "highest activity possible". With these parameters, DAS28 is calculated as follows according to **Prevoo** *et al.* [15].

$$DAS28 = 0.56 \times \sqrt{TEN28} + 0.28 \times \sqrt{SW28} + 0.70 \times \ln(ESR) + 0.014 \times SA$$

E-Radiological investigations: Plain x-ray of hands and feet as well as any affected joints.

F- General Laboratory investigations: Erythrocyte sedimentation rate by vision –B yhlo Biotech. Rheumatoid factor assay by Cobas 6000 (turbidimetric method). CRP assay by Cobas8000. Measurement of serum 25(OH)D level by Cobas 6000, e601 module, by electrochemiluminescence. Anti-cyclic citrullinated peptide (Anti-CCP) by Cobas 6000.

G-Specific Laboratory investigation: Determination of vitamin D receptor gene FokI polymorphism in all patients and controls by direct sequencing using HITACHI 3500 Genetic Analyzer.

Workflow of sequencing reaction: DNA extraction: DNA is extracted from WBCs by a DNA extraction kit. Followed by the detection of the extracted DNA by gel electrophoresis. Polymerase Chain Reaction: amplification of the extracted DNA by using specific primers of VDR gene FokI forward 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and reverse 5'-ATGGAAACACCTTGCTTCTTCTTCTCCCTC-3'. Followed by the detection of the amplified gene by gel electrophoresis. First Purification of the amplified product by purification kit. Measurement of DNA concentration. Cycle sequencing. Secondary Purification of the cycle sequencing product by centri –sep purification kit. Injection of purified amplified

VDR gene FokI in HITACHI 3500 Genetic Analyzer then analysis of the sequencing results.

1) DNA extraction from the white blood cells:
Principle: In the nucleus, the DNA is associated with many different proteins in a structure called chromatin. It is important to remove cellular proteins to extract the DNA. DNA extraction was achieved by the use of the QIAamp DNA blood mini kit (QIAGEN).

2) Selective amplification of VDR gene FokI by PCR technique: Polymerase chain reaction amplicons were generated using the following primer pairs: Forward5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'and reverse 5'-ATGGAAACACCTTGCTTCTTCTTCTCCCTC-3'
PCR amplification was performed in a 30ul reaction mixture, containing 6 ul of genomic DNA, 12ul nuclease-free water, 1ul of 10 pmol of both forward and reverse primers, 10ul of the ready master mix(Top Taq Master Mix Kit QIAGEN). PCR was performed using a thermal cycler (Gene Amp PCR system 2400, Roche diagnostic system) with cycling condition of initial denaturation at (95°C for 1min, 35 cycles of **denaturation** at 95 °C for 15 seconds, **annealing** at 61°C for15 seconds and **extension** at 72°C for 10 seconds, followed by **a final extension** at 72°C for 7 min. PCR products were separated on a 2 % agarose gel. The PCR marker (ladder) 100 bp DNA ladder (Tiangen Biotech Co., LTD) was also loaded into one of the wells.

3) Purification of amplified PCR product: Kit Purification of amplified PCR product achieved by the use of QIAquick PCR Purification (QIAGEN). **The QIAquick principle:** The QIAquick system combines the convenience of spin-column technology with the selective binding properties of the PCR product to a uniquely designed silica membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA and removal of the contaminant in each special application. DNA adsorbs to the silica membrane while contaminants pass through the column. Impurities are efficiently washed away and the pure DNA is eluted with tris buffer or water.

4) Measurement of DNA concentration: After purification of PCR product, DNA concentration was estimated by TheQubit® 3.0 Fluorometer Invitrogen life technologies. The Qubit Fluorometer utilizes specially formulated dyes that bind specifically to DNA.

5) DNA cycle Sequencing: Principle: In the automated fluorescent sequencing, fluorescent dye labels are incorporated into DNA extension products using 3´dye-labeled dideoxynucleotide triphosphates (dye terminators). Extension product termination and labeling occur simultaneously for all bases in one tube. Applied Biosystems DNA sequencer(**HITACHI 3500 Genetic analyzer**) detect fluorescence from four different dyes that are used to identify the A, C, G, and T extension reactions. Each dye emits light at a different wave length when excited by an argon-ion laser. All four colors and therefore all four bases can be detected and distinguished in a single gel lane.

6) Secondary purification using centri – sep spin columns: PRINCIPLE: CENTRI-SEP Columns are used for the fast and efficient purification of nucleic acids from small molecules (nucleotides, buffer salts, etc.). The column design is based on gel filtration for the purification of DNA from nick translation reactions. Each unit consists of a special microfuge tube, dry gel, wash tube, and sample collection tube, all designed for this purpose.

7) DNA sequencing using HITACHI 3500 Genetic Analyzer (Applied Biosystem, USA): It is an automated instrument for analyzing fluorescently labeled DNA fragments by capillary electrophoresis. Electrophoresis is the movement of charged molecules through a polymer in an electrical field. It is used to separate DNA fragments by size. Samples were electrophoretically separated as they travel through the polymer in the capillary. The sequencing reaction sample tubes were placed in an autosampler tray. The autosampler successively brought each sample into contact with the cathode electrode and one end of a glass capillary was felt with a separation polymer. An anode electrode at the other end of the capillary was immersed in buffer. The sample entered the capillary as current flows from the cathode to the anode. The short period of electrophoresis conducted while the capillary and cathode were immersed in the sample is called electrokinetic injection. The sample forms a tight band in the capillary during this injection. The end of the capillary near the cathode was then placed in the buffer. The current was applied again to continue electrophoresis. When the DNA fragments reached a detector window in the capillary, a laser-excited the fluorescent dye labels. Emitted fluorescence from the dyes was collected once per second by a cooled, charge-coupled device (CCD) camera at particular wave length bands (virtual filters) and stored as digital signals on a computer for processing. The Sequencing Analysis software interpreted the result calling the bases from the fluorescence intensity at each data point.

8) Analysis of sequencing results: The sequencing results were examined at the National Center for Biotechnology Information (NCBI), nucleotide BLAST (Basic Local Alignment Search Tool) website for sequencing analysis services and detection of VDR gene FokI polymorphism in the studied samples. The Basic Local Alignment Search Tool found regions of local similarity between sequences. The program compared nucleotide sequences to sequence databases and calculates the statistical significance of matches. We put our sequencing results of VDR gene FokI (rs2228570) in search database genome, homosapiens (human genomic) using megablast (optimized for highly similar sequences).
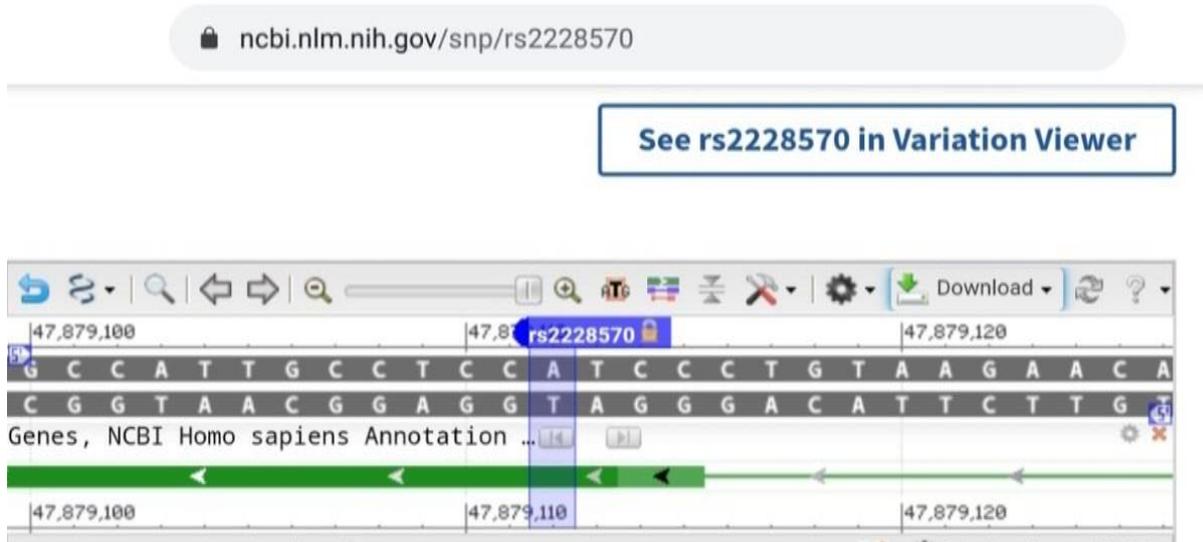
Figure (1): The position of the single nucleotide polymorphism of the VDR gene (FOK1), rs2228570.
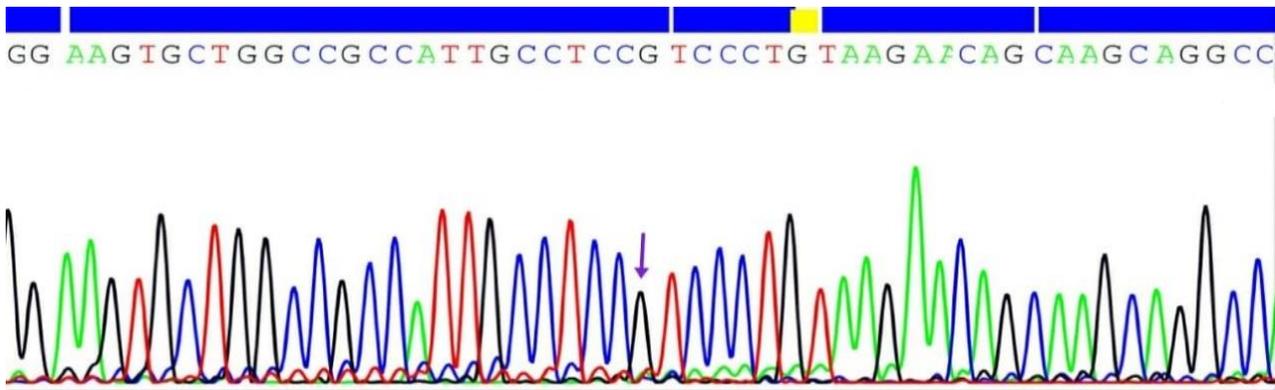
Figure (2): Representative electropherogram sequencing result of the Fok1 VDR gene polymorphism, arrow indicates the presence of homozygous genotype FF (GG) at the polymorphic site.
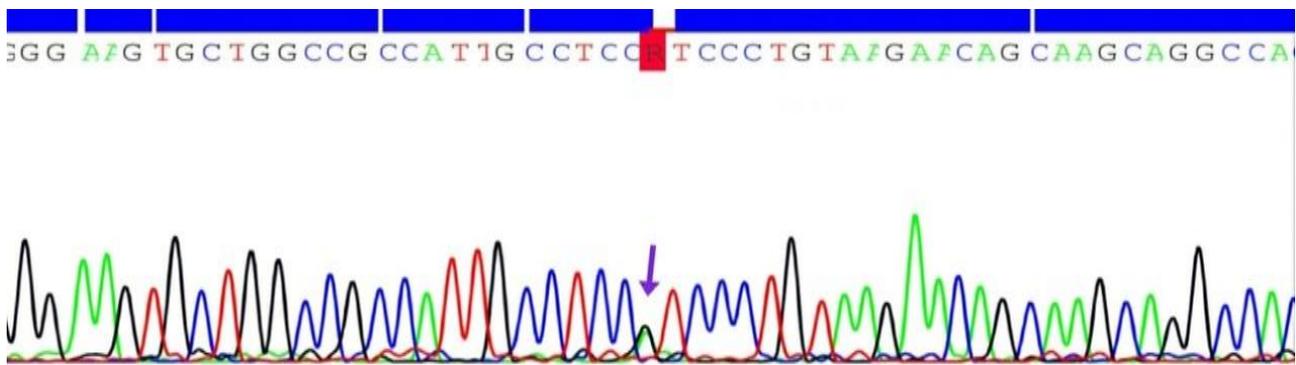
**Figure (3):** Representative electropherogram sequencing result of the Fok1 VDR gene polymorphism, arrow indicates the presence of heterozygous genotype Ff (GA) at the polymorphic site.

Statistical analysis

All data were collected, tabulated, and statistically analyzed using SPSS version 19. Continuous Quantitative variables were expressed as the mean ± SD &median (range), and categorical qualitative variables were expressed as absolute frequencies (number)& relative frequencies (percentage). Mann-Whitney test, Independent student's t-test, Kruskal-Wallis test were used. Categorical data were compared using the chi-square test. The odds ratio was used for risk quantification. The tests were two-sided with a p-value < 0.05 was considered statistically significant (S), p-value < 0.001 was considered highly statistically significant (HS), and p-value ≥ 0.05 was considered statistically insignificant (NS).

RESULTS

There was a non-significant difference between the studied groups regarding age and sex. There was a highly significant difference between the studied groups as regarding vitamin D **(Table 1).**

DAS-28's mean was 4.32 and the mean disease duration was 11.6 years **(Table 2).**

It was noticed that the FF genotype was found to be significantly higher among cases compared to the control group (54.5% versus 18.2%). Also, regarding alleles frequency, the F allele was found to be higher among cases than controls (72.7% versus 47.7%). Having FF and Ff genotypes have almost a higher risk 7.5 times, 1.5 times than having ff genotype to be RA patient. Having an F allele has almost a higher risk three times (2.92) than having an f allele to be a RA patient **(Table 3).**

There was a non-significant difference between the different genotypes as regarding age, however, the difference between them was significant as regarding sex. FF and ff homozygous genotypes were found only in females compared to their male counterparts while Ff heterozygous genotype was found equally among both males and females **(Table 4).**

There were non-significant differences between different genotypes regarding RF and CRP levels. However, the difference between them was significant regarding the Anti-CCP level which was found to be significantly higher among FF genotypes compared to other genotypes**.** There was a non-significant difference between the different genotypes regarding DAS-28 and disease duration. However, the difference between them was significant regarding vitamin D level which was found to be significantly lower among FF genotype compared to other genotypes **(Table 5).**

There was a highly significant strong negative correlation between DAS-28 and vitamin D levels. However, there was a significant positive correlation between DAS-28 and the rheumatoid factor level **(Table 6)**.

Table (1): Comparison of demographic data and vitamin D level among the studied groups

Variable	RA group (N=22)		Control group (N=22)		t-test	P-value
Age (years) Mean ± SD Range	55.04±9.24 35 - 65		50.3± 8.70 40 - 65		1.746	0.088 (NS)
	No	**%**	**No**	**%**	χ^2	**P**
Gender Female Male	18 4	81.8 18.2	15 7	68.2 31.8	1.091	0.296 (NS)

Table (2): Disease characteristics of the studied RA group:

Variable	Studied group (n=22) Mean ± SD
DAS-28	4.32 ± 1.58
Disease duration (years)	11.6 ± 5.93

Table (3): Comparison of genotypes among the studied groups

Variable	RA group (N=22)		Control group (N=22)		OR (95% CI)	P-value
	No	%	No	%		
Genotypes						
FF	12	54.5	4	18.2	**7.5 (1.02-54.9)**	**0.03**
Ff	8	36.4	13	59.1	**1.5 (1.2-9.8)**	**(S)**
ff	2	9.1	5	22.7		
Alleles						
F	32	72.7	21	47.7	**2.92 (1.2-7.10)**	**0.01**
f	12	27.3	23	52.3		**(S)**

Table (4): Relation between genotype polymorphism and demographic data among the RA group

Variable	FF (n=12)		Ff (n=8)		ff (n=2)		F test	P-value
Age (year) Mean ± SD	55.5±8.66		54.7± 8.28		55.1 ± 11.2		0.020	0.980 (NS)
	No	%	No	%	No	%	χ^2	P
Gender Female	12	100	4	50	2	100	**8.55**	**0.01**
Male	0	0	4	50	0	0		**(S)**

Table (5): Relation between genotype polymorphism, laboratory data, vitamin D level, DAS-28, and disease duration among the RA group

Variable	FF (n=12)	Ff (n=8)	ff (n=2)	KW Test	P-value
RF level Median	135.5	163	57.5	0.911	0.634 (NS)
Anti-CCP level Median	170	110	52.5	**3.844**	**0.04 (S)**
CRP level Median	17.5	6	44	3.806	0.149 (NS)
Vitamin D level Median	6	16	9	**8.647**	**0.002 (S)**
DAS-28 Median	3.7	4.3	5.3	3.034	0.219 (NS)
Duration (years) Median	12	10	7.5	1.764	0.414 (NS)

Table (6): The correlation between DAS-28 and different laboratory parameters among the RA studied group

Variable	DAS-28	
	r	P-value
Age(years)	0.164	0.465
RF level	**0.499**	**<0.01****
Anti-CCP level	0.390	0.07
CRP level	0.284	0.201
Vitamin D level	**-0.743**	**<0.001****
Disease duration(years)	0.204	0.364

DISCUSSION

Regarding the serum vitamin D level, there was a significant difference between the studied groups regarding vitamin D levels which were found to be significantly lower among RA patients compared to healthy controls. This agrees with **Rossini et al.,** [16] in the Italian population, **Kerr et al.,** [17] in the US population **& Furuya et al.,** [18] in the Japanese population, who reported that low levels of vitamin D are highly prevalent among RA patients.

In contrary to **Mukhtar et al.,** [19] who reported that vitamin D level was found sufficient among RA, OA, and controls and there was a non-significant difference among the studied groups of Pakistan population.

As regards Disease characteristics of the studied RA group, the DAS-28 ranged between 2.4-7.9 with a mean of 4.32 and the mean disease duration was 11.6 years. The results of this study are supported by findings reported by **Cavalcanti et al.,** [20] as they reported that The DAS28 mean was 3.71 in the South Brazilian RA population, and 5.59 and 3.67 in Southeast and Northeastern Brazil, respectively.

Regarding **genotypes among the studied groups,** there was a significant difference between the studied groups regarding VDR FOK1 gene polymorphism and its alleles. It was noticed that the FF genotype was found to be significantly higher among the RA group compared to the control group (54.5% versus 18.2%). Also, regarding alleles frequency, the F allele was found to be higher among cases than controls (72.7% versus 47.7%). Having FF and Ff genotypes have almost a higher risk 7.5 times, 1.5 times than having ff genotype to be RA patient. Having an F allele has almost a higher risk three times (**2.92**) than having an f allele to be an RA patient. The results of this study are supported by findings reported by **Hitchon et al.,** [21] as they reported that The Vit D receptor Fok1 gene polymorphism was shown to be associated with RA in the North American natives (NAN) population. Compared to the NAN control population, NAN patients with RA were more likely to be homozygous or heterozygous for the F allele. The previous study in the French populations conducted by **Maalej et al.,** [22] have found that the F allele and the genotype FF of the Vit D receptor Fok1 gene appeared to be associated with susceptibility to RA and shown that the F/F genotype was more frequent in RA patients compared to controls (45 RA cases vs 30 controls, P=0.01) among their studied groups.

In addition, a study carried out in Tunisia by **Karray et al.,** [23] reported a significant association of VDR gene polymorphism FokI with susceptibility to rheumatoid arthritis in Tunisian populations.

In contrary to the results of this study, there were no associations between RA and FokI gene polymorphisms by **Ates** [24] in the Turkish populations as well as by **Goertz et al.** [25] in the German population.

As regard **Relationship between genotype polymorphism and demographic data among the RA patients,** there was a non-significant difference between the different genotypes as regarding age, however, the difference between them was significant regarding sex as it was noticed that FF and ff homozygous genotypes were found only in females compared to their male counterparts while Ff heterozygous genotype was found equally among both males and females. **Based on assessing the relation between genotype polymorphism and laboratory data among the RA group,** there were non-significant differences between different genotypes regarding RF and CRP. However, the difference between them was significant regarding Anti-CCP which was found to be significantly higher among FF genotypes compared to other genotypes.

Tobón et al. [26] reported that genetic variations on the VDR gene, at genomic as well as at transcriptional levels, could lead to receptor dysfunction, which in turn could affect immune responses as well as mineral and vitamin D metabolism and bone integrity.

While assessing the relation between genotype polymorphism and vitamin D, DAS-28, and disease duration among the RA group, there was a non-significant difference between the different genotypes regarding DAS-28 and disease duration. However, the difference between them was significant regarding vitamin D which was found to be significantly lower among FF genotype compared to other genotypes. This agrees with **El-Barbary et al.,** [27] as they have shown that the FF homozygote of FokI genotypes was significantly associated with vitamin D deficiency and that they are also associated with high anti-CCP levels. In contrast to **Bhanushali et al.** [28], which showed no correlation was observed concerning FokI gene polymorphism and Vitamin D levels in the Indian population.

Regarding the correlation between DAS-28 and different laboratory parameters among the RA-studied group, there was a highly significant strong negative correlation between DAS-28 and vitamin D levels. However, there was a significant positive correlation between DAS-28 and the rheumatoid factor.

The results of this study are in agreement with findings reported by **Cutolo et al.** [29] that serum Vit D level was negatively associated with DAS28 in RA patients from North and South Europe.

The results of this study are in contrast with a study conducted by **Hanvivadhanakul & Singhea** [30] on the Thai population reported that they did not find an inverse relationship between concentrations of vitamin D level and RA disease activity.

CONCLUSION

Vitamin D receptor gene FokI polymorphism could be considered as genetic risk markers of RA

susceptibility, associated with vitamin D level deficiency which was found to be significantly lower among FF genotype compared to other genotypes. Having FF and Ff genotypes have almost a higher risk than having ff genotype to be RA patient.

REFERENCES

1. **Spinelli F, Pecani A, Ciciarello F** *et al.* **(2017):** Association between antibodies to carbamylated proteins and subclinical atherosclerosis in rheumatoid arthritis patients. BMC Musculoskeletal Disorders BMC Series, 18: 214.
2. **Uhlig T, Moe R, Kvien T (2014):** The burden of disease in rheumatoid arthritis. Pharmacoeconomics, 32: 841-851.
3. **Kuller L, Mackey R, Walitt B** *et al.* **(2014):** Rheumatoid arthritis in the Women's Health Initiative: methods and baseline evaluation. Am J Epidemiol., 179: 917-926.
4. **Boyer J, Gourraud P, Cantagrel A** *et al.* **(2011):** Traditional cardiovascular risk factors in rheumatoid arthritis: a meta-analysis. Joint Bone Spine, 78: 179-183.
5. **Perricone C, Ceccarelli F, Valesini G (2011):** An overview on the genetic of rheumatoid arthritis: a never-ending story. Autoimmun Rev., 10: 599-608.
6. **Song G, Bae S, Lee Y (2012):** Association between vitamin D intake and the risk of rheumatoid arthritis: a meta-analysis. Clin Rheumatol., 31: 1733-1739.
7. **Cutolo M, Otsa K, Uprus M** *et al.* **(2007):** Vitamin D in rheumatoid arthritis. Autoimmun Rev., 7: 59-64.
8. **Haussler M, Whitfield G, Kaneko I** *et al.* **(2013):** Molecular mechanisms of vitamin D action. Calcif Tissue Int., 92: 77-98.
9. **Evans S, Houghton A, Schumaker L** *et al.* **(1996):** Vitamin D receptor and growth inhibition by 1,25-dihydroxyvitamin D3 in human malignant melanoma cell lines. J Surg Res., 61: 127-133.
10. **Gandini S, Gnagnarella P, Serrano D** *et al.* **(2014):** Vitamin D receptor polymorphisms and cancer. Adv Exp Med Biol., 810: 69-105.
11. **Vanessa O, Asani F, Jeffery T** *et al.* **(2013):** Vitamin D receptor gene expression and function in a South African population: ethnicity, Vitamin D and FokI. PLoS One, 8: 663-669.
12. **Ghelani A, Samanta A, Jones A** *et al.* **(2011):** Association analysis of TNFR2, VDR, A2M, GSTT1, GSTM1, and ACE genes with rheumatoid arthritis in South Asians and Caucasians of East Midlands in the United Kingdom. Rheumatol Int., 31: 1355-1361.
13. **Aletaha D, Neogi T, Silman A** *et al.* **(2010):** 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/ European League Against Rheumatism collaborative initiative (PDF). Ann. Rheum. Dis., 69 (9): 1580–8.
14. **Fransen J, Creemers M, Van-Riel P (2004):** Remission in rheumatoid arthritis: agreement of the disease activity score (DAS28) with the ARA preliminary remission criteria. Rheumatology (Oxford), 43(10):1252-5.
15. **Prevoo M, van't Hof M, Kuper H** *et al.* **(1995):** Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum, 38 (1): 44–8.
16. **Rossini M, Maddali B, La Montagna G** *et al.* **(2010):** Vitamin D deficiency in rheumatoid arthritis: prevalence, determinants, and associations with disease activity and disability. Arthritis Res Ther., 12: 216-222.
17. **Kerr G, Sabahi I, Richards J** *et al.* **(2011):** Prevalence of vitamin D insufficiency/deficiency in rheumatoid arthritis and associations with disease severity and activity. J Rheumatol., 38:53–59.
18. **Furuya T, Hosoi T, Tanaka E** *et al.* **(2013)**: Prevalence of and factors associated with vitamin D deficiency in 4793 Japanese patients with rheumatoid arthritis. Clin Rheumatol., 32:1081–1087.
19. **Mukhtar M, Sheikh N, Suqaina S** *et al.* **(2019):** Vitamin D Receptor Gene Polymorphism: An Important Predictor of Arthritis Development. BioMed Research International, 19: 216-223.
20. **Cavalcanti C, de Azevêdo Silva J, de Barros P** *et al.* **(2016):** Vitamin D receptor polymorphisms and expression profile in rheumatoid arthritis Brazilian patients. Molecular Biology Reports, 43(1): 41-51.
21. **Hitchon C, Sun Y, Robinson D** *et al.* **(2012):** Vitamin D receptor polymorphism rs2228570 (Fok1) is associated with rheumatoid arthritis in North American natives. The Journal of Rheumatology, 39(9): 1792-1797.
22. **Maalej A, Petit-Teixeira E, Michou L** *et al.* **(2005):** Association study of VDR gene with rheumatoid arthritis in the French population. Genes and Immunity, 6(8): 707-7011.
23. **Karray I, Abdelghani K** *et al.* **(2012):** Associations of vitamin D receptor gene polymorphisms FokI and BsmI with susceptibility to rheumatoid arthritis and Behçet's disease in Tunisians. Joint Bone Spine, 79(2): 144–148.
24. **Ateş Ö (2011).** Vitamin D Receptor Gene Polymorphisms in Rheumatoid Arthritis. Turkish Journal of Rheumatology, 26(2): 145–149.
25. **Goertz B, Fassbender W, Williams J** *et al.* **(2003):** Vitamin D receptor genotypes are not associated with rheumatoid arthritis or biochemical parameters of bone turnover in German RA patients. Clinical and Experimental Rheumatology, 21(3): 333–339.
26. **Tobón G, Youinou P, Saraux A (2010):** The environment, geo-epidemiology, and autoimmune disease: rheumatoid arthritis. Autoimmun Rev., 9:288–292.
27. **El-Barbary A, Hussein M, Rageh E** *et al.* **(2015):** Vitamin D receptor gene polymorphism in rheumatoid arthritis and its association with atherosclerosis. Egyptian Rheumatology and Rehabilitation, 42(3): 145-149.
28. **Bhanushali A, Lajpal N, Kulkarni S** *et al.* **(2009):** Frequency of fokI and taqI polymorphism of vitamin D receptor gene in Indian population and its association with 25-hydroxyvitamin D levels. Indian Journal of Human Genetics, 15(3): 108-113.
29. **Cutolo M, Otsa K, Laas K** *et al.* **(2006):** vitamin d serum levels and disease activity in rheumatoid arthritis: Northern versus Southern Europe. Clin Exp Rheumatol., 24: 702-4704.
30. **Hanvivadhanakul P, Singhea J (2017):** The Relationship of Serum Vitamin D Level and Disease Activity in Rheumatoid Arthritis Patients. Journal of the Medical Association of Thailand, 100: 107-113.