Chapter 3

MATERIALS AND METHODS
3.1 Study Population:

3.1.1 For CYP2C19 and CYP2C9 genotyping study:
One hundred and thirty nine unrelated healthy subjects residing in Maharashtra (Pune) were recruited for the CYP2C19 and CYP2C9 genotyping study. There were 69 males and 70 females with mean age 23.22 ± 5.3 years.

3.1.2 Genetic basis for concept of Prakriti study:
Initially 489 subjects were screened with the purpose of determining the “Prakriti” status. Out of them 167 subjects having predominant Prakriti were identified. Subsequently from these 167 subjects a total of 132 unrelated ethnically matched healthy subjects (62 males and 70 females) with predominance of either V (26 subjects), P (43) and K (63) were recruited for the genotyping study.

3.1.3 Pharmacogenetics of MTX in RA:
The study was conducted at Centre for Rheumatic Diseases (CRD), Pune. Patients attending Out Patient Department (OPD) Clinic at CRD, Pune were screened to determine the eligibility criteria for inclusion into the study (inclusion/exclusion criteria with other details for efficacy and toxicity assessment given in methods section). RA patient population recruited for pilot study and main study are as follows:

- **Pilot study:**
  36 naive patients completing at least 6 months of supervised MTX, willing and consenting, were selected for the period of March 2006 to June 2006 (16 weeks). Retrospective analysis of the clinical and biochemical data of patients was carried out (from CRD database). Patient recall was used in several instances for the purpose of cross-checking the data.

- **Main Study:**
  A total of 403 RA patients on supervised MTX were screened (according to clinic attendance) from May 2007 to January 2008 (36 weeks). Out of these 403 RA patients; 336 patients having regular follow up and completing at least 1 year of supervised MTX, willing and consenting, were selected for the present study and blood samples were collected from these patients. From these 336 patients; 14 patients having insufficient baseline clinical data were excluded from the study. Thus the study cohort consists of 322 RA patients in which MTX related toxicity analysis was done. From these 322 patients; 227 patients were retrospectively analyzed for their clinical and laboratory data for MTX related efficacy analysis and 94 patients were selected for pharmacokinetic analysis.
Pharmacokinetics analysis of MTX, 70H MTX (metabolite) at 0hr, 2hr, 8 hr and Homocystiene (Hcy) estimation at 0 hr was carried out in these RA patients. Systematic representation of study design is given in the form of flow chart in Figure 4. One hundred and forty four unrelated healthy controls (HC) were also included.

Figure 4: Study design for Pharmacogenetics of MTX response in RA

403 patients screened
336 patients with DNA testing
Study cohort n=322

For toxicity analysis n=322
Pharmacokinetics analysis n=84*

ACR 20 at 3 month
ACR 20 at 6 month
Responder: Patients achieving ACR 50 response at 12 months
ACR 50 at 12 month
Non-responder: ACR response < 80 at 12 months

*Pharmacokinetics analysis of MTX and its metabolite at 0hr, 2hr and 8 hr
* Patient enrolled in a drug trial

3.1.4 Ethical consideration:

The study protocols for all the above studies were approved by the Institutional Ethics Committee and informed consent was obtained from all subjects participating in the study. All invasive procedures were done after obtaining informed consent (Annexure I).
3.2 Materials:

3.2.1 Collection of blood samples:
- 6 ml sterile vacutainer with 10.8 mg Ethylenediamine tetraacetic acid (EDTA) as anti-coagulant (BD VACUTAINER plus).
- Disposable 5 ml syringe with 22 X ½ gauge needle. (Dispovan®)
- Sterile cotton
- Spirit
- Tourniquet
- Sodium hypochloride. (MERCK®) for disposing syringes, cotton.

3.2.2 DNA extraction from whole blood samples:
- Red cell lysis buffer: 1M NH₄Cl solution. (ExcelaR®), 1M NaHCO₃ (Sisco research laboratories)
- Nuclear lysis buffer: 0.5M EDTA solution, 5M NaCl solution. (Sisco research laboratories), 2M Tris (pH – 8)
- 10% SDS: 10g SDS in 100ml distilled water. (Sigma-Aldrich)
- Proteinase K: Stock concentration – 10mg/ml (Bangalore Genei). Working concentration – 100µg/ml
- 4M Ammonium acetate. (Sisco research laboratories)
- Chloroform. (MERCK), Isopropanol. (MERCK), Sodium hypochloride. (MERCK), 70% ethanol. (MERCK)
- Tris-EDTA [T₁₀E₁ (pH 8)]

3.2.3 Amplification of DNA isolated from whole blood samples:
- 10X Taq buffer A (Bangalore Genei) – 10mM Tris (pH 9), 500mM, 15mM MgCl₂, 0.1% gelatin.
- deoxy Nucleotide Tri-Phosphate (dNTPs): Stock concentration – 10mM (Bangalore Genei), Working concentration – 1mM; 2mM
- Taq Polymerase. (Bangalore Genei)
- Primers: Sense and Antisense primers. (Integrated DNA Technology and Life Technology Pvt. Ltd. (Table 3)

3.2.4 Restriction Digestion of amplified product:
- Restriction endonucleases: Smal, BamH1 BamH1, AvaI, NsiI, Kpnl, Hhal, Sau3AI, HaeIII, HinfI, MbolI, DraI, Bsil, HaeIII, NspI (New England Biolabs)
- 10X buffer for restriction digestion
Materials and Methods


Table 3: Primer Sequences used for amplification of PCR fragments in different genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Fragment length (bp)</th>
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<tr>
<td></td>
<td></td>
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<tr>
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<td>R</td>
<td>5' AAA GGG TAA AAT CCA CTG TAA CCG C 3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F – Forward primer; R – reverse primer

3.2.5 Agarose Gel Electrophoresis:

- Agarose powder (Sigma)
- Tris borate EDTA buffer (TBE 5 X): Stock concentration – 0.9M Tris (Sigma), 0.9 M boric acid (Merck), 0.02M EDTA (Sigma), Working concentration – 0.5X
- Gel loading buffer (6X): 0.25% bromophenol blue, 40% (w/v) sucrose in sucrose in water, stored at 4 °C.
- Low molecular weight marker. 100 bp marker (New England Biolabs)
3.2.6 Staining gels:
10 mg/ml ethidium bromide (Sigma). Stored in dark at 4°C.

3.2.7 Real time TaqMan allelic discrimination assay:
- Applied Biosystems 7500 real time PCR system
- SNP assays for ATIC C347G and SHMT1 C1420T
- TaqMan universal master mix
- 96 well plate
- DEPC water

3.3 Pharmacokinetic study:
3.3.1. Reagents:
High Performance Liquid Chromatography (HPLC) grade Methanol and Acetonitrile, Hydrogen peroxide, Heptane sulphonic acid, di-ammonium hydrogen phosphate \((\text{tNH}_{4})_{2}\text{HPO}_{4}\), Tri-Chloro acetic acid (TCA), Chloroform, Potassium di-hydrogen phosphate (KH_{2}PO_{4}), Perchloric acid (PCA) was procured from Merck, India. Tris (2-carboxyethyl) phosphine (TCEP) and 4-fluoro-7-sulfamoyl benzofurazan (ABD-F) was ordered from Sigma-Aldrich, USA.

3.3.2. HPLC standards:
Methotrexate, 7 hydroxy methotrexate (7-OH MTX) and Homocysteine DL were procured from IPCA India, Molcan Corporation Canada and Sigma-Aldrich USA. 0.22 microns filters (Millipore, India) were used to filter the plasma sample before HPLC injection.

3.3.3. Chromatographic system:
Dionex, Germany consisted of P-680 quaternary gradient pump, an ASI 100 autosampler, a universal chromatographic interface UC1-50 and HPLC diode array detector 340U and RF2000 fluorescence detector integrated by Chromeleon Software 6.70

3.3.4. Column:
PpH stable reverse phase C18 column (4.6 ×250mm, Varian, Varian Inc) was used for Hcy determination while Hypersil reverse phase C18 column (4.6 ×250mm, KYA-Tech, Japan) was used for MTX and 7-OH MTX estimation.
3.4 Methods:

3.4.1 Prakriti evaluation:

*Prakriti* of each subject was assessed using a validated questionnaire as per Ayurveda that includes information pertaining to physical, physiological, and psychological characteristics of the individual further supported by clinical judgment (Annexure IV). Physique, skin texture, hunger, thirst, digestive capacity, temperament, memory are some of the attributes evaluated to determine individual constitution (Table 2). The questionnaire also considered information regarding ethnicity, maternal and paternal family history of diseases, past history related to diseases, allergies and dietary habits. Predominant *Prakriti* was allotted if ≥70% dominance of a single *dosha* score was obtained. Only individuals with predominance of either of V, P or K were included in the study. Each subject was also assessed clinically by Ayurvedic physician (*Vaidya*) who independently classified all subjects into V, P or K groups. Finally, subjects were recruited in this study only when over 80% concordance observed in *Prakriti* assessment with questionnaire scores and clinical evaluation by *Vaidya*. In case of borderline cases, opinion of senior *Vaidya* was taken additionally whose decision was considered as final.

3.4.2 Pharmacogenetics of MTX in RA:

**Study design and Patient selection criteria:**

This was cross sectional study conducted at CRD, Pune. To be eligible, the patients (ages ≥ 18 years) had to meet the American College of Rheumatology (ACR) revised classification criteria for RA and had to have received MTX for at least 1 month. Thus the inclusion and exclusion criteria for the present study were as follows:

(A) **Inclusion criteria:**

1. Patients of either sex and in age group 18 – 65 years of age with onset of disease after 16 yrs of age.

2. Patients meeting the ACR criteria (Annexure II) for RA with active disease. Active disease is defined as presence of following two criteria
   - 6 or more painful on motion or tender joint
   - 3 or more swollen joints

And one of the following
   - Morning stiffness for ≥ 45 min
   - ESR ≥ 30 min by Westergren

2. Patients with diagnosed RA of at least 1-year duration.
3. Patients in ACR functional class I, II or III (Annexure III)
4. Willing to come for regular follow-up visits.

(B) Exclusion criteria:
1. Patients suffering from severe RA i.e. ACR functional class IV RA
2. Pregnant and lactating women.
3. Any prior use of anti TNF-α biologics for consecutive 10 weeks prior to the screening visit.
4. Patients with allergy to the compounds similar to chemical class as that of MTX, folic acid.
5. Any history or evidence from physical examination of immunodeficiency syndromes/infection with HIV, TB, hepatitis B, hepatitis C, history of cancer within 5 yrs, CNS-demyelinating events suggestive of Multiple Sclerosis, Systemic Lupus Erythematosus (SLE)
6. Patients with
   - Hepatic Insufficiency: Total Bilirubin > 1.5×upper limit of normal values (ULN) or AST/ALT > 3×ULN
   - Renal insufficiency: Serum Creatinine > 1.5×ULN
   - WBCs < 4000/mm³
   - Thrombocytopenia < 1, 00, 000/ mm³
7. Any major surgery, including joint surgery within 3 months before Informed consent date.

3.4.3 Clinical management in RA:
All patients included in this analysis began treatment with a regimen of oral MTX at a dosage of 7.5 mg weekly, with the dosage increasing to maximum of 20 mg weekly. In the event of insufficient clinical response at each follow up visit, the MTX dosage was increased stepwise, with increase of 2.5 mg every 4 weeks to 20 mg weekly. In case of adverse reactions, MTX was continued at the lowest tolerated dosage. In some patients showing adverse reactions dose was stopped and restarted with lowest tolerable dose. In case of intolerance, MTX could also be given parenteral. If MTX was not tolerated at all, the patient was treated according to the next treatment step. Thus, the dose of MTX and any adjustment in the dose was determined by the rheumatologist based on efficacy and toxicity considerations.
Concomitant medications included NSAIDs, corticosteroids and additional DMARDs. None of the patient received folic acid supplementation throughout the duration of MTX administration. Majority (> 80%) was believed to have consumed folic acid either as a supplement combined with oral iron or a prophylactic during a large duration of MTX administration. Patients were often received folic acid when MTX dose exceeded 12.5 mg/week. The dose of folic acid was mostly 1 mg/day along with iron supplementation in patients with moderately severe anemia (Hb < 9 gm%) beginning MTX. In patients receiving higher doses of MTX (>12.5 mg/week), folic acid was administered as a weekly single dose of 5 mg with the interval of 2-3 days from the day of MTX intake.

(A) Efficacy assessment:
All patients underwent a monthly evaluation. The efficacy of MTX during treatment period was assessed by change from the baseline in ACR core set measures: Tender Joint Count, Swollen Joint Count, Pain in joints Visual Analog Scale (VAS), Health assessment questionnaire (HAQ) score, Patient Global Assessment of Disease Activity, Physician Global Assessment of Disease Activity, Morning stiffness. ESR/CRP (C-Reactive Protein) [Normal range ESR(Westergren): Male < 20mm in 1st hr. Female <30mm in 1st hr; CRP (Nephelometry) <6 mg/L].

ACR core measures were defined as follows:

⇒ Tender joint count and score:
68 Joints was assessed on ordinal scale of 1-3 for tenderness/ pain on motion as follows

0 no pain,

1 positive pain response on question,

2 patient says it is tender and winces,

3 patient says it is tender and withdraws the limb.

Total score and count will be captured in Clinical Record Form (CRF) during each visit.

⇒ Swollen joint count and score:
All the joints excluding hip joints for swelling. Swelling will be evaluated and scored as follows:

0 No swelling

1 Swelling with visible joint contour

2 Swelling with visible joint contour obliterated

3 Swelling with distortion of joint contour
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Bony swelling, nodule formation and joint deformity will be excluded from consideration.

- **Pain in affected joints in preceding 24 hrs:**
Pain in affected joints during the preceding 24 hrs will be marked on a horizontal VAS anchored at 0mm (no pain) and 100 mm (most severe pain).

- **Duration of morning stiffness in min:**
Patients were asked about duration of morning stiffness. The question was asked in consideration of the last 2-3 days. The elapsed time between arising in the morning and maximum mobility without taking pill will be recorded.

- **Health assessment questionnaire:**
The modified CRD, Pune version of HAQ was used. It contains 23 questions under the standard 8 daily activities (hygiene, arising, eating, dressing, walking, reach, grip and house chores/occupation). Any difficulty (0=nil, 1=mild, 2=much, 3 unable) was marked and added to provide a total score (max 24). The highest component score in each category determines the score for the category unless aids or devices are required. A score of 1 was added to the HAQ activity for which the device is being used or assistance is taken from the other person. However, if already the score is 3 i.e. patient is unable to do particular activity but uses the device/take assistance from other person, in that case the score will remain 3 only which is maximum score and not 4. The maximum HAQ score was 24 and the range being 0-24. The HAQ disability index score was not be computed when patient provides answer in fewer than 6 activities.

- **Patient's General Health (GH):**
In this parameter, the patient indicates his/her assessment of the overall health (arthritis + other ailments) by placing a line (mark) on a VAS anchored to 0= perfect health to 100mm= worst possible.

- **Physician Global Assessment of Disease Activity (PHY G):**
In this parameter the investigator asked ‘How would you describe the patient’s disease activity today?’ Investigator takes into consideration Tender joint count, swollen joint count, grip strength, ESR, CRP. Global assessment of disease activity will be measured on a VAS anchored to 0= no activity to 100mm= worst possible health.
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**Patient Global Assessment of Disease Activity (PAT G):**

In this parameter, the patient indicates his/her assessment of the overall activity of his/her arthritis by placing a line (mark) on VAS anchored 0=No activity to 100 mm= extreme activity.

Based on above-mentioned parameters a patient will be given the classification of responder to ACR 50 if the following criteria are met:

> 50% improvement in tender joint count
> 50% improvement in swollen joint count
> 50% improvement in any 3 remaining ACR core set measures
  - Patient assessment of pain (VAS)
  - Patient Global Assessment of Disease Activity
  - Physician Global Assessment of Disease Activity
  - Patient’s self assessed disability (HAQ)
  - Acute phase reactant (1:SR or CRP)

ACR 50 response was considered as the primary efficacy response.

**(B) Toxicity assessments:**

Toxicity was assessed by monitoring for adverse events, physical examination and laboratory parameters for safety evaluation.

**Monitoring for Adverse events:**

Whenever the patient came for follow up during the treatment period, investigator asked open questions to patients regarding any new complaints those were not existing since last follow up and any such complaints were recorded and entered into the CRF. Pre-existing symptoms that increased in severity or frequency during the treatment period was also noted and entered on the CRF. Any one or combinations of the following events were noted as “overall toxicity”. MTX-related side effects were defined as Gastrointestinal which include, Upper GIT (nausea, vomiting, acidity, dyspepsia, anorexia, gastritis), Lower GIT (diarrhea, constipation, anal burning), Pain in abdomen (stomatitis, pancreatitis), Hepatic (Serum SGPT, SGOT > twice the baseline values), Bone marrow (leucopenia < 3,500/mm3, thrombocytopenia < 1,00,000 mm3, anemia, hemoglobin 20% drop from baseline values), Skin rash/aggravated nodules, Mucositis (oral ulcers, glossitis, chelitis, dry mouth), Hairloss, CNS (headache, drowsiness, fatigue, discomfort) and any other known MTX related AE documented in drug literature. Some patients had more than one type of side effect. Leukocyte counts, hemoglobin concentrations, and liver enzyme activities were measured on the day of each study visit, using standard laboratory methods.
The clinical action taken to address MTX toxicity was also recorded; this consisted of “dose maintained with patient under observation,” “dose reduced,” “route of administration changed,” “dose temporarily interrupted,” or “dose permanently discontinued.”

3.4.4 Collection of blood samples for DNA extraction:
5 ml of venous blood was drawn in 6ml sterile plastic vacutainer containing approximately 500ul of 2% EDTA as anti-coagulant. The syringe was discarded in sodium hypochlorite after blood collection.

- DNA extraction from whole blood samples:
DNA extraction from blood samples was carried out in a class II biosafety cabinet in the designed biosafety laboratory, strictly following universal biosafety precautions. All bio-hazardous waste was disposed in separately maintained beakers filled with hypochlorite, autoclaved at 15 lb pressure in an isolated biohazard autoclave and then incinerated. DNA extraction was carried out using modified Miller’s protocol\(^\text{234}\) as follows:

- Collected blood (5ml) was transferred to Oakridge tube.
- Red cell lysis: 40ml of Red Cell Lysis Buffer was added, thoroughly mixed and incubated at room temperature for 30 minutes and then centrifuged (Plastocrafts) at 3500rpm for 20 minutes. The supernatant was drained.
- WBC lysis and DNA isolation: Pellet was resuspended in 3 ml Nuclear Lysis Buffer. 200ul of 10% SDS and 40ul of Proteinase K (Working concentration – 10mg/ml) and mixed thoroughly to disintegrate the pellet. It was incubated at 42\(^\text{0}\) C water bath, overnight.
- 4ml of 4M ammonium acetate was added and mixed thoroughly followed by addition of 3ml of chloroform and slight mixing. It was centrifuged at 3500rpm for 30 minutes.
- Supernatant was transferred into a new Coulter cups (Laxbro).
- 7ml isopropanol was added for selective precipitation of DNA. The cups were swirled gently until the DNA precipitate appears to float as jelly or white mass.
- The floating precipitate was transferred into 1.5ml sterile microfuge tube containing 1 ml ice-cold 70% ethanol.
- The precipitate was washed twice with 70% ethanol to remove any salts trapped with DNA. The tube was drained carefully and the ethanol was evaporated out completely using DNA 120 Speed Vac. (Thermo Savant).
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- 500ul T<sub>10</sub>E<sub>1</sub> was added and the precipitate was dissolved by light finger tip vibration. DNA was kept in T<sub>10</sub>E<sub>1</sub> overnight at room temperature for dissolution.

- Extracted DNA were stored at -70°C until analysis.

- Qualitative detection of DNA:
The extracted human DNA was qualitatively detected on 0.7% agarose gel by using agarose gel electrophoresis technique.

- Quantitative detection of DNA:
The extracted DNA was quantified by taking absorbance at A<sub>260</sub> and it’s purity was checked by A<sub>260</sub> : A<sub>280</sub> ratio, using Nanodrop (JH Bio).

3.4.5 Amplification of DNA isolated from whole blood samples:
DNA amplifications were carried out using Polymerase Chain Reaction (PCR). PCR reactions were standardized for various parameters like dNTP concentration; primer concentration and MgCl<sub>2</sub> concentration. For an example; PCR mix and cycling parameters for amplification of CYP2C19*3 mutant are given in the table 4.

<table>
<thead>
<tr>
<th>Table 3: PCR mix and Cycling Parameters</th>
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<tr>
<td><strong>Amplification of CYP2C19*3</strong></td>
</tr>
<tr>
<td>PCR mix</td>
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<tr>
<td>DNA</td>
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<td>Cycling parameters</td>
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<td>Final extension</td>
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PCR mix was same for all genes except for T<sub>YMS</sub> 5 UTR where additional 5% DMSO and glycerol was added in PCR mix because of high GC content of these primers. Similarly annealing temperature varied according to %GC content of each primer for amplification of different genes.

- Restriction digestion of amplified product:
PCR amplified products were digested using specific restriction enzymes at specific digestion temperature for overnight. The product kept for restriction digestion was covered with the parafilm to prevent evaporation. Restriction digestion temperature and fragment length after restriction digestion for each polymorphism is given in Table 5.
Table 5 Restriction endonucleases, restriction digestion temperature and RFLP fragment sizes resolved in dependence of the wild, heterozygous or variant alleles for the studies polymorphisms

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Restriction enzyme</th>
<th>Digestion temperature</th>
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</tr>
<tr>
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<td>G80A</td>
<td>Hha I</td>
<td>37°C</td>
<td>125, 68, 37</td>
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<tr>
<td>MDR1</td>
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<td>Sau3AI</td>
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<tr>
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<td>HaeIII</td>
<td>37°C</td>
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</tr>
<tr>
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<td>C677T</td>
<td>HinfI</td>
<td>37°C</td>
<td>198</td>
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<td>A1298C</td>
<td>MboIII</td>
<td>37°C</td>
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<td>3'-UTR deletion</td>
<td>Dral</td>
<td>37°C</td>
<td>88, 70</td>
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<td>152, 88, 70</td>
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<tr>
<td>GGH</td>
<td>C401T</td>
<td>Bsu3I</td>
<td>55°C</td>
<td>109</td>
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<td></td>
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<tr>
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<td>A666G</td>
<td>NspI</td>
<td>37°C</td>
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<td></td>
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<td>118, 94, 24</td>
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</table>

- Agarose gel electrophoresis:

2% Agarose gel (2 g in 100ml) was prepared in 0.5X TBE. Ethidium bromide (0.5 μg/ml) was added in the gel itself. The amplified digested products were loaded on the long gel after mixing with gel loading buffer to a working concentration of 1X. 0.5X TBE was used as running buffer. The products were electrophoresed at a constant voltage of 45V at room temperature. Low molecular weight marker was resolved simultaneously.

- Visualization of DNA and Restriction Length Fragment Polymorphism (RFLP) fragments:

Ethidium bromide stained gels were visualized under ultraviolet light. The image was stored in a gel documentation system Biorad. Quantity One Quantitation Software (version 4) and analyzed to determine RFLP profiles.
3.4.6 Real time TaqMan allelic discrimination assay:
Real time TaqMan allelic discrimination method with fluorigenic 3' minor groove binding probes (Assay on demand; Applied Biosystems, Foster city CA) was used for genotyping \textit{ATIC C347G} and \textit{SHMT1 C1420T}.

- Allelic Discrimination (AD) Assays (Applied Biosystems AD user's guide):
An AD assay is a multiplexed (more than one primer/probe pair per reaction), end-point (data is collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the SNP site in a target template sequence. The actual quantity of target sequence is not determined. For each sample in an AD assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan\textsuperscript{®} MGB probes that target an SNP site. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2).

The AD assay classifies unknown samples as:
- Homozygotes (samples having only allele 1 or allele 2)
- Heterozygotes (samples having both allele 1 and allele 2)

The AD assay measures the change in fluorescence of the dyes associated with the probes.

\textbf{Figure 1}: illustrates results from matches and mismatches between target and probe sequences in Assays-on-Demand\textsuperscript{TM} SNP Genotyping Products (Livak \textit{et al.}, 1995).

\begin{table}
\centering
\begin{tabular}{|l|l|}
\hline
A substantial increase in... & Indicates... \\
\hline
VIC dye fluorescence only & Homozygosity for allele 1 \\
FAM\textsuperscript{TM} dye fluorescence only & Homozygosity for allele 2 \\
Both fluorescence signals & Heterozygosity allele 1-allele 2 \\
\hline
\end{tabular}
\end{table}
Materials and Methods

- **AD Experiment Work flow:**
  After we design the experiment and isolate DNA, an AD assay involves performing:
  - A **pre-read run** on an AD plate document to determine the baseline fluorescence associated with primers and probes before amplification.
  - An **amplification run** using an Absolute Quantitation (AQ) plate document to generate real-time PCR data, which can be used to analyze and troubleshoot the PCR data for the AD assay, if needed.
  - A **post-read run** using the original AD plate document, which automatically subtracts the baseline fluorescence determined during the pre-read run, then assigns allele calls (automatically or manually) using the amplified data.

**AD Experiment:**
The experiment uses multiplex PCR. Primers and probes are ordered from Assays on Demand (AB Assay ID C_16218146_10 for ATIC C347G and AB Assay ID C_3063127_10 for SHMT1 C1420T). Reactions are set up for PCR using the TaqMan® Universal PCR Master Mix and appropriate primers and probes.
- Take the extracted DNA. The final concentration of DNA for each sample should be between 50-100ng/L.
- Prepare the reaction mix. The final reaction volume in each well is 10 µL.
  
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL/reaction)</th>
<th>Volume (µL) for 96 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC water</td>
<td>3.5</td>
<td>336</td>
</tr>
<tr>
<td>2× TaqMan Universal PCR Master Mix</td>
<td>5</td>
<td>480</td>
</tr>
<tr>
<td>20× SNP Genotyping Assay Mix</td>
<td>0.5</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>864</td>
</tr>
</tbody>
</table>

Pipette 9 µL of reaction mix into well of a 96-well reaction plate. Make sure to keep 2 wells blank. In one well add reaction mix but no template DNA called as No Template Control (NTC) and in other well no reaction mix but add DEPC water or TE (Tris-EDTA) buffer as control. Add 1µL of sample DNA in remaining wells.
- Perform the pre-read run:
  - Select Start > Programs > Applied Biosystems 7300/7500 > ABI Prism 7300/7500 SDS Software
  - Create an AD plate document.
Materials and Methods

- Enter the sample names and specify tasks in the Well Inspector (View > Well Inspector).
- Perform the AD pre-read run.

Amplify DNA
- Create an AQ plate document for amplifying samples.
- Perform the amplification run.

Perform the post-read run:
- Click the green analysis button to start analysis.
- Assign calls to determine genotype for each sample (details given in result and discussion section).

3.4.7 Pharmacokinetics of methotrexate:
We hypothesized that SNP would affect the metabolism of methotrexate. To evaluate this we decided to study the pharmacokinetics of methotrexate. Patients on various on-going doses were selected. Blood was withdrawn at 0, 2 and 8 hrs considering the reported pharmacokinetics of methotrexate in blood.

Method:
Subjects were given weekly dose of MTX (ranging from 3.75 mg - 17.5 mg) after an overnight fast. 2-3 ml blood sample was collected at following time points:
- 1st interval: 0 hr before MTX administration for baseline values
- 2nd interval: at 2 hrs after MTX administration
- 3rd interval: at 8 hrs after MTX administration

Plasma samples & RBCs were separated within 15 min of collection & stored at -70°C until analyzed. Plasma MTX and its metabolite 7-OH MTX levels were determined using HPLC with post column photoxidation- fluorescence detection. Plasma Hey was estimated at 0 hr in all these patients by HPLC.

Solution preparation:
For MTX and 7 OH MTX:
Stock solution of MTX and 7-OH-MTX approximately 22 μmol L⁻¹ was prepared in 1N NaOH. From this stock further dilutions 0.01 to 1.5 μmol L⁻¹ for standard curve were made in the 0.4M (NH₄)₂HPO₄ pH 6.45. MTX and 7OH MTX stock solution was stored at -70°C.
Mobile phase buffer was prepared from 1M (NH₄)₂HPO₄ stock to 0.04M (NH₄)₂HPO₄ pH 6.45. To this buffer 200 mg of heptane sulphonic acid L⁻¹ was added.

For Hey:
27 mg Hey was dissolved in 1ml 0.1 M HCL to give stock concentration of approximately 199 µmol L⁻¹. From this stock further dilutions 1 to 50 µmol L⁻¹ for standard curve were made in the 0.1 M KH₂PO₄ buffer pH 5.9. Hey stock solution was stored at -70°C.

TCEP stock solutions (10mM) were prepared in 0.1M phosphate buffer pH 5.0. TCEP solution was further diluted to 1 mM for use in the plasma reduction reaction.

For ABD-F, ABD-F stock solutions (44 mM) were prepared in 0.2 M Borate buffer + 1N NH₃OH + H₂O. Sample was mixed well and kept away from light. The final concentration of ABD-F solution in the derivatization reaction was approximately 5mM.

Mobile phase buffer 0.1 M KH₂PO₄ was prepared from 1 M KH₂PO₄ pH 3.05.

Estimation of plasma MTX and 7-OH-MTX by HPLC:
MTX and 7-OH-MTX was analyzed by HPLC with post-column photoxidation-fluorescence detection.

HPLC method for MTX and 7OH MTX:
125µl plasma sample was aliquoted; to this 1ml of chloroform was added to remove the lipids from the plasma. Samples were centrifuged at 12,000rpm for 5 min at 4°C. Supernatant was separated and to this 50µl of 10% ice cold TCA was added to precipitate the proteins. After protein precipitation samples were centrifuged at 12,000 g for 15 min at 4°C. Supernatant was then filtered through 0.22 micron filters to remove any traces of remaining proteins. From this filtered sample 50 µl was injected into a Hypersil reverse phase C18 column (4.6 ×250mm, KYA-Tech. Japan) pre-equilibrated with mobile phase solution containing Acetonitrile-30% hydrogen peroxide- di-ammonium hydrogen phosphate buffer (0.04M) at pH 6.45 (7:3:90) at flow rate of 1.2 mL min⁻¹. The same chromatographic system as mentioned earlier was used in the study with additional apparatus called photoxidation assembly linked between PDA detector and RF2000 fluorescence detector.

MTX and 7 OH MTX were detected by use of postcolumn photoxidation with ultraviolet irradiation in the presence of hydrogen peroxide. A photochemical reactor unit (Septech India Pvt Ltd) equipped with a 254 nm low-pressure mercury ultraviolet lamp and
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containing 1 meter of 1/16-inch (outer diameter) Teflon tubing (0.25 mm internal diameter) assembled as a knitted coil was connected on-line after the analytical column but before the fluorometric detector. Standard curves were produced from stock solutions using concentrations ranging from 0.01 to 1.5 μmol L⁻¹. A flow rate of 1 mL min⁻¹ was used with a running time of 15 min. Retention times and peak areas were monitored at excitation and emission frequencies of 350 and 435 nm, respectively. MTX and 7 OH MTX concentrations were measured by extrapolation of the area values from each run on the calibration curves (Figure 6 - Chromatogram 1, 2 and 3).

Estimation of plasma Homocysteine by HPLC:
Hcy was analyzed by HPLC using a fluorescence detector²³⁷,²³⁸

HPLC method for Hcy:
Briefly, 100μl plasma sample containing hcy were reduced using 10μl of 1mM TCEP in a 10min reaction. This was followed by incubating, the reduced thiols with 15μl of 12mM ABD-F at 55°C for 30min. Then to this ABD-F derivatized sample 50μl of 0.2M buffer was added and proteins were precipitated using 8μl of 70% PCA. After protein precipitation samples were centrifuged at 12,000 g for 15 min at 4°C. Supernatant was then filtered through 0.22 micron filters to remove any traces of remaining proteins. From this filtered sample 50 μl was injected into a pH stable reverse phase C18 column (4.6 ×250mm. Varian, Varian Inc) protected by a guard column and pre-equilibrated with mobile phase solution containing methanol-potassium phosphate buffer (0.1 M) at pH 3.2 (30:70) at flow rate of 1.2 mL min⁻¹. The same chromatographic system as mentioned earlier was used in the study with RF2000 fluorescence detector. The pre-column was changed every 200 injections. Standard curves were produced from stock solutions of low molecular weight thiols using concentrations ranging from 1 to 50 μmol L⁻¹. A flow rate of 1.2 mL min⁻¹ was used with a running time of 6 min. Retention times and peak areas were monitored at excitation and emission frequencies of 380 and 510 nm, respectively. Hcy concentrations were measured by extrapolation of the area values from each run on the calibration curves.

Recovery experiment:
Plasma from healthy individual was separated. To this plasma MTX and 7-OH-MTX were added externally from the stock and then serially diluted to a concentration range of 0.01 to 1.5 μmol L⁻¹. Hcy is a metabolite present in human plasma. Therefore plasma used
Results and Discussions

0.01 to 1.5 \( \mu \text{mol L}^{-1} \). Hcy is a metabolite present in human plasma. Therefore plasma used for recovery experiment should be Hcy free. This was obtained from Dr Yajnik’s Diabetes Unit, KEM hospital Pune. To this plasma, Hcy was added from the stock and then serially diluted in the concentration range of 1 to 50 \( \mu \text{mol L}^{-1} \). These spiked plasma were then deproteinized as per above stated method and then injected in the HPLC system and a standard curve was plotted to estimate the recovery. The recovery observed for methotrexate and 7-OH-methotrexate is given in Table 7.

Table 7 shows the limit of detection (LOD) and limit of quantitation (LOQ) for MTX, 7-OH-MTX and Hcy.

<table>
<thead>
<tr>
<th></th>
<th>MTX</th>
<th>7 OH MTX</th>
<th>Hcy</th>
</tr>
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<tr>
<td>LOD</td>
<td>0.055 ( \mu \text{mol L}^{-1} )</td>
<td>0.011 ( \mu \text{mol L}^{-1} )</td>
<td>1 ( \mu \text{mol L}^{-1} )</td>
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<tr>
<td>LOQ</td>
<td>0.110 ( \mu \text{mol L}^{-1} )</td>
<td>0.021 ( \mu \text{mol L}^{-1} )</td>
<td>2.5 ( \mu \text{mol L}^{-1} )</td>
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Table 8: Intra and Inter day variation

<table>
<thead>
<tr>
<th>C nominal ng/ml</th>
<th>C estimated (ng/ml)</th>
<th>CV (%)</th>
<th>Error</th>
<th>C estimated (ng/ml)</th>
<th>CV (%)</th>
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<td>Intra-day</td>
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<td></td>
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<tr>
<td>100 ul of plasma</td>
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<td>48.35</td>
<td>2.1</td>
<td>0.05</td>
<td>49.2</td>
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<td>96.81</td>
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<td>500</td>
<td>505.5</td>
<td>1.2</td>
<td>0.98</td>
<td>489.1</td>
<td>1.5</td>
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<tr>
<td>Inter-day</td>
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Table 9: Recovery

<table>
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<th>Concentration (ng / ml)</th>
<th>Absolute recovery (%)</th>
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<tr>
<td></td>
<td>Methotrexate</td>
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<tr>
<td>10</td>
<td>79</td>
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<tr>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td>500</td>
<td>105</td>
</tr>
<tr>
<td>1000</td>
<td>106</td>
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3.4.8 Statistical analysis:

Statistical analysis details for each study are as follows:

1. **Genetic polymorphism of CYP2C19 and CYP2C9 in Maharashtrian population:**

Data were compiled according to the genotype and allele frequencies estimated from the observed numbers of each specific allele. The frequency of each allele/genotype in the study population is given together with 95% confidence interval. Differences in allele frequencies and PM genotype frequencies between Maharashtrians and population from various geographical regions were measured by Fisher exact test. Throughout the population comparisons significance is tested at 5% level. The observed genotype frequencies of CYP2C19 and CYP2C9 were compared with expected frequencies according to Hardy-Weinberg law.
2. Genetic basis for concept of Prakriti study:
Using available CYP2C19 genotype data, genotype frequencies were calculated and 2x2 contingency tables were constructed using one Prakriti group against the remaining two groups. Data related to CYP2C19 genotype and three Prakriti types was analyzed using Fisher’s exact probability test; because of presence of very small no of individuals in some of the Prakriti classes having particular genotype.

3. Pharmacogenetics of MTX in RA:

A.1 Pilot study:
Allelic frequencies and genotype distributions among groups (ACR 20 and AE) were compared by Chi-square (χ2) test.

A.2 Single nucleotide polymorphisms in genes coding for Folate - MTX metabolic pathway in Indian (Asian) HC and patients suffering from RA:
Data was analyzed in the same manner as described for CYP2C19 and CYP2C9 study

A.3 Main study:
A.3.1. MTX related toxicity and efficacy analysis:
All quantitative data are expressed as the median (interquartile range) or mean ± SD unless specified otherwise. All qualitative data are expressed as frequencies and percentages. Differences in baseline characteristics were analyzed by Student’s t-test for continuous variables or chi-square test for dichotomous variables. For efficacy and toxicity response, differences in genotype distribution were tested by 3 x2 cross-tabulations for each genotype, and by 2 x 2 cross-tabulations for each possible combination of homozygote and heterozygote genotypes, with the 2-sided chi-square test. When genotype distributions differed between responders and nonresponders or between patients with and without AEs, we used binary logistic analysis to calculate odds ratios (ORs) for achieving good response or experiencing adverse drug events of MTX given by a certain genotype.

Additionally, the possible confounding variables considered to be clinically important were included in the multiple logistic regression models for both efficacy and toxicity, comprising age, sex, duration of disease, MTX dose (mg/week), number of years of treatment with MTX, corticosteroid use, DMARD use, and NSAID use.
Since the integrity of the folate-purine-pyrimidine pathway is critical for cell survival, mutations that produce only subtle alterations in a key enzymatic step may be transmitted across generations (common polymorphism) but are likely to exhibit minimal effects, even in the context of a homozygous variant genotype. Therefore, we calculated a toxicogenetic index as the sum of homozygous variant genotypes carried by an individual. The genotypes which showed statistically significant association (p<0.05) in the binary logistic regression analysis were taken into consideration for calculating toxicogenetic index. The presence of the 4 genotypes was summed as a composite index to constitute a toxicogenetic index for each patient (index range 0–4). Statistical associations were assessed with a multiple logistic regression model, with the occurrence of side effects as the dependent variable and the toxicogenetic index as the main independent variable of interest.

A.3.2. Pharmacokinetics analysis:
The mean plasma concentration of all the groups (each MTX dose group compared against other dose groups) were compared by Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. Pearson Correlation was used to assess the dose dependant increase in MTX and 7-OH MTX concentrations by considering the mean of area under curve of (AUC) versus the different doses of MTX. Independent t-test was used to compare mean plasma concentration of MTX, 7-OH MTX and Hey against MTX dose (<15 mg/week and ≥ 15mg/week), genotypes.

All statistical analyses were performed using SPSS software (version 10.0 for Windows; SPSS, Chicago, IL). P < 0.05 was considered significant.