A STUDY ON THE CLINICAL PROFILE AND PHARMACOGENETICS OF METHOTREXATE TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS.

A dissertation submitted to

THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY
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Submitted by

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APRIL–2014

CERTIFICATE

This is to certify that the dissertation entitled "**A STUDY ON THE CLINICAL PROFILE AND PHARMACOGENETICS OF METHOTREXATE TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS".** Submitted by **Registration No. 261226057,** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacology** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai, is a bonafide record of work carried out by him in the Institute of Pharmacology, Madras Medical College, Chennai during the academic year 2013-2014.

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INTRODUCTION

In a large patient population, a medication that is proven efficacious in many patients often fails to work in some other patients. Furthermore, when it does work, it may cause serious side effects, even death, in a small number of patients. Although large individual variability in drug efficacy and safety has been known to exist since the beginning of human medicine, understanding the origin of individual variation in drug response has proven difficult. On the other hand, the demand to overcome such variation has received more attention now than ever before. It is well documented that large variability of drug efficacy and adverse drug reactions in patients is a major determinant of the clinical use, regulation, and withdrawal-from-market of clinical drugs and a bottleneck in the development of new therapeutic agents.

Genetic variation in humans was recognized as an important determinant of individual variability of drug response from clinical observations in late 1950s.[1] The observation that individual variation of a drug response is often larger among members in a population (population variability) than within the same person at different times (intrapatient variability) further supports inheritance as a major determinant of drug response.[2] These clinical and population-based findings fostered the formation of pharmacogenetics to specifically address genetic contribution to individual variability in drug therapy.

The human genome sequence provides a special record of human evolution that varies among populations and individuals. Sequence variations in drug target proteins, drug-metabolizing enzymes, and drug transporters can alter drug efficacy, drug side effects, or both to cause variable drug responses in individual. [3] From this prospect, the availability of the complete human genome sequence has made it possible to

analyze the impact of variations of the human genome sequence on the pathogenesis of important diseases and the response to drug therapy at an accelerating rate in recent years.

Rheumatoid arthritis has a prevalence of nearly 1% in Indian population [46]. This autoimmune disease is characterised by chronic inflammatory process within the synovial joints, progressive (radiological) joint damage and significant functional impairment. [45] In the last decade patients have been treated with traditional disease modifying anti-rheumatic drugs (DMARDs) including methotrexate, sulphasalazine and leflunomide, or a combination of DMARDs. Most recently growing evidence for the central role of tumour-necrosis factor alpha (TNFα) in the pathogenesis of RA led to the introduction of TNFα inhibitors, such as etanercept, infliximab and adalimumab. These biological DMARDs has proven to play an important role in the treatment of persistant RA in patients, who achieve an incomplete response or develop adverse drug events to traditional DMARDs. In addition, biological with alternate mechanisms of actions such as rituximab, abatacept and tocilizumab have recently been developed. To date, the place of these agents in RA therapy is less established.

Ideally, RA therapy is based on strict monitoring of disease activity and tight control treatment in order to prevent progression of joint damage and functional disability. Namely, it is established that high and variable disease activity is related to increasing joint damage and that effective intervention stops the progression. In current clinical practice, newly diagnosed RA patients are treated with traditional DMARDs, in which MTX is the drug of first choice. In case of unfavourable response, side effects and/or drug toxicity, alteration of dose regimen or drug therapy towards a combination of DMARDs and/or biological is recommended.

Still, different response rates are seen in RA patients treated with MTX. Substantial percentages of 30-40% of RA patients fail to achieve a satisfactory response. Moreover, 15-30% of patients develop adverse drug events. These different responses lead to studies identifying influence of demographic, clinical and immunological variables on treatment outcome with MTX. Next to these factors, genetic influences have also been explored in the last decade. Generally, pharmacogenetics has the potential to increase the drug efficacy and to ameliorate adverse events. Therefore the application might be of great clinical benefit for individuals affected with RA. Studies have reported associations between SNPs in genes encoding enzymes related to the pharmacokinetics and pharmacodynamics of MTX and treatment outcome. The ultimate aim of using pharmacogenetic markers is to predict the probability of a wanted or unwanted drug response in individual patients.

MTX is a structural analogue of folic acid which inhibits dihydofolate reductase, an enzyme responsible for tetrahydrofolate regeneration. MTX may influence several other steps in folate metabolism and cause cellular folate depletion and possibly inhibition of methylenetetrahydrofolate reductase (MTHFR). MTHFR synthesises 5-methyltetrahydrofolate, which acts as the methyl donor for remethylation of homocysteine to methionine.

Several polymorphisms of the MTHFR gene have been described. The most studied C677T polymorphism results in the decreased enzyme activity and hyperhomocysteinaemia in the general population. The recently described A1298C polymorphism is associated with MTHFR activity and may affect plasma homocysteine level.

Our aim in the present study were, first to investigate the distribution of MTHFR A1298C gene polymorphism in MTX treated RA patients compared with a healthy control group; second, to determine the relation between A1298C polymorphism and rheumatoid arthritis activity, methotrexate efficacy, and adverse effects.

AIM AND OBJECTIVES

The aim of the study is to investigate the single nucleotide polymorphism within Methotrexate pathway gene (MTHFR A1298C) related to efficacy and toxicity in Indian Rheumatoid arthritis patients

Primary Objective:

Genotyping assay to study the distribution of different alleles within the study population

Secondary Objectives:

- To assess the efficacy of MTX therapy by DAS and HAQ.
- To evaluate the toxicity of MTX.
- To study the distribution of responders and non-responders to MTX therapy.

REVIEW OF LITERATURE

Rheumatoid arthritis is a common highly inflammatory, destructive poly arthropathy. It affects approximately 0.75% to 1% of the population. 80% of affected patients are disabled after 20 years and life expectancy is reduced by an average of 3 to 18 years. Women are three times more likely than men to develop rheumatoid arthritis. Women typically experience a more severe and delimiting form of disease. Most patients diagnosed are between the ages of 35 and 60.

The process involves an inflammatory response of the capsule around the joints (synovium) secondary to swelling (turgescence) of synovial cells, excess synovial fluid, and the development of fibrous tissue (pannus) in the synovium. The pathology of the disease process often leads to the destruction of articular cartilage and ankylosis (fusion) of the joints. RA can also produce diffuse inflammation in the lungs, the membrane around the heart (pericardium), the membranes of the lung (pleura), and white of the eye (sclera), and also nodular lesions, most common in subcutaneous tissue. Although the cause of RA is unknown, autoimmunity plays a big part, and RA is a systemic autoimmune disease. It is a clinical diagnosis made on the basis of symptoms, physical exam, radiographs (X-rays) and labs.[1]

Treatments are pharmacological and non-pharmacological. Non-pharmacological treatment includes physical therapy, occupational therapy and nutritional therapy but these do not stop the progression of joint destruction. Analgesics and anti-inflammatory drugs, including steroids, suppress symptoms, but do not stop the progression of joint destruction either. Disease-modifying anti-rheumatic drugs (DMARDs) slow or halt the progress of the disease.

Methotrexate is the cornerstone for the therapy of rheumatoid arthritis in spite of the advent of newer biologics. MTX is fast acting and has best efficacy: toxicity ratio and also cheaper. For the treatment of RA it was first introduced in 1951, but after 30years only widespread use in RA came into force in 1985. It is used at the dose of 5-25mg/week in treatment of RA and the dose for its anticancer effect is 5000mg/week. As a gold standard it is started as monotherapy, low dose is safe and well tolerated. For patients unresponsive to NSAIDs it is still the first-line drug for therapy in RA [1].

MTX is taken up by the cells glutamated by Foly-poly glutamyl synthase (FPGS) and there is a competition by Gamma glutamyl hydrolase (GGH), which deconjugates the drug and the free drug is effluxed by ATP-binding cassette (ABC) proteins. Polyglutamation upto 7 subunits takes place and methotrexate-polyglutamates (MTX-PG_{5-7}) roughly correlates with the therapeutic efficacy of the drug. Free MTX is eliminated within 24hrs and a small portion of it is metabolized in liver to 7-hydroxymethotrexate.

At cellular level MTX and MTX-PGs inhibit several enzymes of purine, pyrimidine biosynthesis and also exert anti-inflammatory effect. The key enzymes inhibited are dihydrofolate reductase (DHFR), which causes reduction of dihydrofolate to tetrahydrofolate, essential in synthesis of precursors of DNA; thymidylate synthase (TYMS), a key enzyme involved in pyrimidine synthesis, essential for cellular proliferation and AICAR transformylase is most potently inhibited, which leads to accumulation of 5-aminoimidazole- 4-carboxamide ribonucleotide (AICAR), inhibition of several key enzymes involved such as adenosine monophosphate deaminase (AMPD1) and collectively this leads to accumulation of adenosine, which has anti-inflammatory effect. The other enzymes inhibited by the drug are homocysteine pathway enzymes such as methylenetetrahydrofolate reductase (MTHFR), methionine

synthase reductase (MTRR) and methionine synthase (MS), which could lead to accumulation of homocysteine and related adverse effects [2].

Because of variation in response and toxicity profile 1/3rd of the patients discontinue therapy due to its adverse effects. Folate antagonism leads to anemia, stomatitis, oral ulcers and elevation of transaminases in liver, which could be alleviated by administration of folic or folinic acid. Accumulation of adenosine also leads to GI AEs. The uncommon toxicities are nodulosis, hepatic fibrosis, pulmonary fibrosis, and renal insufficiency [3].

Because of these factors, it is essential to predict the efficacy and adverse effects before administration, to effectively use the drug in treatment of RA. Since the drug is excreted within 24hrs and measurement of MTX-PGs routinely in clinical practice is not feasible, pharmacogenetics could be a useful tool to monitor the treatment outcomes.

PHARMACOGENETICS OF METHOTREXATE

GENES RELATED TO INFLUX AND EFFLUX

ABCB1 C3435T

This is one of the important genes related to P-gp expression, which are best drug transporters in humans. There are several genes and several polymorphisms related to multiple drug resistance and ABCB1 C3435T is widely studied in RA patients and this gene is otherwise called as MDR1 gene.

The distribution of this polymorphism in RA was similar to healthy individuals in a Polish study by Pawlik *et al* [4], but different from Afro-Americans, Chinese, and Japanese healthy individuals [5]. The same group studied this polymorphism in 255

Polish RA patients and reported that carriers of TT genotype were found to be responders [6].

Conversely Takatori *et al* reported that the efflux of MTX was increased in TT genotype and due to this they are non-responders and insisted for increasing the dose during early stages of RA or substitution with biologics (17181924).

Kato *et al* studied the effect of C3435T polymorphism in Japanese RA patients and found that the remission defined by lower DAS score is higher in TT genotype [7].

In a study by Sharma *et al*, CT genotypes were found to be non-responders [8].

RFC-1 G80A

Reduced folate carrier (RFC) is a anion exchanger, transmembrane protein comprising 591 aminoacids and transfers folates across the cell membrane. This polymorphism is otherwise called as solute carrier, SLC19A1 G80A.

Reduced folate carrier influences the entry of methotrexate into the cells and the carriers of AA alleles had increased MTX levels than GG or AG alleles, by increased up take in B and CD4+ cells. This study suggests that this polymorphism is relevant for deciding the dosage of MTX in autoimmune disorders [9].

Drozdzik *et al* reported that the remission in RA patients is increased, when they have the 80AA genotype and the frequency of A allele was higher in the responders group, in a study of 174 RA patients. They also reported increase in transaminase levels in this genotype [10].

Hayashi *et al* reported that the AA allele genotypes had increased intracellular MTX up take, increased efficacy and the need for combination with biologics is less, and this polymorphism influences MTX efficacy in Japanese RA patients [11].

Dervieux *et al* studied the effects of this polymorphism in 226 RA patients and reported that carriers of AA genotype have higher MTX-PG levels and thus influences polyglutamation [12].

GENES RELATED TO POLYGLUTAMATION

GGH

Gamma glutamyl hydrolase is a lysosomal peptidase that catalyzes elimination of gamma linked polyglutamates. Long chain MTX-PGs are converted to short chain MTX-PGs and further converted back to MTX and effluxed from the cell. Since the MTX-PGs are associated with disease activity in rheumatoid arthritis, GGH polymorphisms could influence the therapeutic outcome. Three polymorphisms had been reported in previous studies such as GGH C401T, GGH C452T, & GGH T16C.

Dervieux *et al* C401T in 226 RA patients and found that the carriers of TT genotype had lesser MTX-PG levels when compared to the wild-type and the heterozygous mutants, and thus this polymorphism influences polyglutamation [12].

Chave *et al* studied expression of the polymorphisms in this gene which affects its functional activity in MCF-7 cells and reported that C401T & T124G polymorphisms enhanced hGGH protein expression, which could increase resistance to MTX [13].

Hayashi *et al* reported that in Japanese healthy population, the frequency of C452T polymorphism in 269 healthy Japanese individuals and found that the

distribution of this polymorphism is similar to Afro-Americans and slightly different with Caucasians [14].

In juvenile idiopathic arthritis, Yanagimachi *et al* studied the effect of T16C polymorphism in 92 Japanese patients and reported that TT genotype was associated with higher incidence of liver dysfunction [15].

FPGS

This gene is related to polyglutamation of MTX and is important in one-carbon metabolism.

In a study by Oppeneer *et al* they found this gene is not associated with homocysteine metabolism [16].

Sharma *et al* reported that a polymorphism in this gene (rs1544105) is associated with poor response to MTX therapy in RA [17].

In UK rheumatoid cohort study by Owen *et al*, they found that FPGS polymorphism was associated with adverse effects [18].

GENES RELATED TO HOMOCYSTEINE PATHWAY

MTHFR

This gene encodes an enzyme that catalyzes reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a carbon donor in the metabolism of folate to methionine and the polymorphism leads to reduction in enzyme activity and associated with hyperhomocystinemia.

There were about dozen polymorphisms in this gene and in meta-analysis study of pharmacogenetics of methotrexate by Fischer *et al*, it was identified that MTHFR C677T & A1298C were the widely studied gene polymorphisms [19].

This polymorphism C677T was first reported by Frosst *et al* in 1995 [20] and the second polymorphism A1298C was reported by Weisberg *et al* in 1998 [21]. In C677T heterozygous mutants have ~40% reduction in enzyme activity and homozygous mutants have ~70% reduction and this leads to thermolabile variant of the enzyme. In A1298C polymorphism the homozygous mutants have about ~40% reduction in enzyme activity

The first article related to this polymorphism C677T was published by van Ede *et al* which assessed discontinuation of MTX due to elevation of transaminases. In this study it was concluded that the elevation of liver enzymes is due to homocysteine metabolism and supplementation with folic acid or folinic acid reduced the toxicity-related discontinuation rates [22].

Urano *et al* in 2002 assessed both C677T & A1298C polymorphisms in this gene and found A1298C polymorphism rendered the patients sensitive to MTX treatment, whereas C677T rendered the patients prone for toxicity [23]. In haplotype analysis 677C-1298C were receiving lower dose of MTX and 677T-1298A had a higher frequency of side-effects from MTX. The reported toxicities were elevation of transaminases, gastrointestinal (GI) disturbances, hair loss, fatigue and rash. The same was confirmed in their second study published in the year 2007[24]. In their next study in 2009, the same group also reported that these two polymorphisms are not associated with fracture [25].

Haagsma *et al* reported a persistent increase in homocysteine levels in TT alleles of MTHFR C677T and is associated with GI adverse events (AEs) [26] and elevation of liver enzymes [22]. Concomitant administration of folic acid prevents these adverse effects [27].

Hider *et al* demonstrated that T allele is associated with elevation of liver enzymes in 309 RA patients [28].

Berkun *et al* reported that the allele frequency of 1298CC was higher in RA population and the carriers of 1298AA allele had higher frequency of adverse effects in spite of higher folic acid supplementation and 1298CC may protect against MTX related adverse effects, conducted in 93 RA patients in Israel [29].

Hughes *et al* studied the allelic frequencies of MTHFR C677T, A1298C & rs4846051 in Caucasians and Afro-Americans and highlighted racial or ethnic differences in it. 1298 A allele was associated with MTX-related adverse events in Caucasians, whereas the rs4846051 C allele appears to be related to MTX toxicity in African-Americans, so different alleles in different race may be the markers for response and toxicity [30].

Wessels *et al* reported MTHFR 1298AA was associated with less improvement relative to mutants and MTHFR 1298C allele carriers developed more adverse effects [31].

Aggarwal *et al* studied the effect of C677T polymorphism in north Indians, 150 patients were recruited in the study. All the patients received folic acid supplementation and they concluded that this polymorphism is not predictive of efficacy or toxicity [32].

Kim *et al* studied C677T polymorphism in 385 Korean RA patients, and found the frequency of TT genotypes were higher, the cost of therapy decreased with the probability of continuation with MTX also increased [33].

Kato *et al* studied the effect of MTHFR A1298C and reported that AA genotypes were associated with good response [7].

Brambilla-Tapia *et al* studied this MTHFR C677T in 71 RA patients and carriers of T alleles (TT homozygotes), had lower BMD and reported to have increased risk of osteoporosis and folic acid supplementation is suggested as a prophylactic measure [34].

Graber *et al* reported that MTHFR A1298C polymorphism is protective related to adverse effects of MTX [35].

In a recent study by Davis *et al*, they found that A1298C polymorphism was associated with adverse effects and increased copies of this leads to higher incidence of AEs [36].

GENES RELATED TO PYRIMIDINE PATHWAY

TYMS or TSER *2/*3

Dervieux *et al* assessed this polymorphism and concluded that patients having two tandem repeats had better clinical response than triple repeat [2].

Kumagai *et al* assessed the impact of this polymorphism in Japanese RA patients, and found that triple-repeat allele of the polymorphism (*3/*3) received higher dose of MTX than double repeat allele [37].

TYMS or TSER 3'UTR 6bp deletion TTAAAG

Kumagai *et al* studied this polymorphism in Japanese RA patients and has shown that this 6bp deletion leads to decrease in CRP levels and improvement in response [37].

This deletion polymorphism is associated with decreased expression of mRNA and could increase the drug response in RA patients [38].

TYMS (rs2853539)

Sharma *et al* studied the effect of this polymorphism in RA patients and found that carriers of AA genotype are non-responders [17].

GENES RELATED TO ADENOSINE PATHWAY

Blockade of AICAR affects purine synthesis and leads to accumulation of adenosine and the anti-inflammatory effects of MTX are mediated through this pathway. The polymorphisms in genes influencing anti-inflammatory adenosine release are 5-aminoimidazole- 4-carboxamide ribonucleotide transformylase (ATIC) C347G, inosine triphosphate pyrophosphatase (ITPA) C94A, AMPD1 C34T & adenosine receptors (ADORA) 2a. Methotrexate inhibits the deamination of adenosine and modulates its pharmacokinetics and pharmacodynamics. Adenosine exhibits its anti-inflammatory effect through modulation of inflammatory cells.

Adenosine binds to several receptors such as A1, A2a, A2b, A. ADORA 2a is highly expressed in synovium of RA patients receiving MTX and SNPs in this gene is reported to influence adverse effect profile of MTX [39].

Hider *et al* studied five SNPs in ADORA 2a in 309 RA patients (rs 5760410, rs 2298383, rs 3761422, rs 2267076 & rs 2236624) and found an association with GI AEs. The possible explanations were anti-proliferative effects in the gut and sensitization of chemoreceptors in brain due to this polymorphism and could be alleviated by administration of folic acid and 5HT3 antagonists [40].

Wessels *et al* reported favorable alleles for response as T allele of AMPD1, CC allele of ATIC C347G & CC allele of ITPA C94A. Regarding toxicity, G allele of ATIC C347G was associated with GI AEs [41].

ATIC rs4673993 was assessed and reported as associated with low disease activity in a study [42].

GENES RELATED TO FOLATE METABOLISM

DHFR

This is a key enzyme inhibited by MTX and polymorphisms in it are less studied. It has reported polymorphisms such as rs12517451, rs10072026, and rs1643657, associated with adverse effects [18].

DHFR A317G was studied by Milic *et al* and found that 317AA genotypes were associated with poor response [43].

POLYGENETIC ANALYSIS

Dervieux *et al* studied the combined effect of RFC G80A, ATIC C347G and TSER 2*/3* polymorphisms in 108 RA patients in relation with MTX-PG and efficacy. Favorable alleles were reported to be homozygotes of these polymorphisms (RFC-1 AA, ATIC 347GG, TSER *2/*2) and a Pharmacogenetic index was calculated. Patients

having all the favorable alleles were reported to have increased RBC-MTX PG levels and improvement in disease activity (reduction in pain, tender joint count, swollen joint count & physician's global assessment) compared to non-carriers of all the genotypes. The outcome of the study reports Pharmacogenetic and metabolite measurements are useful in optimizing MTX treatment [2].

In a study with 48 RA patients, the effect of GGH C401T, ATIC C347T, MTHFR A1298C, MTRR A2756G & MS A66G combinations were studied by Dervieux *et al*. The risk alleles were identified as GGH 401CC, ATIC 347GG, MTHFR 1298 AC/CC, MTRR 2756AA & MS 66GG. These genotypes were found to be associated with CNS and gastrointestinal adverse effects [44].

Wessels *et al* studied the polygenetic effect of genes related to adenosine release and reported that the carriers of T allele of AMPD1, CC allele of ATIC C347G & CC allele of ITPA C94A are good responders [41]

Weisman *et al* studied the polygenic effects of MTHFR C677T, TYMS *2/*3, ATIC C34TG & serine hydroxyl methyl transferase (SHMT) C1420T and found the risk genotypes were TT alleles of MTHFR C677T (CNS AE), CC alleles of SHMT C1420T (CNS AE & alopecia),

GG alleles of ATIC C34TG (GI AE) and *2/*2 alleles of TYMS *2/*3 (alopecia) [45] (16447238).

The allelic frequencies were recorded from different studies and represented in Table 2. This table depicts racial and ethnic differences [46, 47].

Table 3.1 – Genes, SNPs & Alleles associated with efficacy and toxicity of Methotrexate in rheumatoid arthritis

Gene symbol	Nucleotide position Polymorphism	Amino acid substitution	Effect of polymorphism	Location	dbSNP	N	Genotype for responders	Genotype for toxicity	Odds ratio	Reference
Influx Genes										
RFC-1	G80A	histidine to arginine	Increased MTX entry into cell	Chr. 21	rs1051266	236	RFC-1 80AA		3.0 (1.3-8.4)	[12]
RFC-1	G80A		Increased MTX entry into cell			174	RFC-1 80AA		3.32 (1.26-8.79)	[10]
Efflux Genes										
ABCB1	C3435T	No amino acid substitution	Involved in MTX transport	Chr.7	rs1045642	225	3435TT		4.65 (1.66-13.05)	[6]
Genes related to polyglutamation										
GGH	C401T		Involved in deconjugation of MTXPGs	Chr.8	rs1154507 8	226	GGH 401TT (non-responder)		4.8 (1.8-13.0)	[12]
GGH	T16C		Reduced activity of GGH		rs1800909	92		GGH 16CC	6.90 (1.38-34.5)	[15]
Homocysteine pathway Genes										
MTHFR	C677T	Alanine to valine	Decreased enzyme activity	Chr.1	rs1801133	236		CT/TT	2.38 (1.06-5.34)	[22]
MTHFR	C677T		Decreased enzyme activity			309		677TT	3.3 (1.05-10.3)	[28]
MTHFR	C677T		Decreased enzyme activity			106		T allele	1.25 (1.05-1.49)	[23]

MTHFR	A1298 C	Glutamine to alanine	Decreased enzyme activity	Chr.1	rs180 1131	93		AA	5.24 (1.38-20)	[29]
MTHFR	A1298 C		Decreased enzyme activity			223		A	15.86 (1.5-16.7)	[30]
MTHFR	A1298 C		Decreased enzyme activity			205		C	2.5 (1.32-4.72)	[31]
MTHFR	A1298 C		Decreased enzyme activity			319		C	0.027 (0.035-0.820)	[36]
Haplotype MTHFR C677T & A1298C			Decreased enzyme activity			186		677C/1298A	2.085 (1.058-4.106)	[33]
Polygenetic Analysis										
RFC1 G80A, ATIC C347G & TYMS *2/*3 analysis – The favorable genotypes for good response are RFC-1 AA , ATIC 347GG , TSER *2/*2						108			3.7 (1.7-9.1)	[2]
GGH C401T, ATIC C347G, MTHFR A1298C, MTRR A2756G & MS A66G analysis – The risk genotypes were found to be GGH 401CC, ATIC 347GG, MTHFR AC/CC, MTRR 2756AA & MS 66GG						48			13.9 (2.6-75.4)	[44]
AMPD1 C34T, ATIC C347G, ITPA C94A analysis – favorable alleles for response are AMPD1 34T, ATIC 347CC & ITPA 94CC						205			27.8 (3.2-250)	[41]
MTHFR C677T, TYMS *2/*3, ATIC C347G, & SHMT C1420T analysis – Risk genotypes were MTHFR 677TT, TSER*2/*2 , ATIC 347GG, & SHMT1 1420CC						214			6.8 (1.52-30.38)	[45]

Table 3.2 – Allelic frequencies of different SNPs in genes associated with Methotrexate Pharmacogenetics [46, 47]

	MTHFR C677T		MTHFR A1298C		TS 5UTR		TS 3UTR		RFC-1 G80A		MTRR A2756G		MS A66G		ATIC C347G		SHMT C1420T		GGH C452T		ABCB1 C3435T	
	C	**T**	**A**	**C**	**2R**	**3R**	**6bp**	**0bp**	**G**	**A**	**A**	**G**	**A**	**G**	**C**	**G**	**C**	**T**	**C**	**T**	**C**	**T**
Indian (H)	0.90	0.10	0.70	0.30	0.36	0.63	0.52	0.46	0.38	0.61	0.66	0.34	0.50	0.50	0.48	0.52	0.20	0.80				
Indian (H)	0.88	0.12					0.51	0.49	0.75	0.25	0.83	0.17					0.51	0.49				
Indian (RA)	0.58	0.42									0.92	0.08										
IndianAsian (H)					0.36	0.64																
European (H)	0.68	0.32	0.72	0.28			0.73	0.27														
African	0.96	0.04	0.87	0.13			0.44	0.56														
Japanese (RA)	0.33	0.67	0.75	0.25	0.85	0.15			0.54	0.46			0.52	0.48	0.60	0.40			0.92	0.07	0.39	0.61
Japanese (H)																			0.89	0.11		
Caucasian (H)					0.49	0.50					0.83	0.17					0.62	0.38				
France (H)					0.40	0.60											0.67	0.33				
Portugese (H)													0.85	0.15			0.73	0.26				
Chinese (H)													0.9	0.08								

													2								
Dutch (RA)	0.51	0.49			0.69	0.31											0.54	0.46	0.83	0.17		
Israel (RA)	0.48	0.52	0.54	0.46																		
Israel (H)	0.41	0.59	0.45	0.55																		
American (RA)			0.67	0.33	0.81	0.19			0.56	0.44					0.63	0.37						
Poland (RA)									0.31	0.69												

MATERIALS USED

List of chemicals used for the DNA isolation, PCR and RFLP

Chemicals	**Manufacturer**
10x PCR buffer	Invitrogen
50bp ladder DNA	Fermentas
50mm $MgCl_2$	Invitrogen
Agarose	Lonza
Ammonium acetate	Merc
Ammonium chloride	Srl
Boric acid	Srl
Chloroform	Srl
dNTPs	Biolabs
EDTA	Srl
Ethanol	Hayman specality products
Ethidium bromide	Genei
Hydrochloric acid	Srl
Isoamyl alcohol	Srl
Mbo II enzyme	Thermo scientific
Milli Q water	Sigma
MTHFR FP	Sigma
MTHFR RP	Sigma
Phenol	Srl
Potassium bicarbonate	Loba chemie

Premix loading dye	Thermo scientific
Proteinase k	Invitrogen
SDS	Srl
Sodium chloride	Srl
Sodium hydroxide	Srl
Taq DNA polymerase	Invitrogen
Tris	Srl

Lab-wares and Instruments

Lab-wares / Instruments	**Manufacturer**
Sterile K3EDTA Vacutainer	BD
96 Wellplate	Pipettman
Centrifuge Tubes	Torsons
Effendorf Tubes	Torsons
Micro pipets	Thermo Scientific
Seal mat	Pipettman
Single use Syringe 2ml	Dispovan
Centifuge	Remi equipments
Electrophoresis chamber	BroViga
GelDoc Imager	BioRad
Incubator	Remi equipments
Magnetic Stirrer	SPINIT
Microwave oven	LG

Mini centrifuge	cubee
NanoDrop	Remi equipments
PCR (Mod.no.2720)	Applied biosciences
pH meter	Remi equipments
Refrigirator	LG
Roto mixer	Remi equipments
Vortex micxer (Model No CM101)	Remi equipments
Water bath	Amersham
Weighing balance (CX 220)	Citizen

METHODOLOGY

STUDY DESIGN

Pharmacogenetic study

STUDY TYPE

Prospective, non- interventional study

STUDY CENTER

- Department of rheumatology, Rajiv Gandhi Government General Hospital, Chennai-03.
- Institute of pharmacology, Madras Medical College, Chennai-03.
- Department of Molecular Genetics, MDRF Siruseri, Chennai-103.

STUDY PERIOD

Feb 2013 to Feb 2014.

STUDY POPULATION

Rheumatoid arthritis patients on MTX therapy attending Rheumatology department, Rajiv Gandhi Government General Hospital, Chennai.

SAMPLE SIZE

100 patients with RA and 50 age matched healthy volunteers. (Faculty members, Staffs and Students from Madras Medical College, Chennai those who met the age group served as control group)

SUBJECT SELECTION

Inclusion Criteria:

- Age : ≥ 18 years
- Sex : Both genders
- Patients willing to give written informed consent
- Recently diagnosed Rheumatoid arthritis patients receiving MTX therapy and/or patients on MTX therapy for less than 2 years

Exclusion Criteria

- Patients with liver function abnormalities, GI disturbances, Cardiac and Renal diseases.

STUDY PROCEDURE

Ethical consideration

The protocol was prepared and submitted to the Institutional Ethics Committee, Madras Medical College, Chennai and approval was obtained. (IEC approval NO.18032013)

Selection of patients

Patients who were diagnosed with history of RA and admitted in the ward of Rheumatology department were explained about the study procedure and purpose and those who were willing to participate were enrolled.

Those who met inclusion criteria were recruited and informed consent was obtained prior to any study related procedure.

Clinical investigations

Detailed medical history, Clinical examination and baseline laboratory investigations were documented. The following reports were documented from their medical records during baseline visit and also during subsequent visits.

Disease characteristics

Morning stiffness

Pain scale

TJC, SJC, HAQ and DAS

Drug characteristics

Dose of MTX

Biochemical characteristics

SGOT, SGPT and ESR

Adverse effects

Adverse effects were noted and recorded.

Those who fulfilled the inclusion and exclusion criteria were enrolled and the demographic details and vitals were recorder. Then 2ml of blood was drawn for genetic analysis.

Blood sample collection

2ml of blood Sample was drawn by disposable syringe and transferred into BD vacutainer tubes containing EDTA. They were stored at 4˚C until transportation to Department of Molecular Genetics, MDRF, Siruseri, Chennai. The blood samples were stored at 4˚C until processing.

DETERMINATION OF GENOTYPE OF THE PATIENTS

The genotyping of blood samples involved the following steps

1. Extraction of DNA from whole blood.
2. Quality checking and quantification by using spectrophotometer.
3. Polymerase Chain Reaction (PCR) for MTHFR gene.
4. Identification of genotype with Restriction Fragment Length Polymorphism (RFLP) using Agarose gel electrophoresis.

DNA isolation and purification:

The phenol chloroform method of DNA isolation was used in this study. This frequently used method for DNA isolation removes proteins and other cellular components from nucleic acids, resulting in relatively pure DNA preparations.

Principle:

The concept of isolation of DNA is that, all the other components of the cell and chromatin are removed using suitable methods to leave behind the DNA. In general the isolation of DNA from mammalian tissues follows four different steps.

1. Lysis of cells with a detergent like sodium dodecyl sulphate (SDS).
2. Digestion of proteins with enzyme (Proteinase-K).

3. Extraction of DNA by phenol chloroform method.
4. Precipitation of DNA with isopropyl alcohol or 100% ethanol.

Reagents and their functions:

a) 10 x Lysis buffer

0.77M Ammonium chloride - 41.18 g

0.046M KHCO3 - 4.6 g

Make up to 1000 ml with distilled water. For the DNA extraction, pH

b) 2X Lysis Mix

200ml of 10X Lysis made up to 1000ml with Milli-Q water.

c) 500mM EDTA

EDTA -186.1g

Distilled water -1000ml

186.1g of EDTA with 800ml water (Use magnetic stirrer). Dissolve it using NaOH pellets and Con.HCl to bring the pH 7.5 and then volume adjusted to 1000ml with water.

d) SALT / EDTA Buffer

0.075M NaCl - 4.39g

0.025M EDTA - 50ml of 500mM EDTA

Distilled water - up to 1000ml

e) 10% SDS solution

10g of SDS is dissolved in 100ml distilled water

f) Proteinase K

20mg in 1ml distilled water.

g) Ammonium Acetate

96.35g of Ammonium Acetate dissolved in 250ml of deionised water.

h) 1M Tris

121.4gm of Tris is dissolved in 700ml Milli-Q water. Adjust the pH to 8 by using NaOH or Con HCl and then volume made up to 1000ml with water.

Roles of chemicals in DNA isolation

NH_4Cl

For the DNA extraction, pH should be in 7-8 range, so these compounds are used to maintain the pH of the solution. They act as a buffer in Lysis.

NaCl, $KHCO_3$

Salt would attract the phosphate ends of DNA; it pulls away the DNA from other substances present in the sample and protects the DNA from surroundings.

SDS

A detergent used to lyse the cells.

Proteinase K

This protease enzyme used to digest the cell surface proteins. When cell surface proteins are digested, the integrity of the cell membrane is compromised leading to cell Lysis.

EDTA

Chelates divalent cations required for DNAse activity, protecting the DNA from degradation.

Phenol and Chloroform

Extract proteins and lipids away from the DNA.

Cold alcohol and Ammonium acetate

Chilled absolute ethanol precipitates the DNA, the last step in a traditional DNA extraction. Ammonium acetate aids the DNA extraction.

Tris

Buffer used to maintain the pH. Additionally, it plays an important role in cell Lysis.

PROCEDURE OF GENOMIC DNA ISOLATION

1. The blood samples were carefully transferred to a new graduated centrifuge tube and 2x Lysis buffer was added to the sample. (three times of the volume of blood)

2. These tubes were mixed in rotator at 25 rpm for 10 minutes and kept in the refrigerator for 25 minutes.
3. The samples were centrifuged at 2000 rpm for 25 minutes and the supernatant was discarded, the pellet then treated with 1 ml salt EDTA, 0.1 ml SDS and 0.01ml proteinase k. then vortexed, mixed well and incubated at 37 C for 12 hours.
4. The incubated samples then added twice the volume of phenol and mixed by rotator for 10 minutes and spun at 2000 rpm for 10 minutes.
5. The supernatant again subjected to the previous step.
6. From the above mixture the supernatant were collected and transferred to a new tube and 2ml of chloroform: Isoamyl alcohol (24:1) was added and mixed by rotator and spun at 2000 rpm for 10 minutes.
7. The supernatant again subjected to the previous step.
8. The upper aqueous phase alone carefully collected with the help of wide bore tips without disturbing the other layers and transferred to a new tube.
9. To this aqueous phase, 0.75 ml of ammonium acetate and twice the volume of chilled absolute ethanol were added and tubes were inverted gently for several times.
10. The DNA will be visible like a thread and will assume the shape of a cotton ball.
11. The DNA was transferred to an eppendorf tube and was air-dried in a sterile place for 3 hours to remove any trace of residual ethanol.
12. Appropriate amount of 1X TBE was added according to the amount of the DNA, allowed to dissolve and stored at 4°C.

QUALITY CHECK AND QUANTIFICATION OF DNA

The integrity of the DNA was assessed by running it in 0.7% Agarose gel. Further the quantification and quality check of DNA was performed by subjecting the DNA to spectrophotometry.

Principle:

The concept of quality check of DNA is to find out the purity of the extracted DNA. The extracted DNA may contain impurities like phenol, proteins and others. The integrity of the DNA is checked by agarose gel electrophoresis. The intact high molecular weight DNA will appear as sharp band without smearing.

Reagents

a) **TAE buffer (10x)**

Tris base - 48.4g

Glacial acetic acid - 11.42ml

0.5M EDTA (pH 8.0) - 20ml

Distilled water was added and made up to 1000ml. autoclaved and stored at room temperature.

b) **Sodium Borate Buffer (20x)**

Sodium Hydroxide (200mM) - 8g

Boric Acid (760mM) - 47g

In 800ml of distilled water, the above components added and dissolved. pH adjusted to 8.2 using NaOH and made up to 1000ml. sterilized by autoclaving and stored at room temperature.

c) Gel loading dye- Type III (6x)

Bomophenol blue - 0.25% (w/v)

Xylene cyanol FF - 0.25% (w/v)

Glycerol in water - 30% (v/v)

These compounds were mixed well by stirring and stored as 1ml aliquots at -20˚C.

d) Ethidium bromide (10µg/µl)

10mg of Ethidium bromide was added to 1ml of sterile distilled water and mixed well to ensure that the dye has dissolved completely. The tube was wrapped in aluminium foil and stored at 4˚C.

PROCEDURE FOR AGAROSE GEL ELECTROPHORESIS:

1. 0.7% agarose gel (For Genomic DNA) and 3% agarose gel (for PCR and RFLP products) were made using 0.5x TAE buffer (For Genomic DNA) and 1x TAE buffer (for PCR/RFLP Products) 5µl of ethidium bromide (10µg/µl) for 100ml of agarose gel added and mixed well. After polymerization the gel was placed and immersed in the electrophoresis tank with respective buffer.
2. 1µl of each Genomic DNA sample was taken and mixed with 2µl of 6x loading dye and 8µl of sterile double distilled water prior loading.
3. The PCR products or RFLP products were mixed with 2µl of 6x loading dye prior loading.
4. The samples were loaded into the wells and resolved at 100V-135V for 20min-35min in Agarose Gel electrophoresis unit.

5. 5µl of 50bp DNA ladder loaded for reference while resolving PCR/RFLP Products.
6. After the complete run, gel was documented in UV-Gel Doc system and the image was stored as jpeg file.

PROCEDURE FOR UV-SPECTROPHOTOMETRY:

The nucleic acid sample was analysed at 260nm and 280nm by using (Nano Drop) Spectrophotometer. The concentration and purity of the sample was analysed using the following formula,

a) Concentration of DNA:

$$\text{Concentration of double stranded DNA sample (µg/µl)} = \frac{A_{260} \times 50}{1000}$$

b) Purity of DNA:

Pure DNA = $A_{260} / A_{280} \geq 1.8$

$A_{260} / A_{280} < 1.8$ indicates protein and phenol contamination.

$A_{260} / A_{280} > 2.0$ indicates the possible contamination with RNA.

2. POLYMERASE CHAIN REACTION:

The polymerase chain reaction (PCR) is used to amplify a desired region of the genome enzymatically without using a living organism. The concentration of the desired target sequence theoretically increases from one molecule to several million

copies. There are three steps to any polymerase chain reaction which are cycled about 25-35 times, which are:

a. **Denaturation:** This step occurs at 95˚C and entails the uncoiling of double stranded DNA into 2 single strands by breaking apart the hydrogen bonds.

b. **Annealing:** This occurs at 55-65˚C. A pair of short (17-20) oligonucleotide sequences called primers anneal to the ends of the template strands of DNA and begin the reaction. The temperature of this stage depends on the primers and is usually 5˚C below their melting temperature.

c. **Extension:** This occurs at 72˚C and entails the extension of the primers to form a new strand that is complimentary to the template strand. This occurs in the presence of the Taq DNA polymerase, a DNA polymerase isolated from the organism Thermus aquaticus, a bacterium that can survive high temperature without denaturation.

COMPONENT OF PCR:

The following components are used for the PCR mixture.

a. 10X PCR buffer
b. Magnesium chloride
c. dNTp: dATP + dTTP + dCTP + dGTP
d. Forward and Reverse primers
e. Taq DNA polymerase

PCR STANDARDIZATION

The protocol has to be standardized for the following parameters

a. **Annealing temperature:** Using the Ta obtained from the equation [Ta = Tm - 5˚C, where Tm = 2(A+T) + 4(G+C), the protocol is run at Ta±3˚ and the Ta with best results is chosen.

b. **Magnesium chloride concentration**: The dNTPs require MgCl2 to facilitate the cleavage of nucleotide from the tri-phosphate group. At the same time in the presence of excess MgCl2, the dNTPs intercalate and are no longer available for the PCR.

c. **Cycle time and cycle number:** The protocol must also be standardized for the number of cycles and the time for each step in the cycle. The usual cycle times tested are that of 30 and 45s respectively. The number of cycles varies from 25 – 35.

PROCEDURE

1. Prepare a master mix (cocktail) for the no. of samples required in a 1.5 ml Eppendorf tube as follows

Components	Volume per reaction
10X Buffer	2.5µl
$MgCl_2$	0.75 µl
dNTPs	0.5 µl
Taq polymerase	0.1 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Distilled water	18.15 µl
Total	**24 µl**

2. Add 1µl of DNA template to the tubes on the work bench.
3. Place tubes in Thermal Cycler and run the cycler for:

Initial denaturation	- 94˚C – 5 min	
Denaturation	-94˚C – 45 sec	30 cycles
Annealing	-55˚C – 54 sec	
Extension	-72˚C – 45 sec	
Final Extension	-72˚C – 10 min	
Incubate at	-4˚C	

Following PCR, positive amplification is checked by running 5µl of the amplified product mixed with bromophenol blue-xylene cyanol dye, on an ethidium bromide stained 3% agarose gel.

Primers used in this study

SNP	**PRIMERS**
MTHFR 1298 A>C	FP: 5'-CAAGGAGGAGCTGCTGAAGA RP: 5' –CCACTCCAGCATCACTCACT

Primer dilution

The primer was obtained as lyophilized powder and was reconstituted in appropriate volume of sterile distilled water to a concentration of 100µM. A working stock of 5pM /µl was prepared and all the stocks of primers stored at -20°C.

RESTRICTION FRAGMENT LENTH POLYMORPHISM (RFLP)

Principle:

In this method, DNA sequence variation is identified by amplification of the region using polymerase chain reaction followed by digestion of the amplified product with a restriction endonuclease known to be capable of distinguishing the polymorphic patterns. The restriction fragments vary in size and can be revealed as different bands on gel electrophoresis.

Restriction Digestion Composition

Components	**Volume per reaction**
Mbo II enzyme	0.4µl
10X buffer	1.5µl
Distilled water	3.1µl
Total	**5.0µl**

Procedure

- Pipette PCR product separately to each labelled tube and prepare master mix of the remaining items for the required number of samples.
- Dispense 5µl of master mix into each tube containing PCR product.
- Spin the tubes briefly to collect the contents at the bottom and incubate at 37˚C overnight in a water bath.

Restriction Digestionbased genotype:

After the completion of the restriction digestion, the samples were resolved in 3% agarose gel using Tris borate buffer at 135V for 35 min and the UV-Gel document was used to reveal the genotype of each sample based on their restriction pattern as

SNP annotation and ID	**PCR product (bp)**	**Enzyme for RFLP and condition**	**Digestion pattern and Genotype**
MTHFR 1298 A>C	128	Mbo II at 37˚C overnight	28,28 &72bp(AA) 28 & 100bp (CC) 28,72&100bp(AC)

RESULTS

A total of 100 RA and 50 age-matched control subjects were enrolled in the study. The characteristics of RA and control subjects are presented in **Table 1**. There was no significant difference between mean age and gender among control and RA patients ($p>0.05$). Most of the patients were at the early stages of the disease with duration of ~ 2yrs. The mean duration of MTX treatment was less than 2yrs and the mean dose were found to be less than 10mg/week, orally. All the patients received 5mg folic acid on all the days except the drug day.

Successful genotyping of MTHFR A1298C was observed in 96 RA patients and 44 control subjects. The frequency of MTHFR A1298C polymorphism was determined in RA patients and control subjects and presented in **Table 2**. The heterozygous polymorphism AC was found to be higher in control subjects (51%) and in RA patients (46.87%).

After 3months follow-up period 42 RA patients completed the study. There were 13 wild-type, 29 heterozygous genotypes and 10 homozygous genotypes. In order to study the relationship between MTX-related efficacy and toxicity, their disease characteristics, dose profile, biochemical parameters and adverse effect profiles were studied. **(Table 3, Table 4, Table 5 and Table 6)**

The efficacy related parameters comprised of morning stiffness, pain scale, tender and swollen-joint counts, and disease activity score(DAS), health assessment questionnaire(HAQ) scoring and weekly dose of MTX were studied.

The relief in morning stiffness was found to be significantly improved among AC ($p<0.001$) and AA genotypes ($p<0.05$) **Fig 1& 4**. Even though the morning stiffness decreased among the CC genotypes, it was not statistically significant.

There was a significant decrease in tender joint count in AA genotypes ($p<0.05$) **Fig 2**.

There was no significant difference in pain scale among all the genotypes.

Swollen joint count was significantly increased in CC genotypes when compared to AC genotypes ($p<0.05$) **Fig 16**.

The DAS was significantly increased ($p<0.01$) in CC genotypes in final visit **Fig 7**.

There was no significant difference among AA and AC genotypes between the visits.

Disability index measured by health assessment questionnaire was compared in all the visits. AC and CC genotypes, showed statistically significance ($p<0.001$) when compared to AA **Fig 5, 8, 12, 13, 20 & 21**.

In the CC genotypes the dose of MTX required for remission increased significantly when compared to AA and AC genotypes ($p<0.05$) **Fig 17.**

Among biochemical profile, ESR was significantly elevated in the CC genotypes **Fig 9**.

Regarding adverse effects, elevation of transaminases was taken as a marker of liver toxicity and nausea, vomiting was considered as gastrointestinal (GI) side effects. Other adverse effects like alopecia, pulmonary fibrosis and bone marrow depression were looked for, but they were not detected or reported by the patients who had completed the follow-up phase of the study. **(Table 3, Table 4, Table 5 and Table 6)**

When compared to the initial visit SGOT was significantly increased in the wild-type ($p<0.05$) and AC genotypes ($p<0.05$) **Fig 3 & 6**. There was a marked elevation of SGOT and SGPT in the CC genotypes in the final visit ($p<0.01$) **Fig 10 & 11**.

In comparison of adverse effect profile of AC and CC genotypes with wild-type, the elevation of transaminases was significantly higher in the AC &CC genotypes ($p<0.05$) **Fig 18 & 19**.

The CC genotypes had significant increase in SGOT ($p<0.001$) and SGPT ($p<0.001$) in final visit, when compared to AA and AC genotypes in final visit **Fig 23 & 24**.

Table 1- Demographic and Clinical characteristics of RA patients and controls

	RA patients	**Controls**
Age (in years)	43.53 ± 8.98	44.14 ± 10.19
Gender (Female/Male)	75/25	32/18
Duration of disease (in years)	2 ± 0.69	
Duration of MTX treatment (in years)	1.38 ± 0.47	
Dose of MTX (mg/week)	9.25 ± 2.5	

Table 2 – Frequency Distribution of MTHFR A1289C (AA, AC & CC)

	Control (N=43)	**Rheumatoid arthritis (N=96)**
AA (Wild-type)	14 (32.6%)	30 (31.3%)
AC (Heterozygous Mutants)	22 (51.16%)	45 (46.87%)
CC (Homozygous Mutants	7 (16.28%)	21 (21.88%)

Table 3 – Comparison of Initial and Final visits of Wild-type, Heterozygous Mutants & Homozygous Mutants

	Wild-type			Heterozygous Mutants			Homozygous Mutants		
	Initial visit	**Final visit**	**P value**	**Initial visit**	**Final visit**	**P value**	**Initial visit**	**Final visit**	**P value**
Disease characteristics									
Morning stiffness	53.8 ± 14.7	46.5 ± 10.7*	0.0459	51.4 ± 14.0	38.3 ± 11.6***	0.001	56.0 ± 20.0	49.0 ± 15.8	0.1108
Pain scale	6.0 ± 1.6	6.7 ± 0.9	0.0821	6.5 ± 1.3	6.7 ± 1.4	0.3597	6.5 ± 1.3	7.3 ± 1.1	0.0697
TJC	4.5 ± 1.4	3.8 ± 1.2*	0.0395	4.2 ± 1.4	3.8 ± 1.0	0.1100	4.3 ± 1.8	4.3 ± 1.4	1.0000
SJC	2.2 ± 0.8	2.2 ± 1.1	0.8372	1.9 ± 0.7	1.7 ± 0.9	0.3306	2.1 ± 1.2	2.6 ± 0.8	0.1773
DAS	4.4 ± 0.6	4.4 ± 0.5	0.8414	4.3 ± 0.5	4.3 ± 0.5	0.9529	4.01 ± 0.4	4.6 ± 0.4**	0.0048
HAQ	0.76 ± 0.13	0.70 ± 0.09	0.2456	1.48 ± 0.14	2.01 ± 0.19***	0.001	1.51 ± 0.23	1.94 ± 0.16***	0.001
Drug characteristics									
Dose of MTX	9.0 ± 2.2	9.6 ± 2.2	0.3870	9.1 ± 2.5	9.5 ± 2.0	0.5461	10.75 ± 2.4	12 ± 3.9	0.3221
Biochemical characteristics									
ESR	34.4 ± 25.8	37.0 ± 18.1	0.7546	27.0 ± 13.9	32.7 ± 17.9	0.3042	19 ± 4.6	39.3 ± 13.8**	0.0022
Toxicity									
Liver toxicity									
SGOT	18.5 ± 4.4	22.8 ± 5.4*	0.0227	22.5 ± 8.2	27.3 ± 8.4	0.0867	22.6 ± 11.0	64.88 ± 31**	0.0065
SGPT	18.1 ± 5.5	21.7 ± 3.9	0.1066	19.3 ± 6.7	27.4 ± 7.7*	0.0100	19.9 ± 5.1	72.13 ± 40.2**	0.0063
GI disturbances	6 (46%)	3 (23%)		14(73.68%)	12 (63.15%)		8 (80%)	7 (70%)	

TJC = Tender Joint Count, SJC = Swollen Joint Count, DAS = Disease activity Score, MTX = Methotrexate, HAQ= Health assessment questionnaire, ESR = Erythrocyte Sedimentation Rate, SGOT= Serum glutamatic oxaloacetic transaminase or Aspartate transaminase, SGPT = Serum glutamatic pyruvate transaminase or alanine transaminase.

Table 4 – Wild Vs Mutants

	Initial visit			Final visit		
	Wild-type	**Mutants**	**P value**	**Wild-type**	**Mutants**	**P value**
Disease characteristics						
Morning stiffness	53.8 ± 14.7	53 ±14.5	0.6770	46.5 ± 10.7	42.1 ± 17	0.3217
Pain scale	6.0 ± 1.6	6.5 ± 1.3	0.2714	6.7 ± 0.9	6.9 ± 1.4	0.5580
TJC	4.5 ± 1.4	4.2 ± 1.6	0.6057	3.8 ± 1.2	4.0 ± 1.4	0.7654
SJC	2.2 ± 0.8	2.0 ± 0.9	0.3742	2.2 ± 1.1	2.0 ± 1.0	0.6541
DAS	4.4 ± 0.6	4.2 ± 0.5	0.2418	4.4 ± 0.5	4.4 ± 0.6	0.8607
HAQ	0.76 ± 0.13	1.5 ± 0.17***	0.001	0.70 ± 0.09	2.0 ± 0.18***	0.001
Drug characteristics						
Dose of MTX	9.0 ± 2.2	9.7 ± 2.4	0.4558	9.6 ± 2.0	10.3 ± 3.8	0.4254
Biochemical characteristics						
ESR	34.4 ± 25.8	24.1 ± 13.0	0.0880	37.0 ± 18.1	35.1 ± 15.5	0.6500
Toxicity						
Liver toxicity						
SGOT	18.5 ± 4.4	22.5 ± 9.7	0.1528	22.8 ± 5.4	38.4 ± 33.7 *	0.0316
SGPT	18.1 ± 5.5	19.5 ± 5.1	0.4858	21.7 ± 3.9	41.5 ± 40.9 *	0.0318
GI disturbances	6 (46%)	22 (75%)		3 (23%)	19 (65%)	

TJC = Tender Joint Count, SJC = Swollen Joint Count, DAS = Disease activity Score, MTX = Methotrexate, HAQ= Health assessment questionnaire, ESR = Erythrocyte Sedimentation Rate, SGOT= Serum glutamatic oxaloacetic transaminase or Aspartate transaminase, SGPT = Serum glutamatic pyruvate transaminase or alanine transaminase.

Table 5 –Homozygous Mutants Vs Heterozygous Mutants

	Initial visit			Final visit		
	Heterozygous Mutants	Homozygous mutants	P value	Heterozygous Mutants	Homozygous mutants	P value
Disease characteristics						
Morning stiffness	51.4 ± 14.0	56.0 ± 20.0	0.4803	38.3 ± 11.6	49.0 ± 15.8	0.0509
Pain scale	6.5 ± 1.3	6.5 ± 1.3	0.9589	6.7 ± 1.4	7.3 ± 1.1	0.2781
TJC	4.2 ± 1.4	4.3 ± 1.8	0.8130	3.8 ± 1.0	4.3 ± 1.4	0.2758
SJC	1.9 ± 0.7	2.1 ± 1.2	0.5713	1.7 ± 0.9	2.6 ± 0.8*	0.0161
DAS	4.3 ± 0.5	4.01 ± 0.4	0.1770	4.3 ± 0.5	4.6 ± 0.4	0.0654
HAQ	1.48 ± 0.14	1.51 ± 0.23	0.6303	2.01 ± 0.19	1.94 ± 0.16	0.2806
Drug characteristics						
Dose of MTX	9.1 ± 2.5	10.75 ± 2.4	0.0957	9.5 ± 2.0	12.0 ± 3.9*	0.0264
Biochemical characteristics						
ESR	27.0 ± 13.9	19.0 ± 4.6	0.0907	32.7 ± 17.9	39.3 ± 13.8	0.3248
Toxicity						
Liver toxicity						
SGOT	22.5 ± 8.2	22.6 ± 11.0	0.9723	27.3 ± 8.4	64.9 ± 31.0 ***	0.0001
SGPT	19.3 ± 6.7	19.9 ± 5.1	0.8113	27.4 ± 7.7	72.13 ± 40.2 ***	0.0001
GI disturbances	14 (74%)	8 (80%)		12 (63%)	7 (70%)	

TJC = Tender Joint Count, SJC = Swollen Joint Count, DAS = Disease activity Score, MTX = Methotrexate, HAQ= Health assessment questionnaire, ESR = Erythrocyte Sedimentation Rate, SGOT= Serum glutamatic oxaloacetic transaminase or Aspartate transaminase, SGPT = Serum glutamatic pyruvate transaminase or alanine transaminase.

Table 6 – Wild Vs Homozygous Mutants Vs Heterozygous Mutants

	Initial visit				Final visit			
	Wild-type	**Heterozygous Mutants**	**Homozygous mutants**	**P value**	**Wild-type**	**Heterozygous Mutants**	**Homozygous mutants**	**P value**
Disease characteristics								
Morning stiffness	53.8 ± 14.7	51.4 ± 14.0	56.0 ± 20.0	0.7544	46.5 ± 10.7	38.3 ± 11.6	49.0 ± 15.8	0.0617
Pain scale	6.0 ± 1.6	6.5 ± 1.3	6.5 ± 1.3	0.5498	6.7 ± 0.9	6.7 ± 1.4	7.3 ± 1.1	0.4177
TJC	4.5 ± 1.4	4.2 ± 1.4	4.3 ± 1.8	0.8508	3.8 ± 1.2	3.8 ± 1.0	4.3 ± 1.4	0.5269
SJC	2.2 ± 0.8	1.9 ± 0.7	2.1 ± 1.2	0.5696	2.2 ± 1.1	1.7 ± 0.9	2.6 ± 0.8	0.0576
DAS	4.4 ± 0.6	4.3 ± 0.5	4.01 ± 0.4		4.4 ± 0.5	4.3 ± 0.5	4.6 ± 0.4	0.1961
HAQ	0.76 ± 0.13	1.48 ± 0.14 ###	1.51 ± 0.23 ###	0.001	0.70 ± 0.09	2.01 ± 0.19 ###	1.94 ± 0.16 ###	0.001
Drug characteristics								
Dose of MTX	9.0 ± 2.2	9.1 ± 2.5	10.75 ± 2.4	0.1628	9.6 ± 2.0	9.5 ± 2.0	12.0 ± 3.9*	0.0369
Biochemical characteristics								
ESR	34.4 ± 25.8	27.0 ± 13.9	19.0 ± 4.6	0.1217	37.0 ± 18.1	32.7 ± 17.9	39.3 ± 13.8	0.5921
Toxicity								
Liver toxicity								
SGOT	18.5 ± 4.4	22.5 ± 8.2	22.6 ± 11.0	0.3644	22.8 ± 5.4	27.3 ± 8.4	64.9 ± 31.0 ### ***	0.0001
SGPT	18.1 ± 5.5	19.3 ± 6.7	19.9 ± 5.1	0.7628	21.7 ± 3.9	27.4 ± 7.7	72.13 ± 40.2 ### ***	0.0001
GI disturbances	6 (46%)	14 (74%)	8 (80%)		3 (23%)	12 (63%)	7 (70%)	

TJC = Tender Joint Count, SJC = Swollen Joint Count, DAS = Disease activity Score, MTX = Methotrexate, HAQ= Health assessment questionnaire, ESR = Erythrocyte Sedimentation Rate, SGOT= Serum glutamatic oxaloacetic transaminase or Aspartate transaminase, SGPT = Serum glutamatic pyruvate transaminase or alanine transaminase.

Comparison of Initial and Final visits of Wild-type, Heterozygous Mutants & Homozygous Mutants (Figure 1 to 11 and the values from Table 3)

Wild type (AA Genotype)

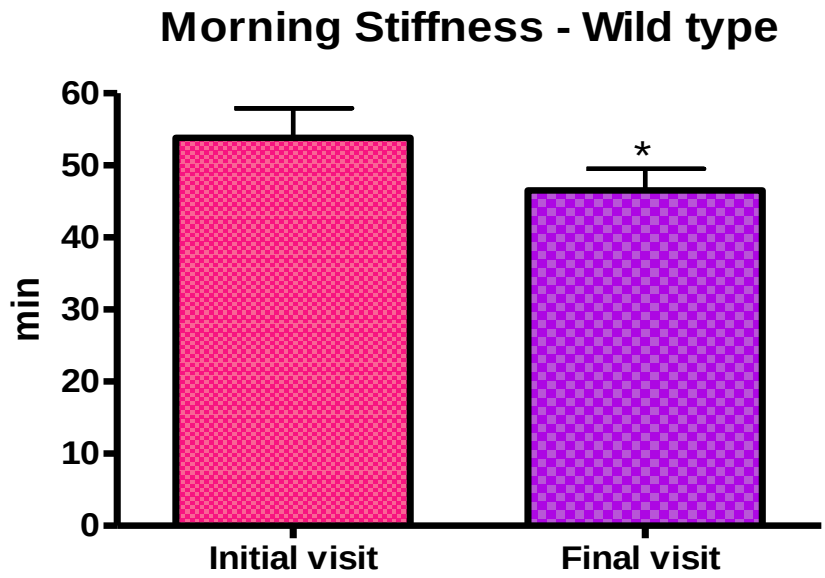


Figure 1: Comparison of morning stiffness of AA genotype during initial and final visit was 53.8 ± 14.7 and 46.5 ± 10.7 (*****p<0.05) and considered statistically significant

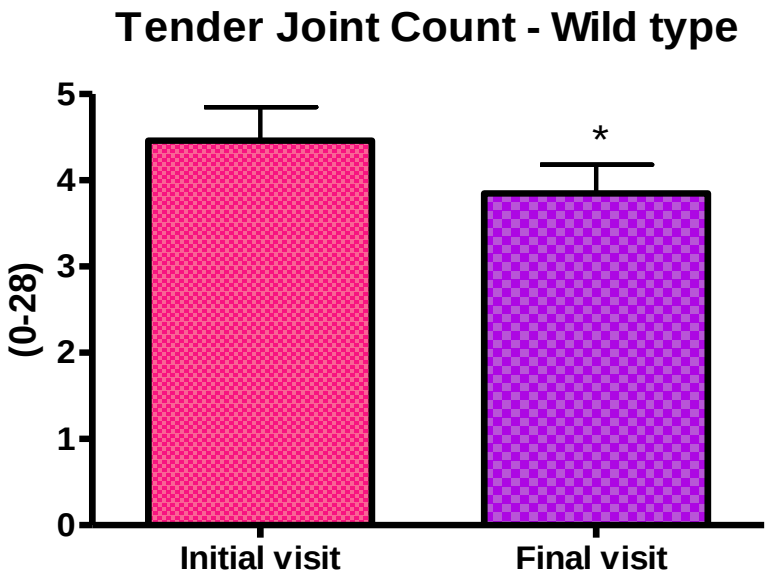


Figure 2: Comparison of tender joint count of AA genotype during initial and final visit was 4.5 ± 1.4 and 3.8 ± 1.2(*****p<0.05) and considered statistically significant

Figure 3: Comparison of tender joint count of AA genotype during initial and final visit levels were 18.5 ± 4.4 and 22.8 ± 5.4 **(***p<0.05) and considered statistically significant

Heterozygous Mutants (AC Genotype)

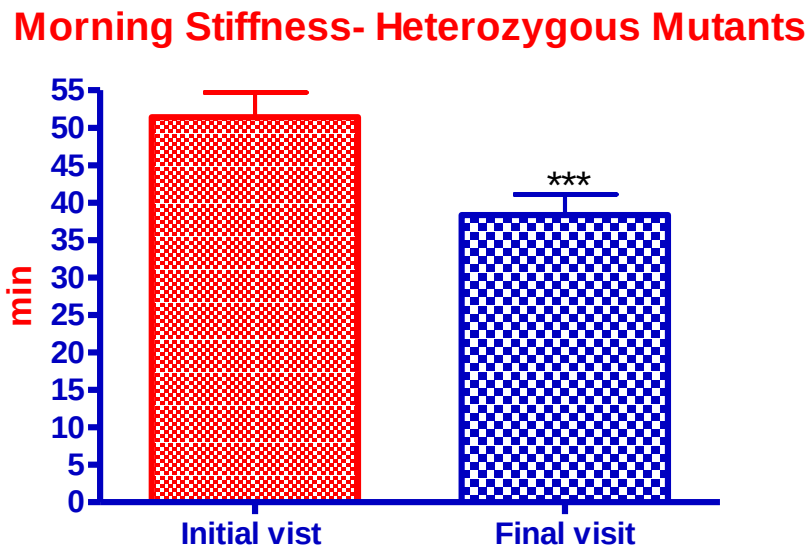


Figure 4: Comparison of morning stiffness of AC genotype during initial and final visit was 51.4 ± 14.0 and 38.3 ± 11.6 (*******p<0.001) and considered statistically significant

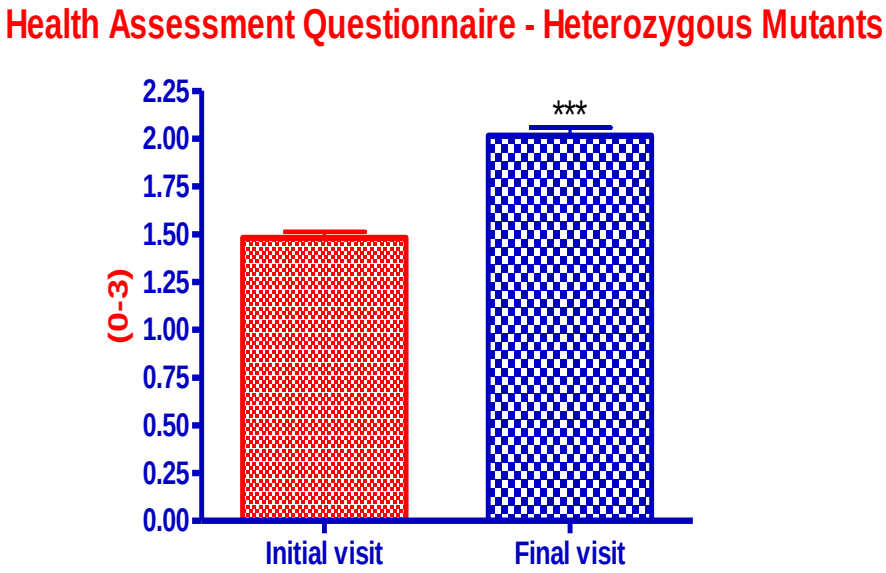


Figure 5: Comparison of Health Assessment Questionnaire of AC genotype during initial and final visit was 1.48 ± 0.14 and 2.01 ± 0.19 **(*****p<0.001) and considered statistically significant

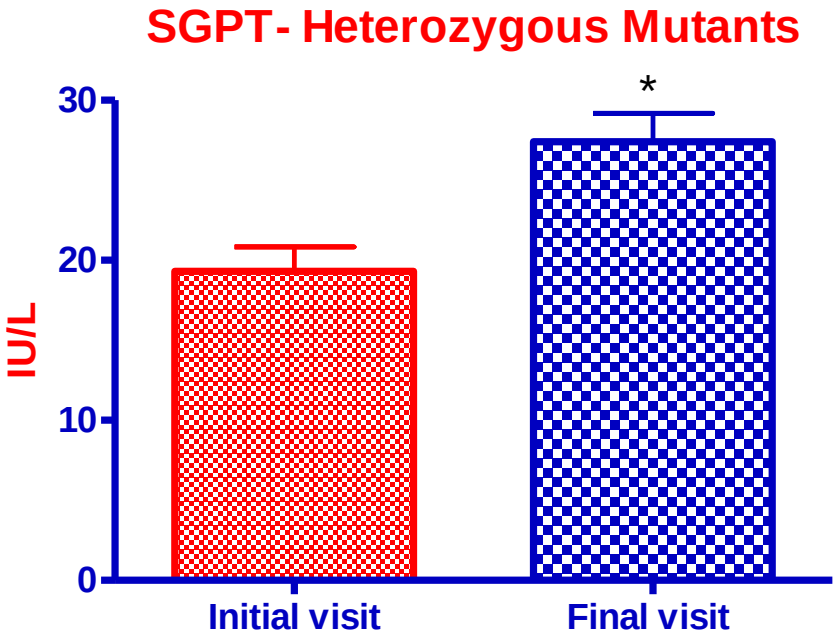


Figure 6: Comparison of SGPT of AC genotype during initial and final visit was 19.3 ± 6.7 and 27.4 ± 7.7 **(***p<0.05) and considered statistically significant

Homozygous Mutants (CC Genotype)

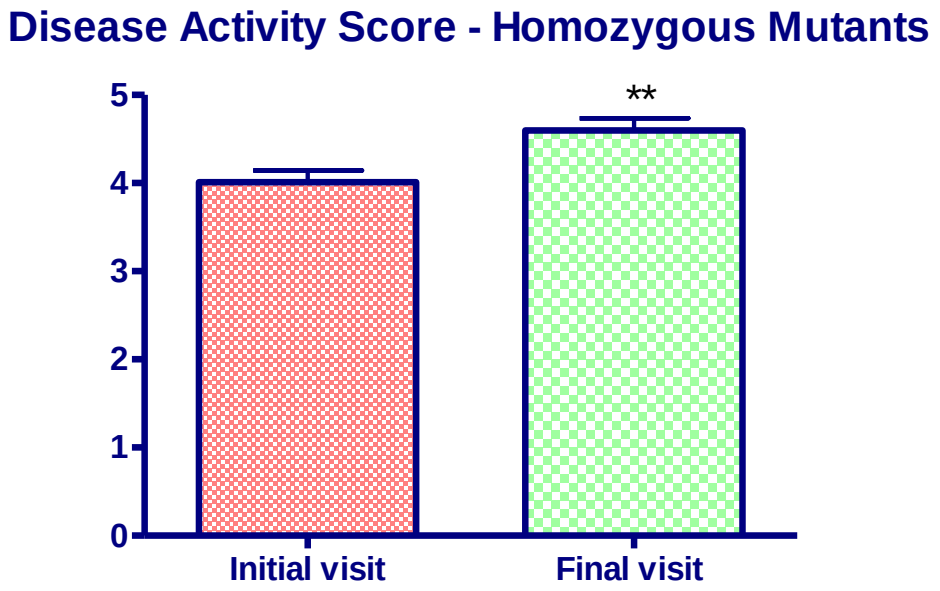


Figure 7: Comparison of DAS of CC genotype during initial and final visit was 4.01 ± 0.4 and 4.6 ± 0.4 **(**p<0.01) and considered statistically significant

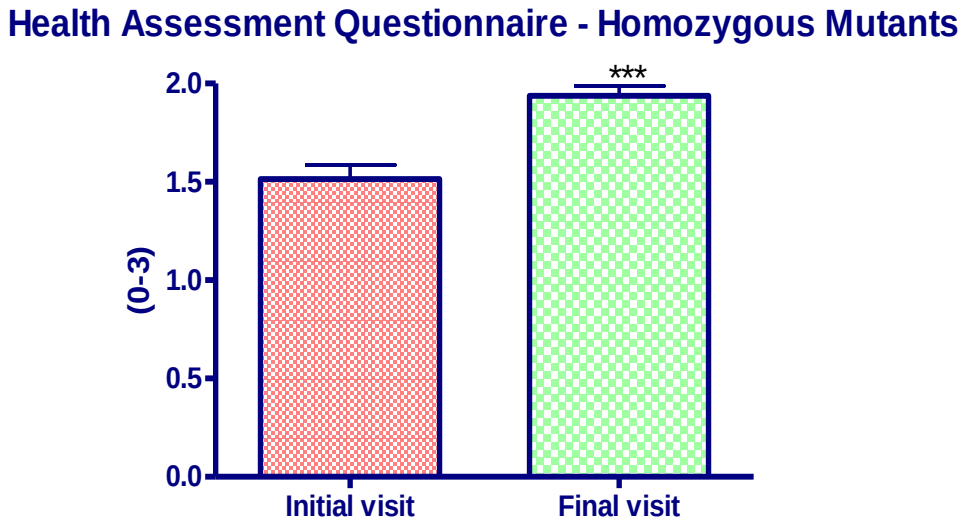


Figure 8: Comparison of HAQ of CC genotype during initial and final visit was 1.51 ± 0.23 and 1.94 ± 0.16 (***p<0.001) and considered statistically significant

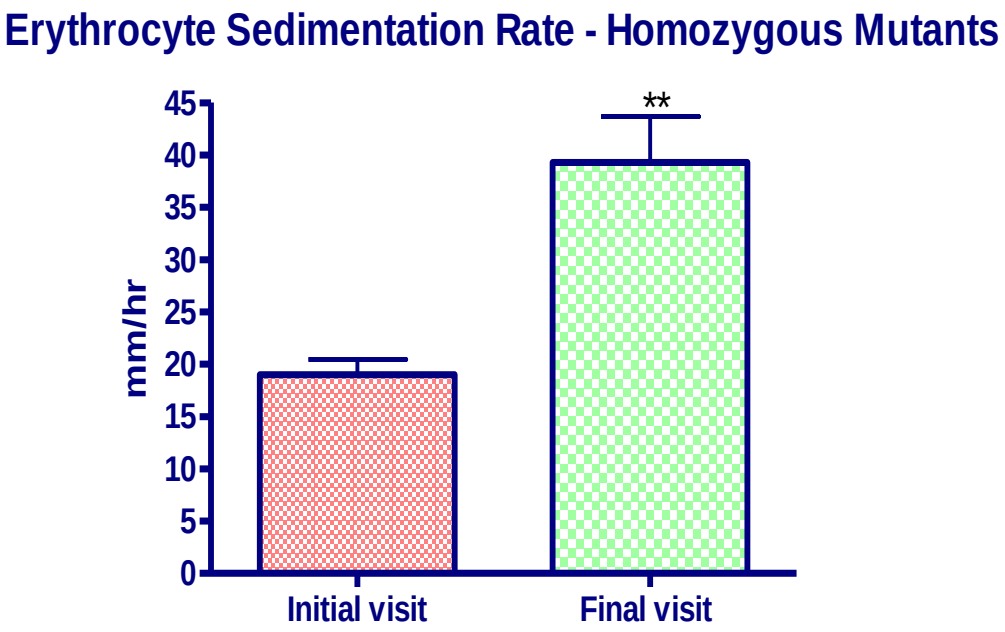


Figure 9: Comparison of ESR of CC genotype during initial and final visit was 19 ± 4.6 and 39.3 ± 13.8 **(****p<0.01) and considered statistically significant

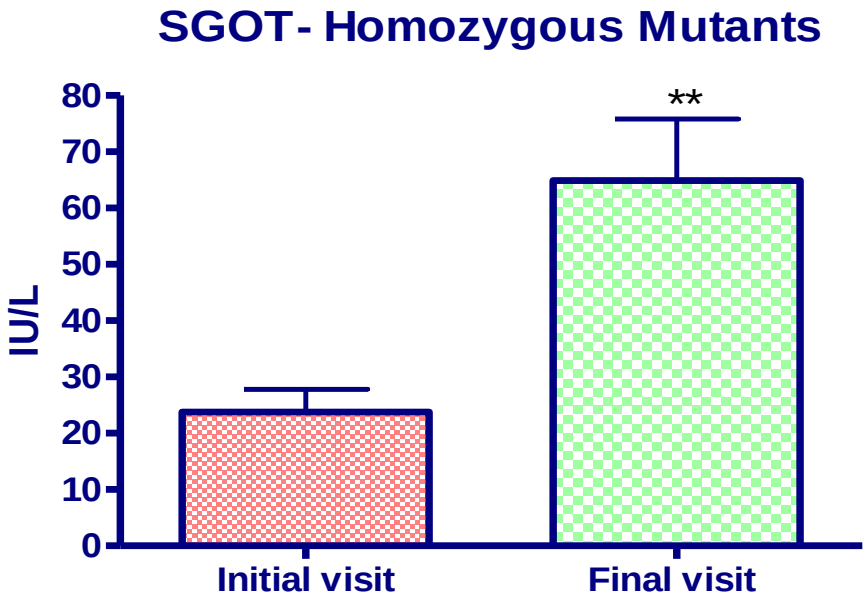


Figure 10: Comparison of SGOT of CC genotype during initial and final visit was 22.6 ± 11.0 and 64.88 ± 31 (**p<0.01) and considered statistically significant

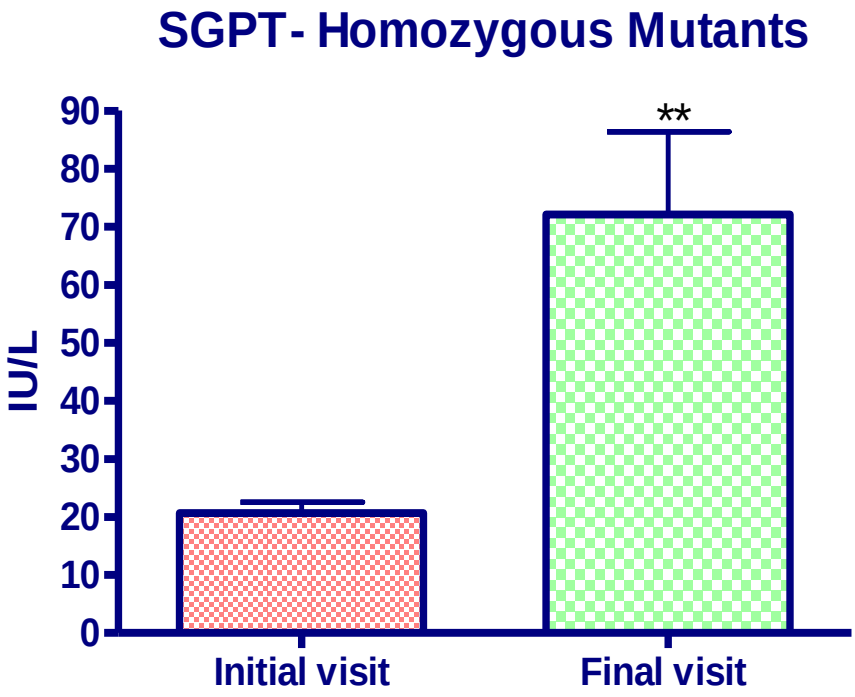


Figure 11: Comparison of SGPT of CC genotype during initial and final visit was 19.9 ± 5.1 and 72.13 ± 40.2 (**p<0.01) and considered statistically significant

Comparison of Initial and Final visits of Wild-type Vs Mutants (Heterozygous Mutants & Homozygous Mutants) (Figure 12 to 15 and the values from Table 4)

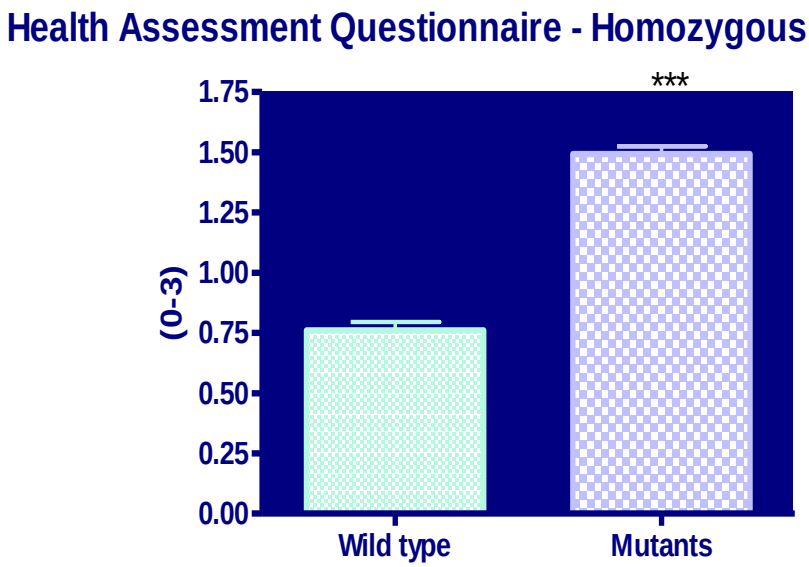


Figure 12: Comparison of HAQ of AA (Vs.) CC +AC genotype during initial visit was 0.76 ± 0.13 and 1.5 ± 0.17 (***p<0.001) and considered statistically significant

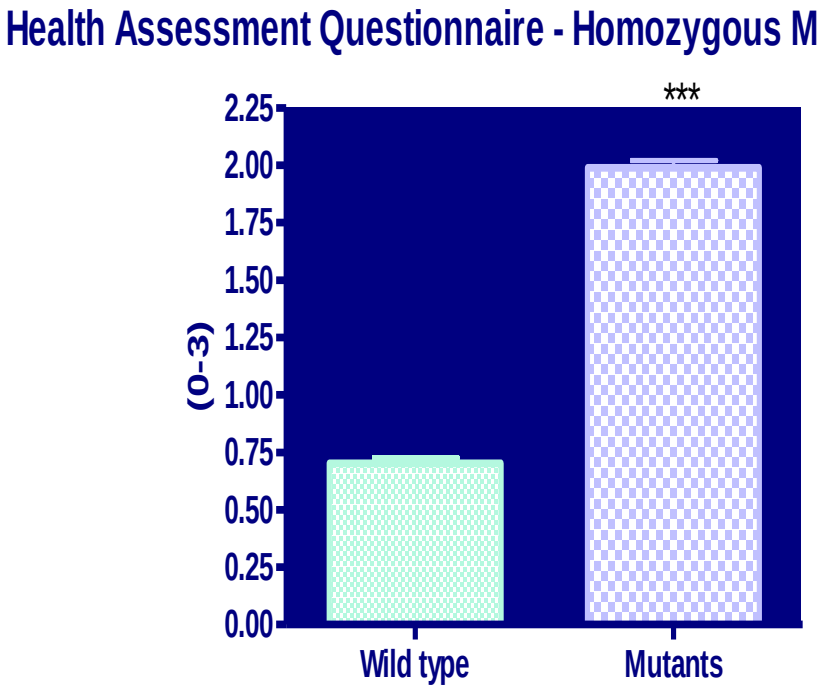


Figure 13: Comparison of HAQ of AA (Vs.) CC+ AC genotype during Final visit was 0.70 ± 0.09 and 2.0 ± 0.18 (*p<0.001) and considered statistically significant

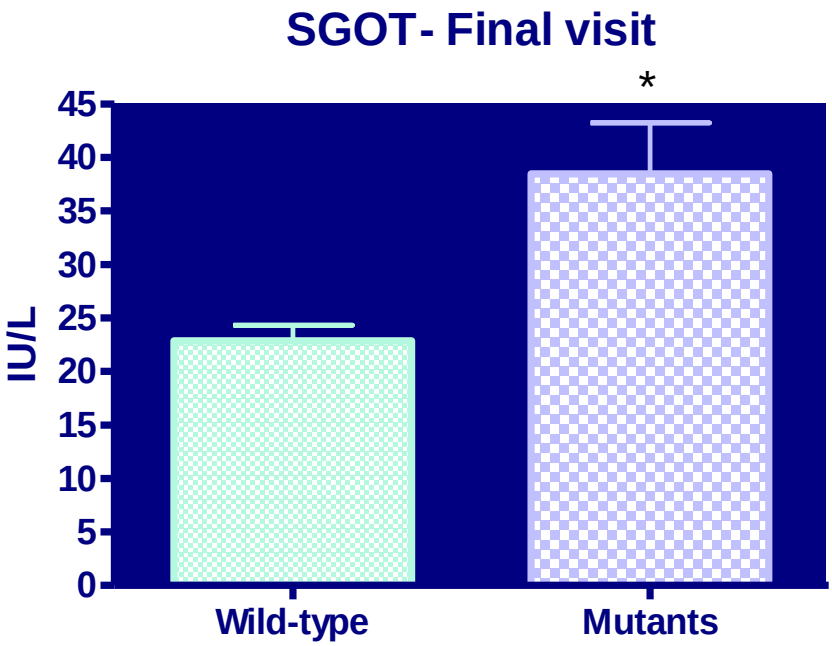


Figure 14: Comparison of SGPT of AA (Vs.) CC + AC genotype during Final visit was 22.8 ± 5.4 and 38.4 ± 33.7 (*p<0.05) and considered statistically significant

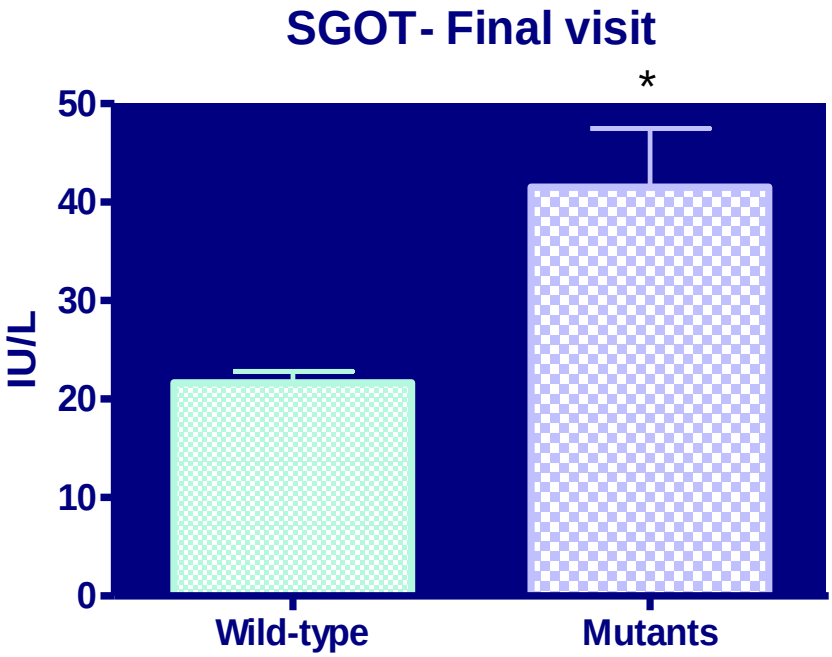


Figure 15: Comparison of SGOT of AA (Vs.) CC + AC genotype during Final visit was 21.7 ± 3.9 and 41.5 ± 40.9 (*p<0.05) was considered statistically significant

Comparison of Initial and Final visits of Heterozygous Mutants Vs Homozygous Mutants (Figure 16 to 19 and values from Table 5)

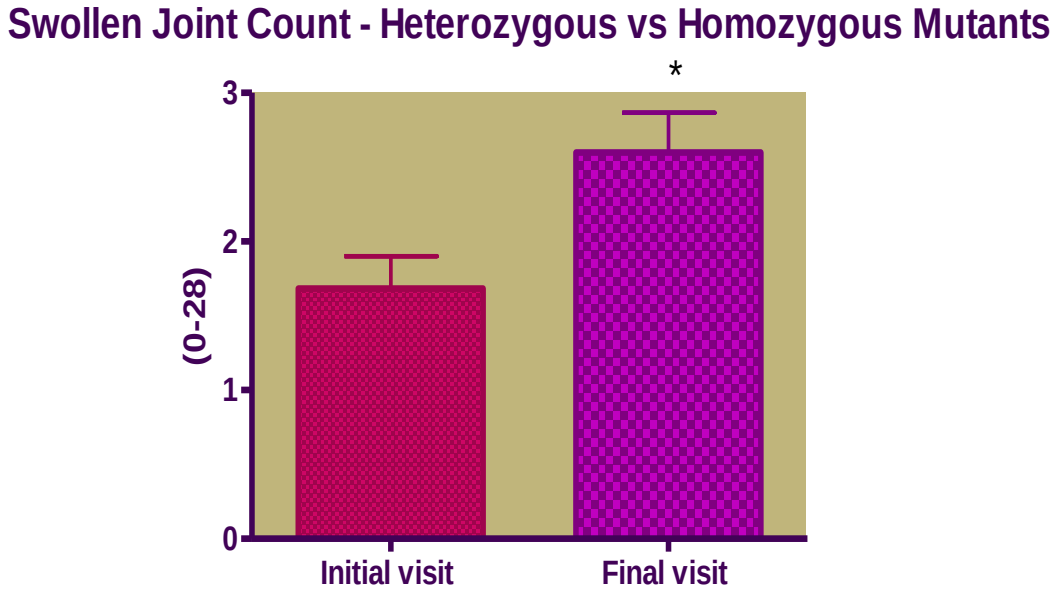


Figure 16: Comparison of Swollen joint count of AC and CC genotype during Final visit was 1.7 ± 0.9 and 2.6 ± 0.8 (*p<0.05) and considered statistically significant

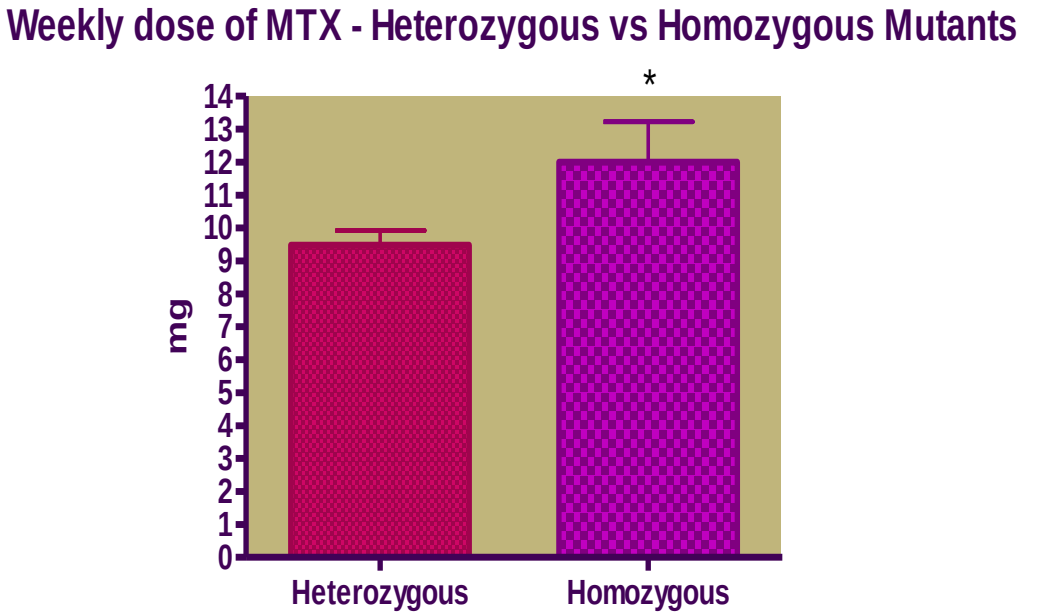


Figure 17: Comparison of weekly dose of MTX of AC and CC genotype during final visit was 9.5 ± 2.0 and 12.0 ± 3.9 (*p<0.05) and considered statistically significant

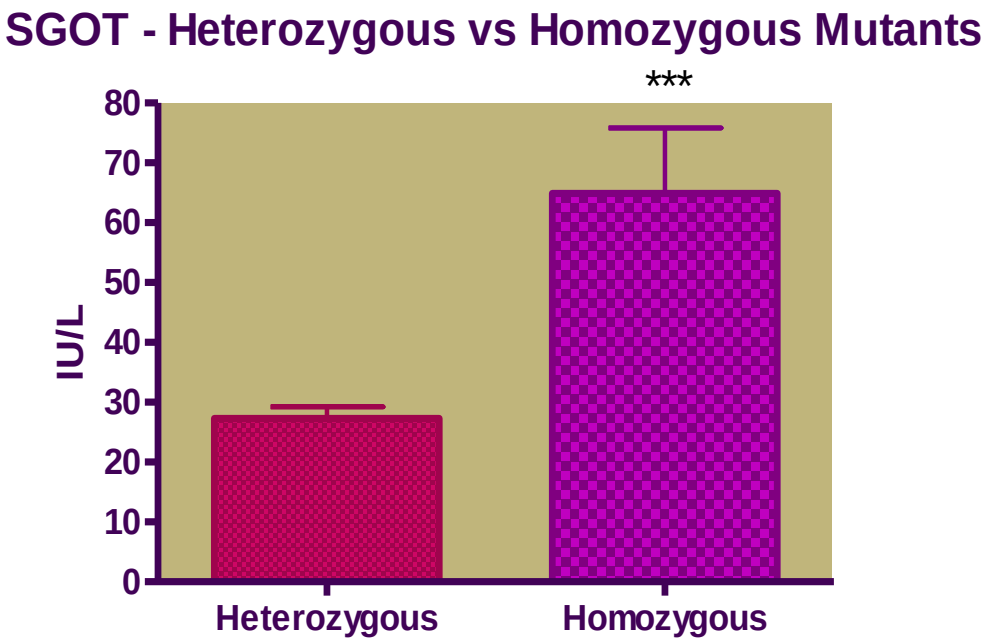


Figure 18: Comparison of SGOT of AC and CC genotype during final visit was 27.3 ± 8.4 and 64.9 ± 31.0 (***p<0.001) and considered statistically significant

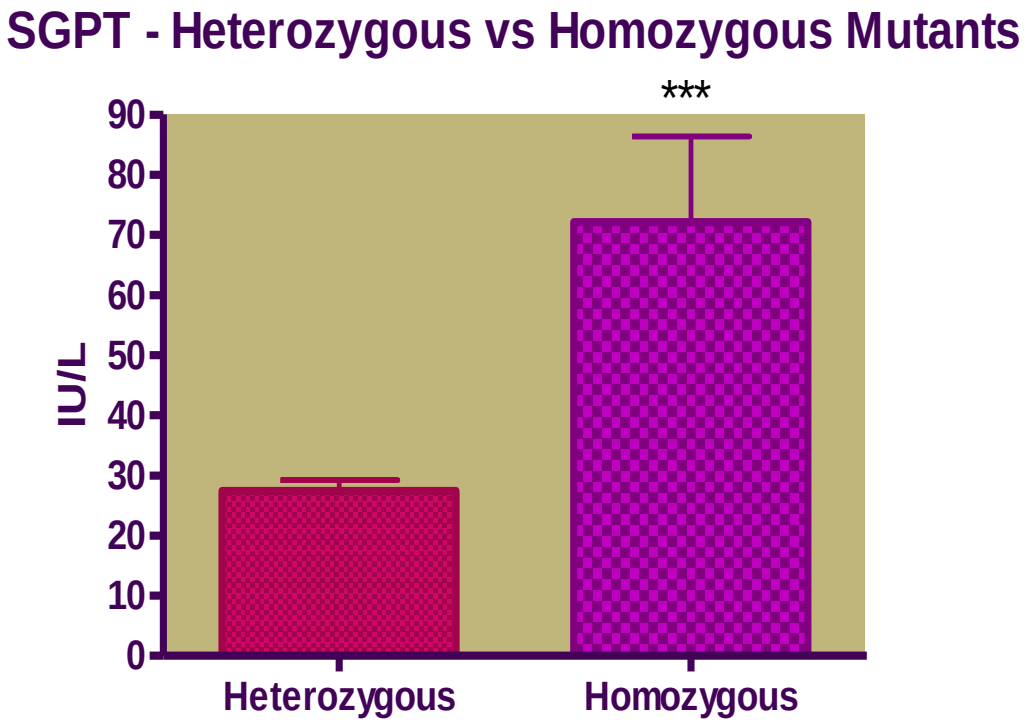


Figure 19: Comparison of SGPT of AC and CC genotype during final visit was 27.4 ± 7.7 and 72.13 ± 40.2 (***p<0.001) and considered statistically significant

Comparison of Initial and Final visits of Wild Vs Homozygous Mutants Vs Heterozygous Mutants (Figures 20 to 24 and values from Table 6)

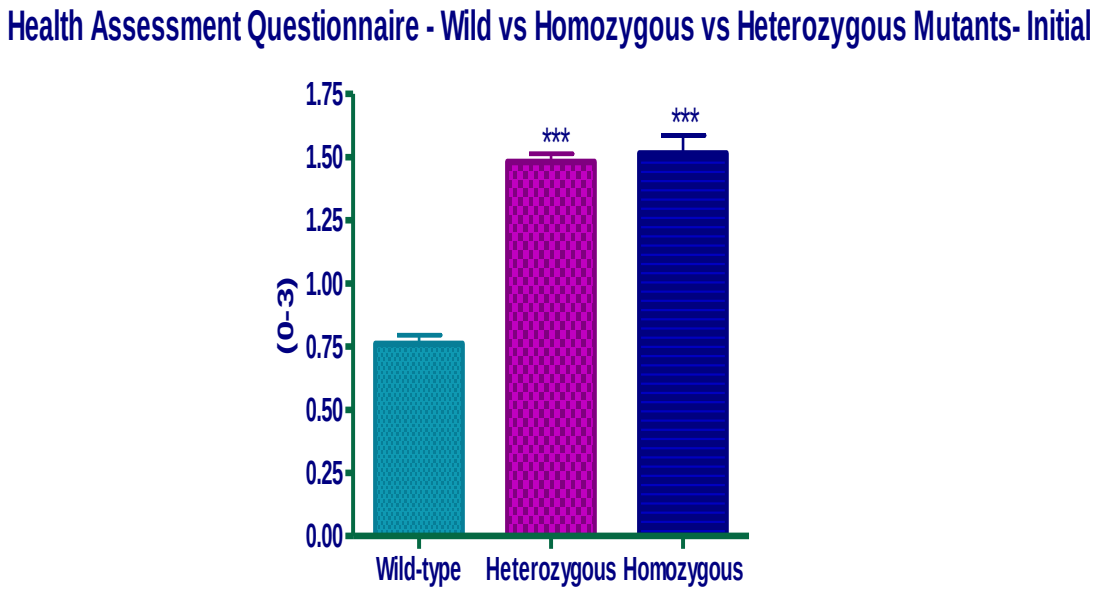


Figure 20: Comparison of HAQ of AA, AC and CC genotype during initial visit was 0.76 ± 0.13, 1.48 ± 0.14 and 1.51 ± 0.23 (***p<0.001) and considered statistically significant

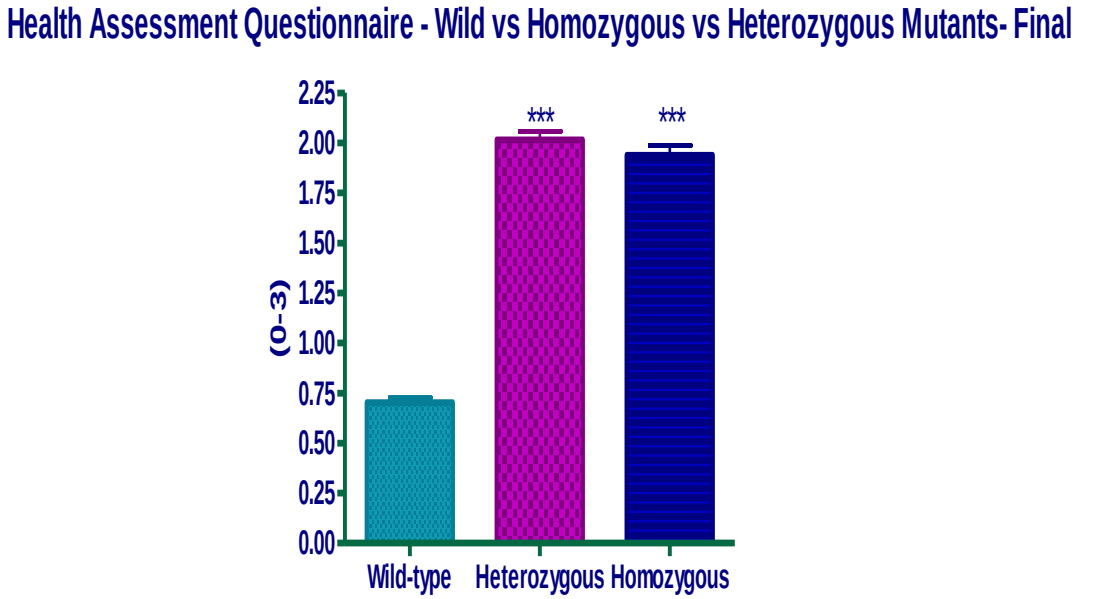


Figure 21: Comparison of HAQ of AA, AC and CC genotype during final visit was 0.70 ± 0.09, 2.01 ± 0.19 and 1.94 ± 0.16 (***p<0.001) and considered statistically significant

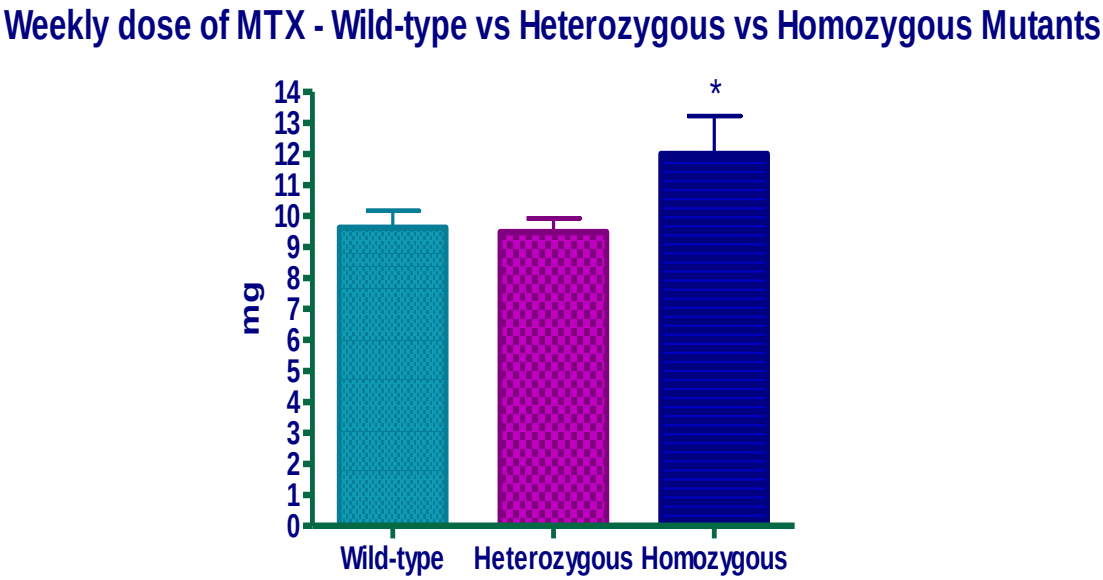


Figure 22: Comparison of Weekly dose of MTX of AA, AC and CC genotype during final visit was 9.6 ± 2.0, 9.5 ± 2.0 and 12.0 ± 3.9 (*p<0.05) and considered statistically significant

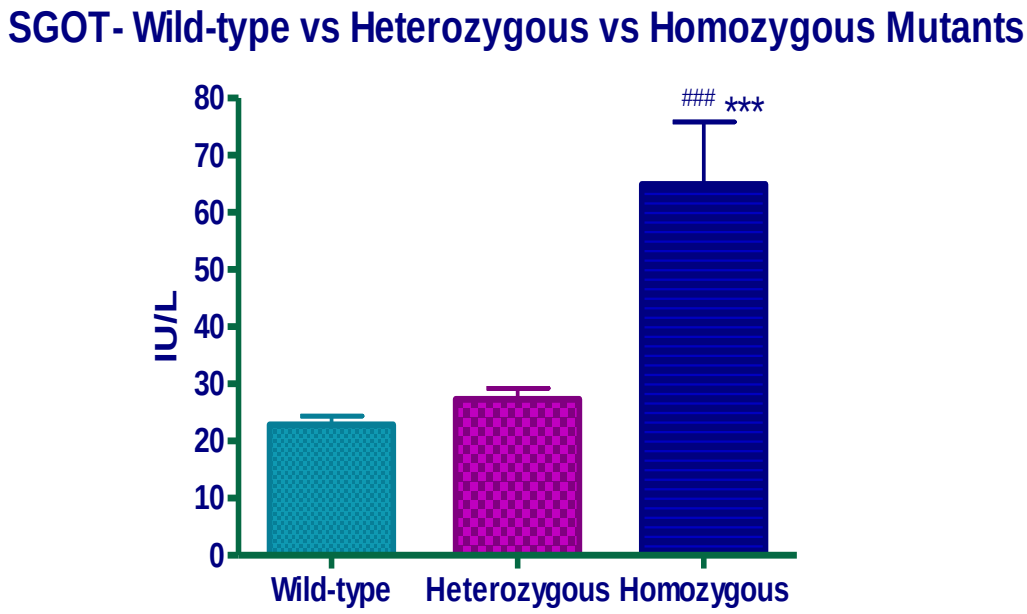


Figure 23: Comparison of SGOT of AA, AC and CC genotype during final visit was 22.8 ± 5.4, 27.3 ± 8.4 and 64.9 ± 31.0 (***p<0.001, ###p<0.001) and considered statistically significant

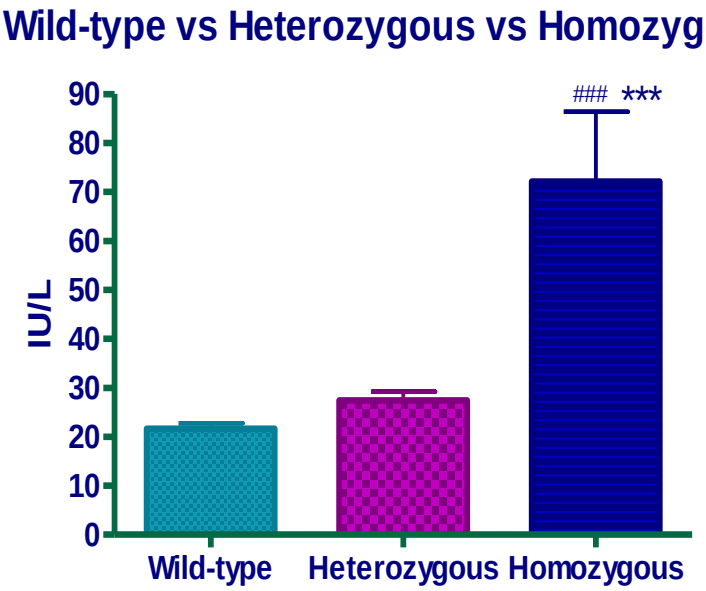


Figure 24: Comparison of SGOT of AA, AC and CC genotype during final visit was 21.7 ± 3.9, 27.4 ± 7.7 and 72.13 ± 40.2 (*******p<0.001, ###p<0.001) and considered statistically significant

PCR and RFLP Products of MTHFR A1298C gene

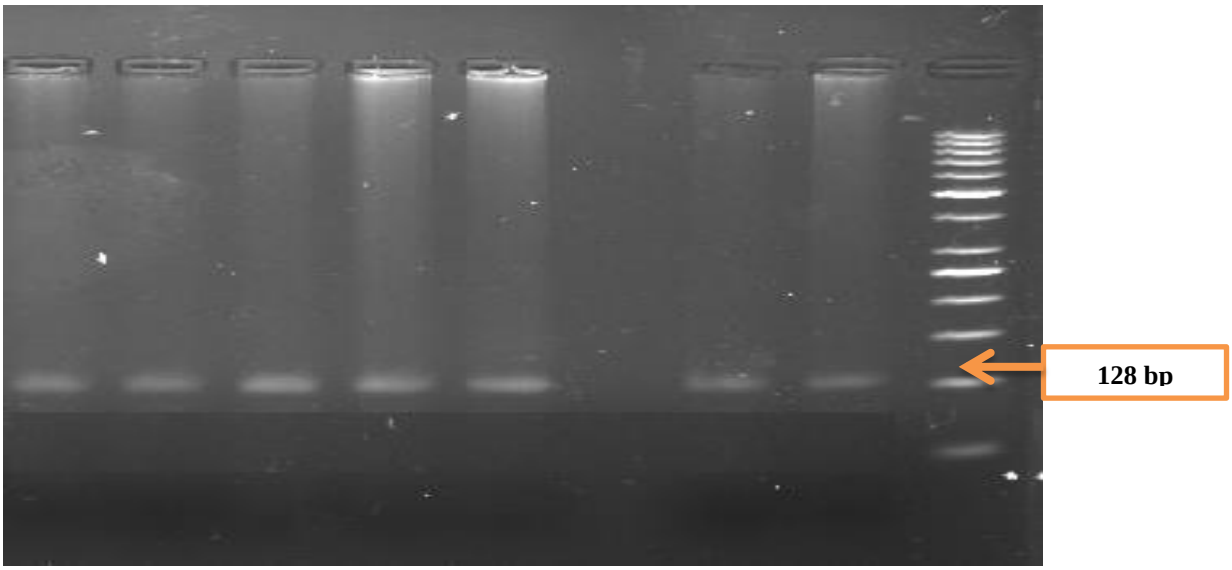


Figure 25: UV-Trans-illuminated agarose gel photo of the MTHFR 1298 A>C genes PCR Product and samples resolved in 3% agarose gel using 1XTBE buffer in 135V for 30mins

L 1 2 3 4 5 6 7 L

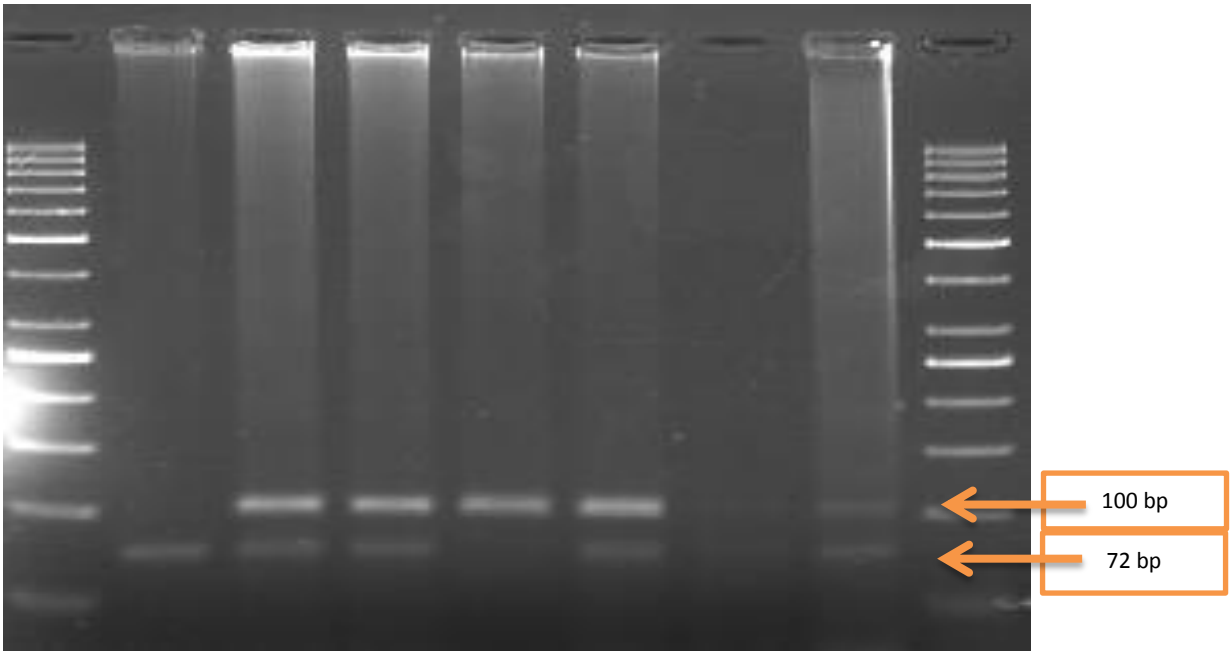


Figure 25: UV-Trans-illuminated agarose gel photo of the MTHFR 1298 A>C genes PCR-RFLP Product using Mbo II enzyme and samples Dissolved in 3% agarose gel using 1XTBE buffer in 135V for 30mins.

L- Ladder gene (50bp); 1- Wild type (AA Genotype); 2,3,5 and 7- Heterozygous Mutant (AC genotype); 4- Homozygous Mutant (CC genotype)

DISCUSSION

Rheumatoid arthritis is an autoimmune disease that results in a chronic, Systemic inflammatory disorder that affects many tissues and organs, but principally affects the flexible (synovial) joints. It can be a disabling and painful condition, which can lead to substantial loss of functioning and mobility if not adequately treated.

Methotrexate is the cornerstone for the therapy of rheumatoid arthritis in spite of the advent of newer biologics. MTX is fast acting and has best efficacy: toxicity ratio and also cheaper. Due to the variation in response and toxicity profile, 1/3rd of the patients discontinue therapy due to its adverse effects.

At cellular level MTX and MTX-PGs (metabolite of MTX) inhibit several enzymes of purine, pyrimidine biosynthesis and also exert anti-inflammatory effect. MTHFR is one of such enzyme inhibited by MTX. So the study in the polymorphism of MTHFR gene can be a better tool to reveal the efficacy: toxicity of MTX.

This study was done in the Department of Rheumatology at Rajiv Gandhi Government General Hospital, Madras Medical College, Chennai. A total of 100 patients were enrolled for this study and after 3months follow-up period 42 RA patients completed the study. Successful genotyping of MTHFR A1298C was observed in 96 RA patients and 44 control subjects. The frequency of MTHFR A1298C polymorphism was determined in RA patients and control subjects.

During enrolment patients were assessed by clinical examination and lab investigations and they were repeated after 3 months. Data were compiled and results analysed statistically.

The efficacy related parameters comprised of morning stiffness, pain scale, tender and swollen-joint counts, and disease activity score, health assessment questionnaire scoring and weekly dose of MTX. Toxicity related parameters were SGOT, SGPT and GI symptoms.

The most widely reported polymorphisms were C677T and A1298C, in MTHFR gene which is associated with efficacy and adverse effects. In 1998, MTHFR A1298C has been reported by Weisberg *et al*, which causes glutamine to alanine substitution and renders reduced activity of the enzyme. In A1298C polymorphism the homozygous mutants have about ~40% reduction in enzyme activity.

In our study, the frequency distribution of genetic polymorphism in **south Indian healthy subjects** is 32.6% and 67.44% respectively for wild-type and mutants, which is contradictory to a study conducted at Pune, European and African population. In Pune study the frequency of this polymorphism was reported to be 70% and 30% for wild-type and mutants respectively, in a total of one hundred and forty four unrelated healthy subjects, which is similar to European population. In African healthy subjects, the frequency was reported to be 87% and 13% by Yogita *et al*. [59] The reason for this difference could be due to Ethnic variation.

In our study the frequency distribution of polymorphism in **RA patients** were observed as 31.3 **:** 68.75 for A and C alleles respectively, which is contradictory to a study conducted in Japanese RA patients by Sachie Inoue *et al*,[55] the frequency of A:C were 76:24 respectively. A study in Israel RA patients by Berkun *et al*,[49] it was reported as 53.8 **:** 46.2 for A and C alleles respectively. A study in Afro-Americans by Hughes *et al*.,[53] it

was found to be 74 **:** 26 and in Caucasians it was reported as 45 **:** 55. This proves racial differences in the allelic frequency.

Berkun *et al*,[49] reported that the allele frequency of 1298CC was higher in RA population and the carriers of 1298AA allele had higher frequency of adverse effects in spite of higher folic acid supplementation and 1298CC may protect against MTX related adverse effects, conducted in 93 RA patients in Israel. In our study, similarly 1298CC was higher in RA population but the frequency of adverse effects was higher in the CC genotype and is not protective of MTX-related adverse effects. Instead, CC genotype is predictive of adverse effects in spite of folate supplementation. The reason for this difference could be due to Ethnic variation and probably be attributed to smaller sample size of the study.

In our study 1298AA genotypes were associated with better response to MTX treatment and lower incidence of adverse effects, whereas the 1298C allele carriers are prone for adverse effects. In a study by Wessels *et al*,[58] who reported that MTHFR 1298AA was associated with less improvement relative to mutants, which is contradictory to our study and MTHFR 1298C allele carriers, developed more adverse effects. Whereas Graber *et al* reported that MTHFR A1298C polymorphism is protective related to adverse effects of MTX, which is a contradictory to above finding. Polygenetic analysis will address this limitation.

In our study, the elevation in transaminases and GI adverse events in spite of folate supplementation was found to be higher in 1298CC genotype than the 1298AC and this

polymorphism warrants susceptibility to MTX toxicity. This is similar to the results of the study conducted by Davis *et al*.[52]

In our study the proportion of patients experiencing toxicity is found to be higher in 1298CC genotypes than 1298AA genotypes and this is similar to the study conducted by Choe *et al*,[51] in Korean RA patients.

In our study the 1298AA genotype shows better improvement comparable to 1298CC genotypes. The DAS score and HAQ score increased insignificantly for 1298CC genotype and the dose required for remission has also increased and they could become non-responders if the drug is administered chronically. This is similar to the results of study conducted by Kato *et al.*[54]

Urano *et al*,[56] in 2002 assessed both C677T & A1298C polymorphisms in this gene and found A1298C polymorphism rendered the patients sensitive to MTX treatment, whereas C677T rendered the patients prone for toxicity. In haplotype analysis 677C-1298C were receiving lower dose of MTX and 677T-1298A had a higher frequency of side-effects from MTX. The reported toxicities were elevation of transaminases, gastrointestinal (GI) disturbances, hair loss, fatigue and rash. The same was confirmed in their second study published in the year 2007. In their subsequent study in 2009, the same group also reported that these two polymorphisms are not associated with the occurrence of fracture. In our study, haplotype-analysis was not done and further studies are essential to study the interaction of these two polymorphisms in the MTHFR gene.

Assessment of the genotypes could be useful to identify the subset of genotypes such as 1298CC and their relative phenotypes and appropriate dose decisions with track of

adverse effects could be useful to assess better clinical response. This study proves pharmacogenetics could be a tool for assessing the treatment outcomes. Larger sample size, haplotype analysis and investigation of other genes contributing to the enzymatic pathway of methotrexate could guide clinicians in tackling variations in clinical toxicity and efficacy in rheumatoid arthritis.

CONCLUSION

The study concludes that

- The frequency distribution of genetic polymorphism of MTHFR A1298C gene was different among control and RA patients. The 1298CC genotype was higher in RA patients compared to control group.
- The 1298AA genotype shows better efficacy and lower incidence of toxicity to MTX therapy in the RA patients.
- The 1298CC genotype shows elevation in transaminases and GI adverse events in spite of folate supplementation to MTX therapy in the RA patients.
- The RA patients with 1298AA genotype found were to be good responders and 1298CC genotype patients show poor response to MTX therapy in RA.

REFERENCES

1. Chan ES, Cronstein BN. Mechanisms of action of methotrexate. Bull Hosp Jt Dis (2013). 2013;71 Suppl 1:S5-8.

2. Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M, Kremer J. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004;50(9):2766-74.

3. Tian H, Cronstein BN. Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. Bull NYU Hosp Jt Dis. 2007;65(3):168-73.

4. Pawlik A, Wrzesniewska J, Fiedorowicz-Fabrycy I, Gawronska-Szklarz B. The MDR1 3435 polymorphism in patients with rheumatoid arthritis. Int J Clin Pharmacol Ther. 2004;42(9):496-503.

5. Kurzawski M, Pawlik A, Górnik W, Droździk M. Frequency of common MDR1 gene variants in a Polish population. Pharmacol Rep. 2006;58(1):35-40.

6. Drozdzik M, Rudas T, Pawlik A, Kurzawski M, Czerny B, Gornik W, Herczynska M. The effect of 3435C>T MDR1 gene polymorphism on rheumatoid arthritis treatment with disease-modifying antirheumatic drugs. Eur J Clin Pharmacol. 2006;62(11):933-7.

7. Kato T, Hamada A, Mori S, Saito H. Genetic polymorphisms in metabolic and cellular transport pathway of methotrexate impact clinical outcome of methotrexate monotherapy in Japanese patients with rheumatoid arthritis. Drug Metab Pharmacokinet. 2012;27(2):192-9.

8. Sharma S, Das M, Kumar A, Marwaha V, Shankar S, Aneja R, Grover R, Arya V, Dhir V, Gupta R, Kumar U, Juyal RC, B K T. Interaction of genes from

influx-metabolism-efflux pathway and their influence on methotrexate efficacy in rheumatoid arthritis patients among Indians. Pharmacogenet Genomics. 2008;18(12):1041-9.

9. Baslund B, Gregers J, Nielsen CH. Reduced folate carrier polymorphism determines methotrexate uptake by B cells and CD4+ T cells. Rheumatology (Oxford). 2008;47(4):451-3.

10. Drozdzik M, Rudas T, Pawlik A, Gornik W, Kurzawski M, Herczynska M. Reduced folate carrier-1 80G>A polymorphism affects methotrexate treatment outcome in rheumatoid arthritis. Pharmacogenomics J. 2007 Dec;7(6):404-7.

11. Hayashi H, Tazoe Y, Tsuboi S, Horino M, Morishita M, Arai T, Ohshima M, Matsuyama T, Kosuge K, Yamada H, Tsuji D, Inoue K, Itoh K. A single nucleotide polymorphism of reduced folate carrier 1 predicts methotrexate efficacy in Japanese patients with rheumatoid arthritis. Drug Metab Pharmacokinet. 2013;28(2):164-8.

12. Dervieux T, Kremer J, Lein DO, Capps R, Barham R, Meyer G, Smith K, Caldwell J, Furst DE. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamylhydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics. 2004;14(11):733-9.

13. Chave KJ, Ryan TJ, Chmura SE, Galivan J.Identification of single nucleotide polymorphisms in the human gamma-glutamyl hydrolase gene and characterization of promoter polymorphisms. Gene. 2003;319:167-75.

14. Hayashi H, Fujimaki C, Daimon T, Tsuboi S, Matsuyama T, Itoh K. Genetic polymorphisms in folate pathway enzymes as a possible marker for predicting the outcome of methotrexate therapy in Japanese patients with rheumatoid arthritis. J Clin Pharm Ther. 2009;34(3):355-61.

15. Yanagimachi M, Naruto T, Hara T, Kikuchi M, Hara R, Miyamae T, Imagawa T, Mori M, Kaneko T, Morita S, Goto H, Yokota S. Influence of polymorphisms within the methotrexate pathway genes on the toxicity and efficacy of methotrexate in patients with juvenile idiopathic arthritis. Br J Clin Pharmacol. 2011;71(2):237-43.

16. Oppeneer SJ, Ross JA, Koh WP, Yuan JM, Robien K. Genetic variation in folylpolyglutamate synthase and gamma-glutamyl hydrolase and plasma homocysteine levels in the Singapore Chinese Health Study. Mol Genet Metab. 2012;105(1):73-8.

17. Sharma S, Das M, Kumar A, Marwaha V, Shankar S, Singh P, Raghu P, Aneja R, Grover R, Arya V, Dhir V, Gupta R, Kumar U, Juyal RC, K TB. Purine biosynthetic pathway genes and methotrexate response in rheumatoid arthritis patients among north Indians. Pharmacogenet Genomics. 2009;19(10):823-8.

18. Owen SA, Hider SL, Martin P, Bruce IN, Barton A, Thomson W. Genetic polymorphisms in key methotrexate pathway genes are associated with response to treatment in rheumatoid arthritis patients. Pharmacogenomics J. 2013;13(3):227-34.

19. Fisher MC, Cronstein BN. Metaanalysis of methylenetetrahydrofolate reductase (MTHFR) polymorphisms affecting methotrexate toxicity. J Rheumatol. 2009;36(3):539-45.

20. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 1995;10(1):111-3.

21. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab. 1998;64(3):169-72.

22. van Ede AE, Laan RF, Blom HJ, Huizinga TW, Haagsma CJ, Giesendorf BA, de Boo TM, van de Putte LB.The C677T mutation in the methylenetetrahydrofolate reductase gene: a genetic risk factor for methotrexate-related elevation of liver enzymes in rheumatoid arthritis patients. Arthritis Rheum. 2001;44(11):2525-30.

23. Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y, Akama H, Kitamura Y, Kamatani N. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics. 2002;12(3):183-90.

24. Taniguchi A, Urano W, Tanaka E, Furihata S, Kamitsuji S, Inoue E, Yamanaka M, Yamanaka H, Kamatani N. Validation of the associations between single nucleotide polymorphisms or haplotypes and responses to disease-modifying antirheumatic drugs in patients with rheumatoid arthritis: a proposal for prospective pharmacogenomic study in clinical practice. Pharmacogenet Genomics. 2007;17(6):383-90.

25. Urano W, Furuya T, Inoue E, Taniguchi A, Urano T, Kotake S, Sekita C, Inoue S, Hara M, Momohara S, Kamatani N, Yamanaka H. Associations between methotrexate treatment and methylenetetrahydrofolate reductase gene polymorphisms with incident fractures in Japanese female rheumatoid arthritis patients. J Bone Miner Metab. 2009;27(5):574-83.

26. Haagsma CJ, Blom HJ, van Riel PL, van't Hof MA, Giesendorf BA, van Oppenraaij-Emmerzaal D, van de Putte LB. Influence of sulphasalazine, methotrexate, and the combination of both on plasma homocysteine concentrations in patients with rheumatoid arthritis. Ann Rheum Dis. 1999;58(2):79-84.

27. van Ede AE, Laan RF, Blom HJ, Boers GH, Haagsma CJ, Thomas CM, De Boo TM, van de Putte LB. Homocysteine and folate status in methotrexate-treated patients with rheumatoid arthritis. Rheumatology (Oxford). 2002;41(6):658-65.

28. Hider SL, Bruce IN, Thomson W.The pharmacogenetics of methotrexate. Rheumatology (Oxford). 2007;46(10):1520-4.

29. Berkun Y, Levartovsky D, Rubinow A, Orbach H, Aamar S, Grenader T, Abou Atta I, Mevorach D, Friedman G, Ben-Yehuda A. Methotrexate related adverse effects in patients with rheumatoid arthritis are associated with the A1298C polymorphism of the MTHFR gene. Ann Rheum Dis. 2004;63(10):1227-31.

30. Hughes LB, Beasley TM, Patel H, Tiwari HK, Morgan SL, Baggott JE, Saag KG, McNicholl J, Moreland LW, Alarcón GS, Bridges SL Jr. Racial or ethnic differences in allele frequencies of single-nucleotide polymorphisms in the methylenetetrahydrofolate reductase gene and their influence on response to methotrexate in rheumatoid arthritis. Ann Rheum Dis. 2006;65(9):1213-8.

31. Wessels JA, de Vries-Bouwstra JK, Heijmans BT, Slagboom PE, Goekoop-Ruiterman YP, Allaart CF, Kerstens PJ, van Zeben D, Breedveld FC, Dijkmans BA, Huizinga TW, Guchelaar HJ. Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis Rheum. 2006;54(4):1087-95.

32. Aggarwal P, Naik S, Mishra KP, Aggarwal A, Misra R. Correlation between methotrexate efficacy & toxicity with C677T polymorphism of the methylenetetrahydrofolate gene in rheumatoid arthritis patients on folate supplementation. Indian J Med Res. 2006;124(5):521-6.

33. Choe JY, Lee H, Jung HY, Park SH, Bae SC, Kim SK. Methylenetetrahydrofolate reductase polymorphisms, C677T and A1298C, are associated with methotrexate-related toxicities in Korean patients with rheumatoid arthritis. Rheumatol Int. 2012;32(6):1837-42.

34. Brambila-Tapia AJ, Durán-González J, Sandoval-Ramírez L, Mena JP, Salazar-Páramo M, Gámez-Nava JI, González-López L, Lazalde-Medina B B, Dávalos NO, Peralta-Leal V, Vázquez del Mercado M, Beltrán-Miranda CP, Dávalos IP. MTHFR C677T, MTHFR A1298C, and OPG A163G polymorphisms in Mexican patients with rheumatoid arthritis and osteoporosis. Dis Markers. 2012;32(2):109-14.

35. Bohanec Grabar P, Logar D, Lestan B, Dolzan V. Genetic determinants of methotrexate toxicity in rheumatoid arthritis patients: a study of polymorphisms affecting methotrexate transport and folate metabolism. Eur J Clin Pharmacol. 2008;64(11):1057-68.

36. Davis LA, Polk B, Mann A, Wolff RK, Kerr GS, Reimold AM, Cannon GW, Mikuls TR, Caplan L. Folic acid pathway single nucleotide polymorphisms associated with methotrexate significant adverse events in United States veterans with rheumatoid arthritis. Clin Exp Rheumatol. 2014 Jan 20. [Epub ahead of print]

37. Kumagai K, Hiyama K, Oyama T, Maeda H, Kohno N. Polymorphisms in the thymidylate synthase and methylenetetrahydrofolate reductase genes and sensitivity to the low-dose methotrexate therapy in patients with rheumatoid arthritis. Int J Mol Med. 2003;11(5):593-600.

38. Mandola MV, Stoehlmacher J, Zhang W, Groshen S, Yu MC, Iqbal S, Lenz HJ, Ladner RD. A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. Pharmacogenetics. 2004;14(5):319-27.

39. Stamp LK, Hazlett J, Roberts RL, Frampton C, Highton J, Hessian PA. Adenosine receptor expression in rheumatoid synovium: a basis for methotrexate action. Arthritis Res Ther. 2012;14(3):R138.

40. Hider SL, Thomson W, Mack LF, Armstrong DJ, Shadforth M, Bruce IN. Polymorphisms within the adenosine receptor 2a gene are associated with

adverse events in RA patients treated with MTX. Rheumatology (Oxford). 2008;47(8):1156-9.

41. Wessels JA, Kooloos WM, De Jonge R, De Vries-Bouwstra JK, Allaart CF, Linssen A, Collee G, De Sonnaville P, Lindemans J, Huizinga TW, Guchelaar HJ. Relationship between genetic variants in the adenosine pathway and outcome of methotrexate treatment in patients with recent-onset rheumatoid arthritis. Arthritis Rheum. 2006;54(9):2830-9.

42. Lee YC, Cui J, Costenbader KH, Shadick NA, Weinblatt ME, Karlson EW. Investigation of candidate polymorphisms and disease activity in rheumatoid arthritis patients on methotrexate. Rheumatology (Oxford). 2009;48(6):613-7.

43. Milic V, Jekic B, Lukovic L, Bunjevacki V, Milasin J, Novakovic I, Damnjanovic T, Popovic B, Maksimovic N, Damjanov N, Radunovic G, Pejnovic N, Krajinovic M. Association of dihydrofolate reductase (DHFR) -317AA genotype with poor response to methotrexate in patients with rheumatoid arthritis. Clin Exp Rheumatol. 2012;30(2):178-83.

44. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006;54(10):3095-103.

45. Weisman MH, Furst DE, Park GS, Kremer JM, Smith KM, Wallace DJ, Caldwell JR, Dervieux T.Risk genotypes in folate-dependent enzymes and their association with methotrexate-related side effects in rheumatoid arthritis. Arthritis Rheum. 2006;54(2):607-12.

46. Padmalatha S Rai, Murali TS, Vasudevan TG, Sharma K Prasada, Ashok Kumar Bhagavath, Pranita Pai, Gopinath PM, Satyamoorthy K. Genetic variations in genes involved in folate and drug metabolism in a south Indian population. Ind J Hum Genetics 2011; 17(1): S48-53

47. Vandana SR, Upendra Y, Pradeep K. Prevalence of methylenetetrahydrofolate reductase C677T polymorphism in eastern Uttar Pradesh. Ind J Hum Genetics 2012; 18(1):43-6

48. Aggarwal P, Naik S, Mishra KP, Aggarwal A, Misra R. Correlation between methotrexate efficacy & toxicity with C677T polymorphism of the methylenetetrahydrofolate gene in rheumatoid arthritis patients on folate supplementation. Indian J Med Res. 2006;124(5):521-6.

49. Berkun Y, Levartovsky D, Rubinow A, Orbach H, Aamar S, Grenader T, Abou Atta I, Mevorach D, Friedman G, Ben-Yehuda A. Methotrexate related adverse effects in patients with rheumatoid arthritis are associated with the A1298C polymorphism of the MTHFR gene. Ann Rheum Dis. 2004;63(10):1227-31.

50. Bohanec Grabar P, Logar D, Lestan B, Dolzan V. Genetic determinants of methotrexate toxicity in rheumatoid arthritis patients: a study of polymorphisms affecting methotrexate transport and folate metabolism. Eur J Clin Pharmacol. 2008;64(11):1057-68.

51. Choe JY, Lee H, Jung HY, Park SH, Bae SC, Kim SK. Methylenetetrahydrofolate reductase polymorphisms, C677T and A1298C, are associated with methotrexate-related toxicities in Korean patients with rheumatoid arthritis. Rheumatol Int. 2012;32(6):1837-42.

52. Davis LA, Polk B, Mann A, Wolff RK, Kerr GS, Reimold AM, Cannon GW, Mikuls TR, Caplan L. Folic acid pathway single nucleotide polymorphisms associated with methotrexate significant adverse events in United States veterans with rheumatoid arthritis. Clin Exp Rheumatol. 2014 Jan 20. [Epub ahead of print]

53. Hughes LB, Beasley TM, Patel H, Tiwari HK, Morgan SL, Baggott JE, Saag KG, McNicholl J, Moreland LW, Alarcón GS, Bridges SL Jr. Racial or ethnic differences in allele frequencies of single-nucleotide polymorphisms in the methylenetetrahydrofolate reductase gene and their influence on response to methotrexate in rheumatoid arthritis. Ann Rheum Dis. 2006;65(9):1213-8.

54. Kato T, Hamada A, Mori S, Saito H. Genetic polymorphisms in metabolic and cellular transport pathway of methotrexate impact clinical outcome of methotrexate monotherapy in Japanese patients with rheumatoid arthritis. Drug Metab Pharmacokinet. 2012;27(2):192-9.

55. Sachie I, Mayasukhi H, Kenji T, Shinichi K, and Mayumi M. Preliminary study to identify the predictive factors for the response to Methotrexate therapy in patients with rheumatoid arthritis. Yakugaku Zasshi. 2009; 129(7): 843-849.

56. Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y, Akama H, Kitamura Y, Kamatani N. Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics. 2002;12(3):183-90.

57. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab. 1998;64(3):169-72.

58. Wessels JA, de Vries-Bouwstra JK, Heijmans BT, Slagboom PE, Goekoop-Ruiterman YP, Allaart CF, Kerstens PJ, van Zeben D, Breedveld FC, Dijkmans BA, Huizinga TW, Guchelaar HJ. Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis Rheum. 2006;54(4):1087-95.

59. Yogita G, Aravind C, Amrutesh P, Kalpana J, Bhushan P. Profiling single nucleotide polymorphisms (SNPs) across intracellular folate metabolic pathway in healthy Indians. Indian J Med Res. 2011; 133: 274-279.

PROFORMA

Baseline visit (Visit 1)

Visit Date

RCC.No:

OP/IP No:

Demographic data

State: _____

District: _____

Name: _____

DOB/AGE: _____

Gender: | M | F |

Marital Status: _____

Weight: _____ Kg

Height: _____ cm _____

Employment/Income: _____

Smoker /Alcoholic

Family History of RA:

Previous history of Jaundice:

MEDICAL HISTORY

Duration of RA:

Concomitant illness: SHT/DM/CHD/Dyslipidimea/Obesity

Others

Concomitant treatment | Yes | No |

Details of concomitant treatment

MTX dose (mg/week) : _____

Folic acid dose : _____

Any other drug : CQS/SSZ/Prednisolone/NSAID

LABORATORY ASSESSMENTS

Clinical pathology

Hb _____ g/dl

TC _____ (cells/cu.mm)

DC N% L% M% E%

ESR: _____ mm/Hr

Platelets _____ (cells/cu.mm)

Peripheral smear _____

Urine Albumin _____

Sugar _____

Deposits _____

Biochemistry

RBS _____ (mg/dl)

Urea _____ (mg/dl)

Creatinine _____ (mg/dl)

T.B/D.B _____ (mg/dl)

Total protein _____ (g/dl)

Albumin _____ (g/dl)

SGOT _____ (U/L)

SGPT _____ (U/L)

SAP _____ (U/L)

Immunology

RF _____ (IU/ml)

CRP _____ (mg/L)

ADVERSE DRUG REACTION

GI disturbances (nausea, vomiting, stomatitis)

Abnormalities

Any others

CLINICAL EVALUATION

Morning stiffness

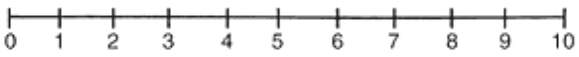
Duration of morning stiffness in minutes:_____

Grip strength:

Reading in mmHg

	1	2	3	Average
Right				
Left				

Pain Visual analogue scale



	No Pain	Slight	Moderate	Severe	Excruciating
Patient's assessment:					
Doctor's assessment:					

Genetic information (MTHFR A1298C)

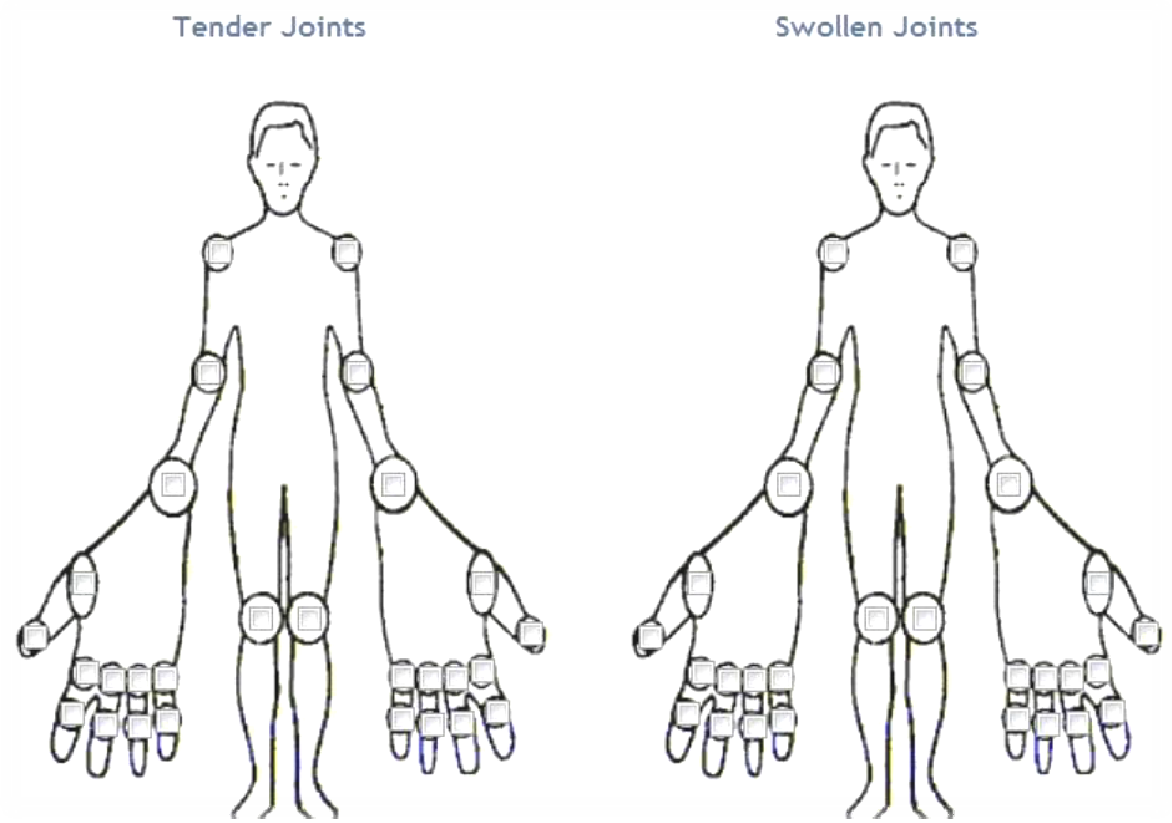
Allele : AA (wild-life)

CA (Heterozygous mutant)

CC (Homozygous mutant)

Comments:

Number of affected Joints:



Patient Global Health:

INFORMED CONSENT FORM

Title of the study: " A study on the clinical profile and pharmacogenetics of methotrexate treatment in patients with rheumatoid arthritis ".

Name of the Participant :
Name of the Principal investigator : J.Sivaraman
Name of the Institution : Madras Medical College,Chennai-03.

I _____ have read the information in the form (or it has been read to me). I was free to ask any questions and they have been answered. I am over 18 years of age and, exercising my free power of choice, hereby give my consent to be included as a participant in " A study on the clinical profile and pharmacogenetics of methotrexate treatment in patients with Rheumatoid arthritis "

1. I have read and understood this consent form and the information provided to me.
2. I have had the consent document explained to me.
3. I have been explained about the nature of the study.
4. I have been explained about my rights and responsibilities by the investigator.
5. I have been informed the investigator of all the treatments I am taking or have taken in the past 6 months including any native (alternative) treatment.
6. I have been advised about the risks associated with my participation in this study.
7. I agree to cooperate with the investigator and I will inform him/her immediately if I suffer unusual symptoms.
8. I have not participated in any research study within the past 6 month(s).
9. I have not donated blood within the past 6 months
10. I am aware of the fact that I can opt out of the study at any time without having to give any reason and this will not affect my future treatment in this hospital.
11. I am also aware that the investigator may terminate my participation in the study at any time, for any reason, without my consent.
12. I hereby give permission to the investigators to release the information obtained from me as a result of participation in this study to the sponsors, regulatory authorities, Govt. agencies, and IEC. I understand that they are publicly presented.
13. I have understood that my identity will be kept confidential if my data are publicly presented
14. I have had my questions answered to my satisfaction.
15. I have decided to be in the research study.

I am aware that if I have any question during this study, I should contact the investigator. By signing this consent form I attest that the information given in this document has been clearly explained to me and understood by me, I will be given a copy of this consent document.

Name and signature / thumb impression of the participant (or legal representative if participant incompetent)

Name _____ Signature_____ Date_____

Name and Signature of impartial witness (required for illiterate patients):

Name _____ Signature_____ Date_____

Address and contact number of the impartial witness:

Name and Signature of the investigator or his representative obtaining consent:

Name _____ Signature_____ Date_____

.

Information to Participants

Principle Investigator : J.Sivaraman
Name of Participant :

Title: "A STUDY ON THE CLINICAL PROFILE AND PHARMACOGENETICS OF METHOTREXATE TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS"

You are invited to take part in this study. The information in this document is meant to help you decide whether or not to take part. Please feel free to ask if you have any queries or concerns.

You are being asked to participate in this study being conducted in Madras Medical College and Government General Hospital.

This study will be done on the patients with RHEUMATOID ARTHRITIS who are on a drug called Methotrexate therapy alone. If you are eligible to participate in this study, you will be enrolled. We will obtain some of your medical/treatment data from your records. You will have to provide 5ml Blood sample for pharmacogenetic testing

We have obtained approval from the Institutional Ethics Committee, Madras Medical College.

Pharmacogenetics means the study of genetic variability in drug response. So analysis of genes responsible for efficacy and toxicity can be analyzed in every individual who are participating in this study. This study data may gives you the optimized treatment.

Improvement of your condition will be assessed by DAS and HAQ scoring and also by examining your blood sample for C-Reactive protein and Erythrocyte sedimentation rate. For this you may be required to provide 5ml of blood at 6 month interval for 1 year.

If you notice any adverse effects to the treatment drug (MTX), you may report to us and it will be recorded. 5ml of blood will be collected at 3 months interval to assess the toxic effects of MTX on liver functions. So the total amount of blood will be collected through out of the study will be 30ml.

This study involves only analysis of blood and collection of data. There will be no risk involved. You will not be charged for the analysis of blood samples. Pharmacogenetic analysis may provide benefits to the society in terms of advancements of medical knowledge and/or therapeutic benefits for management of RHEUMATOID ARTHRITIS

You have the right to confidentiality regarding the privacy of your medical information (personal details, results of physical examinations, investigations, and your medical history). By signing this document, you will be allowing the research team investigators, other study personnel, sponsors, IEC and any person or agency required by law like the Drug Controller General of India to view your data, if required.

The information from this study, if published in scientific journals or presented at scientific meetings, will not reveal your identity.

Your decisions to not participate in this research study will not affect your medical care or your relationship with investigator or the institution. Your doctor will still take care of you and you will not loose any benefits to which you are entitled.

The participation in this research is purely voluntary and you have the right to withdrawal from this study at any time during course of the study without giving any reasons.

.

Name of the participant:	Name of the Investigator:
Signature:	Signature:
Date:	Date:

LIST OF ABBREVIATIONS USED

ABCB1	ATP-binding cassette proteins
ADA	Adenosine deaminase
ADORA	Adenosine receptors
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole- 4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPD	Adenosine monophosphate deaminase
ATIC	5-aminoimidazole- 4-carboxamide ribonucleotide transformylase
ATP	Adenosine triphosphate
bp	Base pair
CAD	Coronary artery diseases
CQS	Chloroquine
CRP	C-Reactive protein
dATP	Deoxy adenine triphosphate
DC	Differential count
dCTP	Deoxy cytosine triphosphate
dGTP	Deoxy guanine triphosphate
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DM	Diabetes mellitus
DNA	Deoxy-ribo Nucleic Acid

dNTp	Deoxy nucleotide tri phosphate
dTMP	Deoxy thymidine monophosphate
dUMP	Deoxy uridine monophosphate
EDTA	Ethylene Diamine Tetra acetic acid
ESR	Erythrocytes sedimentation rate
FAICAR	10, formyl AICAR
FP	Forward primer
FPGS	Folylpolyglutamate synthase
GGH	Gamma-glutamyl hydrolase
H	Healthy individuals
HB	Haemoglobin
IEC	Institutional ethics committee
IMP	Inosine monophosphate
ITP	Inosine triphosphate
ITPA	Inosine triphosphate pyrophosphatase
IU	International Units
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
MTRR	Methionine synthase reductase
MTX	Methotrexate
MTX-PGs	Polyglutamated MTX
NSAID	Non-steroidal anti-inflammatory drugs
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis

RBC	Red Blood Cells
RBS	Random blood sugar
RF	Rheumatoid factor
RFC	Reduced folate carrier
RFLP	Restriction fragment length polymorphism
RNA	Ribo Nucleic Acid
RP	Reverse primer
RPM	Revolutions per minute
SDS	Sodium dodecyl sulphate
SGOT	Serum glutamate oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SHMT	Serine hydroxyl methyl transferase
SJC	Swollen joint count
SNP	Single nucleotide polymorphisms
SSZ	Sulfasalazine
TC	Total count
THF	Tetrahydrofolate
TJC	Tender Joint count
TYMS or TSER	Thymidylate synthase
WBC	White Blood Cells

LIST OF CLINICAL PARAMETERS USED

DISEASE CHARACTERISTICS

- **Morning stiffness (in Minutes)**
  - Expressed in minutes.
- **Pain scale(0-10)**
  - 0 indicates no pain and the value 10 indicates Extreme pain.
- **TJC (0-28)**
  - Tender joint count ranges from 0 to 28.
- **SJC (0-20)**
  - Swollen joint count ranges from 0 to 28
- **DAS (calculated by using three variables TJC, SJC and ESR)**
  - DAS score can be calculated by using the following formula

    DAS = 0.56* sqrt (TJC28) + 0.28* sqrt (SJC28) + 0.70* In(ESR)
- **HAQ(0-3)**
  - Health Assessment Questionnaire values are between 0-3. The value 0 indicates activity of daily living without any difficulty and the value 3 indicates extreme difficulty in daily activities

DRUG CHARACTERISTICS

- **Dose of MTX**
  - Dose of methotrexate expressed as mg/week.

BIOCHEMICAL CHARACTERISTICS

- **ESR**
  - Expressed as mm/Hr. (Male: 0- 15mm/Hr., Female: 0-20mm/Hr.)
- **SGOT**
  - Expressed as U/L (Normal value 5 to 40 U/ L)
- **SGPT**
  - Expressed as U/L (Normal value 7 to 56 U/L)

GI SYMPTOMS

This includes Nausea and Vomiting.