Supplementary material 3
FIMD domain wise calculations for model validation

The proposed study was hypothesized to develop an animal model which can give a better insight of Rheumatoid Arthritis with pathophysiological interventions similar to human etiology of the disease. The design of study was progressed with the complexity of the disease similar to the human disease events. Here, different inducing agents were selected for the induction of Rheumatoid Arthritis and the extra organ manifestations similar to human conditions.

CFA (Complete Freund's Adjuvant) was taken as primary inducing agent as this is the most frequently used agent for development of RA in rats. To see the advancement in disease stages CIA (Collagen type II) was opted as secondary inducing agent to cross link the inflammatory pathway with the immunological manifestations. LPS (Lipopolysachcharide) and HFD (High Fat Diet) was incorporated to mimic the cardiovascular complications generally occurring in RA. Clinically, in patients taking NSAIDs for long term or patients having stable RA progressed CVD complications with alteration of autoimmune and inflammatory responses.

The study was carried out using Wistar male rats. Different groups were evaluated for selected inducing agents alone and in combination. All the groups were primarily evaluated using ANOVA as a statistical tool for two situations 1) Rheumatoid Arthritis induction; and 2) Cardiovascular complication associated with Rheumatoid Arthritis. After getting the suitable groups, these optimized groups were used for the validation of models to check the human resemblance of the disease generated in rats for the two above-mentioned conditions.

Validation

A decision regarding the selection of an optimum/appropriate dose of LPS (among three concentrations 0.1, 0.5 and 10 μg/mL) was made by processing the obtained data for significance using ANOVA as a primary evaluation tool for pharmacological comparison. The selected models were further validated on the basis of Framework to Identify Models of Disease (FIMD) given by Guilherme S. Ferreira et al.(2020) This is a questionnaire-based validation system where the models were further compared and evaluated for eight domains: Epidemiology, Symptomatology and Natural History – SNH, Biochemical validation, Aetiological validation, Pharmacological validation, Histological validation, Endpoint validation, and Genetic validation.

The framework was adopted and the questions were framed using reference of the framework, but some extra points were added according to the need of the study. The questions were more focused on the Rheumatoid Arthritis and Cardiovascular complications in the selected models with the questions of clinical relevance.

Instructions given for scoring and calculations for the final radar plot reading were followed to fit the validation criteria in the proposed models of RA and RA along with CVD using following steps:

1. The questions were framed according to disease severity and progression using the mentioned domains.

2. Each domain has given the same weight (score), which was calculated using 100 as the total score and equally divided 100 into 8 domains, which comes down to 12.5 for each domain.
3. Now all the domains may have subsections, so the same weight for each domain (12.5) was equally divided to get the score for each subsection.

   Suppose question no. 1 has two subsections, so the score will be:
   1- Weighing (score) 12.5
   1.1 score 6.25
   1.2 score 6.25
4. The subsections may also have other sub-subsections, as question 1.1 has the total score of 6.25, but it has 2 more sub-subsections, so the score will be calculated as follows:
   1.1 score is 6.25
      1.1.1 score is 3.125
      1.1.2 score is 3.125
5. All the section, subsection and sub-subsection total should be 100.
6. Now how to give the answer to the questions mentioned in each domain and sections:
   1. There are total five options to answer each question with their weighing score in percentage:
      I. Yes- which has 100% score
         If your Answer to question 1.1.1 is yes, you have to take percentage of the given score (3.125) with 100
      II. Yes completely- It has a perfect score of 100%, but it also has a grading system that requires us to assign a grade t each question based on its weight or degree of severity of disease. Three grades were obtained for this study; A (70%), A^{+} (80%) and A^{++} (90%)

If your answer is yes completely with 70%, you have to calculate 70% of obtained score, and if it is A^{+} or A^{++} with 80 or 90 % weight, you have to calculate the percentage accordingly.

      III. Yes Partially has 50% weight, so you have to calculate 50% of the obtained score
      IV. No has 10% weight
      V. Unclear 0%
7. After answering the questions with suitable grade, all the factors were added to get the final score, and the final score was subtracted from the actual domain score to get a ratio for a plotting radar chart.
   Que. 1 Weighing score for domain – 12.5
   1.1 score 6.25
   1.1.1 score 3.125. Answer is yes partially, so the factor will be 50%, and the final score will be 3.125*50/100= 1.56
   1.1.2 score 3.125. Answer is A, then the factor will be 70%, and the final score will be 3.125*70/100= 2.18

8. Now to get the Radar value (Ratio), all the final scores obtained after calculating the percentage were added and subtracted from the score of the domain, i.e. 12.5
   So the Radar value for Que. 1 will be: 1.56+2.18+6.25/12.5= 0.7

How to prepare a Radar Plot

Radar chart or web chart is one of the comparative tools for analyzing multivariate data. Here the radar chart gives each domain an axis and we can compare the models by putting the ratio obtained after giving a suitable score to each question and calculated through the steps mentioned above.

How to interpret a Radar plot

- Here, Microsoft excel was used to generate a Radar plot and values (calculated ratio) for each domain were analyzed.
- While we put the ratio in radar plot using excel data sheet, we get the graph.
- In this graph, we can see the domains on different axis as this graph is a two-dimensional representation.
- On the basis of the ratio value which moves towards the axis and the dispersion from the axis, we can get the results in the form of similarity factor and uncertainty factor.
- The values moving towards the axis and having the similar intersecting points with other domains values have the interconnectivity between them.
- The values which are not intersecting each other and have higher dispersion from the axis towards the edges of the graph have a higher ratio and they have higher dissimilarity or uncertainty with the compared groups.

Design of questionnaire

Questionnaire was made for two situations as per the need of the study:

1) **Rheumatoid Arthritis model**
   - Questionnaire 1 (Model I RA induction with CFA 0.1 mL + LPS 10 μg/mL)
   - Questionnaire 2 (Model II RA induction with CIA 0.1 mL+ LPS 10 μg/mL)
2) **Rheumatoid Arthritis with co morbid conditions**
   - Questionnaire 3 (Model III RA and CVD induction with CFA 0.1 mL+ LPS 10 μg/mL + HFD)
   - Questionnaire 4(Model IV RA and CVD induction with CIA 0.1 ml + LPS 10 μg/mL + HFD)

The FIMD questionnaire was prepared and each section and subsection was answered using the following instructions to get the final radar score.

How to answer the questions

Here in this study, the questions were answered by using five criteria **Yes, Yes completely, Yes partially, No** and **Unclear**. In this section, each domain wise answer and the justification for suitable answer are given with details.

1. Epidemiological Validation

This section is similar for both the models RA as well as RA and associated complications.

1.1 Nature of population (Inbred/Out bred)

Here the question is answered with ***Yes partially***.

Justification

The animals were obtained from the different research organizations which are registered breeders, and the animals were bred for the experimental purpose only. The place where the experiment was conducted is different from their breeding place, but for this laboratory, the animals were outbred, so the answer is ***Yes partially.***

1.2 Is the model able to simulate the disease in relevant age groups (e.g. juvenile, adult or ageing)

Answer to the question is ***Unclear***

Justification

In humans for clinical presentation of symptoms, age is one of the criteria to see the prevalence of disease in a particular age (e.g. juvenile, adult and elderly). Some diseases have onset at a particular stage of life in clinical situations. It is important to incorporate such criteria in preclinical situation, but it will not be able to give similar results as the life span of a rat and a human is different, but we can compare that whether the disease develops totally, partially or not at all in the same developmental phases. In the present study, RA was developed in the rats which had been selected on the basis of their maturity, but age was not the major criterion when compared to the human rheumatoid conditions where elder people and adults are more prone towards the disease.

Here Wistar male rats taken for the studies were approximately of 6-8 wks old. But the specific age was not adopted for the model development and comparison between the models, so the *in vivo* data were not sufficient to answer this question; hence the answer is ***Unclear***

1.3 Is the model able to simulate the disease in the relevant genders?

Here the question was answered differently for different questionnaires:

- In **RA only using CFA and LPS** as inducing agents, this question was answered as ***Yes partially***

Justification

This particular study was performed on Wistar male rats, but the data for the RA development were present in both the genders using CFA and CIA. Here in the model in which RA was induced with CFA 0.1 mL sub planter and CIA 0.1 mL sub planter, the answer was ***Yes partially*** because the literature search suggests that female rats are equally prone towards the disease induction using the same inducing agent. LPS had no any accounts with either of these inducing agents.

Clinically, also firstly RA was considered as the disease of females and elder people, but as the clinical manifestations occurred equally also in the adult males now, RA is no longer considered as a gender or age specific disease.

- In other models of RA along with cardiovascular complications (questionnaires 3, 4), this question was answered as ***Unclear***.

Justification

All the models have incorporation of LPS with CFA or CIA with or without HFD, which is uniqueness of this model and no such models have come to our knowledge for inducing these situations in Wistar male rats. Moreover, here the study was performed on male rats only and data of this particular combination are not available for female rats, so these questions were answered as ***Unclear*** in questionnaires 3 and 4 in RA along with CVD models.

2. Symptomatology and natural history

This section has different subsections and sub subsections on the basis of the models and questionnaire prepared for that particular model.

2.1 Is model able to mimic human disease symptoms, if so, which ones?

In this section, the major symptoms according to the progression of disease in the model developed for Rheumatoid Arthritis, and the questions were framed accordingly. Inflammation is one of the major markers in RA and immunological intervention is a connecting link between RA and extra organ manifestations.

2.1.1 Inflammatory markers

Inflammation is the primary indication of RA as the definition says RA is a chronic inflammatory autoimmune disorder. Here in this part, the core symptoms generated in a clinical situation are to be compared. Physical inflammatory markers like paw volume and biochemical markers like ESR and CRP were measured for assessment of inflammatory responses.

In questionnaire 1 (RA only with CFA and LPS), the answer was ***Yes partially*** because the inflammation was not constant throughout the study period and, when compared with other groups, it was less than in the CIA induced groups.

In **questionnaires 2 and 3,** in which collagen was used as an inducing agent, the answer was ***Yes*** due to high grade persistent inflammation in these two groups.

In **questionnaire 4,** the question was answered as ***Yes completely*** as the inflammation was very high, and it was similar with clinical situations which represent walking.

2.1.2 Immunological markers

Immunological markers such as TNF-α, IL-6 and ACCP were measured for assessment. Where:

In questionnaire 1 (Model I), the answer was ***Yes partially*** because the immune markers were expressed but not significantly different from those in the normal control and test groups.

In **questionnaire 2 and 3** in which collagen was used as an inducing agent were, the answer was ***Yes*** with A^+ (70 %) grade as the expression of immune markers in these two groups was high when compared to other groups.

In **questionnaire 4.** the question was answered as ***Yes completely*** (100%), as the immune responses were very high, and it was similar with clinical situations.

2.1.3 Cross-linking markers for cardiovascular complications

Cross-linking markers such as homocysteine and TLR-4 were estimated in both the situations, which are the indication of initiation of extra organ manifestations in stable RA.

In Model I and II, there was no significant estimation observed, so the answer was given as ***Unclear*** in these groups.

In model III, the values of these markers were expressed to some extent, so this model was answered as ***Yes partially.***

In model IV, the expression of these markers was significantly high, so this question was answered in this group as ***Yes*** with grade A

2.2 Natural History criteria matching with human disease onset

As the patient history cannot be taken in the animal models due to species specific criteria, this question was assessed and answered basing on the observational measures only. In this section, the questions were kept similar with clinical conditions and on the basis of observation made by a researcher, they were answered for all four models.

2.2.1 Time of onset of disease generation

The duration in which disease generation initiated and turned to moderate and severe was observed and according to the days of disease occurrence this question was answered.

This question was addressed based on the time of onset of disease that was observed to have begun, progressed to a moderate state, and finally reached a severe state.

The time of onset of disease is different in models due to different inducing agents which changes the answers of the questions accordingly.

Yes partially was answered in CFA induced models as this model design is of 28 days, and the disease specific markers were significantly elevated after day 14, so the model was practically induced in a later phase of the study, and the disease was not persistent throughout the study period.

In model III, as the onset of disease was earlier when compared to group I, but it did not match completely human conditions, so it was scored with grade A (70%) and the answer was ***Yes.***

In model IV, the disease induction was fast and persistent throughout the study, and the progression of disease was just like a human condition; primary and secondary lesions were noticed prominently, so this group was scored with 100% grade with ***Yes completely.***

2.2.2 Disease Progression

Disease progression in model I matched partially human conditions so the answer was ***Yes partially.***

In model II, the progression was high when compared to group I, so the answer was ***Yes,*** but with grade A (70%) as the progression was fluctuating.

In model III, again the progression was partial (on /off) effects in symptoms, so the answer was ***Yes partially***. Rats in a CFA model do not show immunological intervention as it was seen in CIA model, so the answer for the specific group will be ***Yes partially***. As the species are different, this answer will not be counted as bias.

In group IV, this question was answered with ***Yes completely*** as the progression was symmetrical, persistent and constant, which fully mimics the clinical situations. But the bias will be there as humans and rats belong to different species, and exact physiological changes are not possible.

2.2.3 Duration of Symptoms

Disease symptoms in model I matched partially human conditions so the answer was ***Yes partially.***

In model II, the progression was high when compared to group I, so the answer was ***Yes,*** but with grade A^+ (80%) as they are not constant throughout the study. Models III A (70%) and IV also showed the higher duration of symptoms with grade A^+ (80%).

2.2.4 Severity

Describe whether the severity of the symptoms manifested in the model is similar to the ones manifested in humans. This shall be done comparatively, with a brief description of both the human and animal situations.

This question was answered as ***Yes partially*** in model I; ***Yes*** – with 70% in model II; in model III – ***Yes partially*** 50%, and in model IV – ***Yes*** with 80%.

2.2.5 Metabolic dysbiosis (obesity, TG, TC, LDL, HDL and fat accumulation in stool)

This question was answered ***Unclear*** in model I and II, where diet modification was not done, and these models were developed only for RA.

In model III, this question was answered with ***Yes partially*** as the changes were 50% similar to human conditions, and in model IV, the question was answered with ***Yes*** with grade A.

2.3 Are co-morbid conditions replicated in models similar to human conditions? If yes, which ones?

In this section, the models were compared with clinical situations in terms of severity, extra organ manifestation and constant pathological changes.

2.3.1 Secondary lesions

Secondary lesions were observed on day 21 in CFA-induced groups, and on days 21 and 35 in CIA-induced groups. In this section, the model I did not resemble the secondary lesions, only primary lesions were seen in these models, so the answer was ***Unclear***.
In models II and III, these changes were observed with grade A^+ (80%), so the answer was ***yes.***

3. Biochemical Validations

3.1 Pharmacodynamic biomarkers mimic the pathophysiology of the human disease

Biochemical validation was done for different biomarker elevation with the physical changes which occurred in the animals with the progression of disease. In these sections, the questions were framed for inflammatory markers (CRP, ESR, and Arthritic Index), immunological markers (TNF-α, IL-6, ACCP, and Hyc), and atherogenic markers (TG, TC, Cholesterol, Atherosclerotic index).

How to score

In this section, the models were scored on the basis of answers given – ***Yes*** and ***Yes completely*** on the basis of elevation of disease parameters evaluated during the study period.

3.2 Do these PD markers behave similarly to humans?

The comparison of the human condition with 100% symptomatology is not possible, hence the answer was ***Yes partially*** and ***Yes*** in the respective groups.

3.3 Known prognostic markers related to pathophysiology of the disease

This section was to assess the physical, biochemical and perceptive changes in the models in context to RA as well as RA with CVD in form of body weight, paw volume, biochemical elevation with inflammation, nodule formation, symmetric progression of disease and increase in secretions and walking disability in animals of different groups.

4. Aetiological Validation

Aetiological validation was done for both the situations; RA and RA with CVD for different situations in the form of different physical, perceptive and biochemical changes.
In this section, the questions were designed for Cytokine Activation (TNF-α, IL-6) Cell infiltration (ACCP generation) and Radiographic changes seen in the models, and they were answered according to the results for different models of RA.
Disability, Receptor Activation (TLR-4), Extra organ manifestation, Fibre length of Vistus medialis, Fibre length of Biceps Femoris were framed for RA with CVD.

The Aetiology of the disease in the model is similar to that in humans regarding both genetic and environmental (including lifestyle) factors. The hypothesized biomarker activation is also

included in this section for comparison of progression of disease via different sensitizing agents. A brief review of what is known of the aetiology of the disease shall be included together with a comparative discussion on the animal model's disease aetiology. Genetic factors shall be cited in reference to the Genetic Validation domain, and environmental factors shall also be described.

4.1 If there are known Pharmacodynamic (PD) biomarkers related to the pathophysiology of the disease, are they also present in the model?

The known Pharmacodynamic markers for RA were taken from literature survey, and they were analyzed in the developed model to see the mimicking in the model with resemblance with the human condition. The most relevant markers in pathophysiology of the disease in humans were selected for the comparison (ACCP, CRP, Hcy) because these are the molecules which can describe the current state of the disease. These markers can also be prognostic biomarkers, which can give a better insight of the disease, and we can also define the severity of the disease by evaluating them at different time points.

How to score?

If more than one biomarker is identified, a subsection shall be created for each relevant biomarker and individually analyzed. The total score for this question shall be calculated as the sum of each subsection (which can be answered according to the pre-specified answers).

Examples

Yes.

Here we can take C-RP as one of the markers: if it is high and thus similar to human disease conditions, the answer will be ***Yes***

No.

If the C-RP levels were not increased, the answer will be ***No***

4.2 Do these PD biomarkers behave similarly to humans'?

Describe whether the behavior of such biomarkers (higher or lower levels) is similar to the one seen in humans. If relevant, temporal (i.e. relevant only during a specific stage of development of disease phase) and spatial (i.e. present only in specific tissues) aspects of expression must also be considered.

How to score?

If more than one biomarker is identified, a subsection shall be created for each relevant biomarker and individually analyzed. The total score for this question shall be calculated as the sum of each subsection (which can be answered according to the pre-specified answers).

Examples

Yes.

Like humans, rats have increased levels ACCP.

No.

Unlike humans, rats have normal levels of ACCP.

4.3 If there are known prognostic biomarkers related to the pathophysiology of the disease, are they also present in the model?

Prognostic markers similar to human condition of RA and RA along with CVD were identified with different quantitative and qualitative methods which, were analyzed during model development, and they are kept as subsections and sub subsections in the questionnaire

How to score?

If more than one biomarker is identified, a subsection shall be created for each relevant biomarker and individually analyzed. The total score for this question shall be calculated as the sum of each subsection (which can be answered according to the pre-specified answers).

4.4 Do these prognostic biomarkers behave similarly to humans'?

Describe whether the behavior of such biomarkers (higher or lower levels) is similar to the one seen in humans. If relevant, temporal (i.e. relevant only during a specific stage of development of a disease phase) and spatial (i.e. present only in specific tissues) aspects of expression must also be considered.

How to score?

If more than one biomarker is identified, a subsection shall be created for each relevant biomarker and individually analyzed. The total score for this question shall be calculated as the sum of each subsection (which can be answered according to the pre-specified answers).

In this section, the hypothesized biomarker activation: TLR 4 or NLRP activation – were answered as ***Yes partially*****.**

And the known biomarkers like ACCP and Metabolic dysbiosis, walking difficulties, Arthritic Index, symmetric progression of disease were answered as ***Yes completely*****,** according to suitable grading.

5. Pharmacological Validation

Pharmacological validation was done on the basis of evaluation of test drugs in different groups.

In this study, three herbal drugs were evaluated against the models given in this study. And all the treatment groups were also evaluated on the parameters mentioned in different sections in the questionnaire.

As the treatment protocol was not part of this study, and the study was more focused on model comparison, the results for the treatment groups were not presented in this study. And the code for the test drugs was given as Test drug 1 (MPTN02), Test drug 2 (MPTN01) and Test drug 3 (MPTN04). These test compounds were evaluated individually as well as in the combination in the finalized models to evaluate their ameliorative effects on RA and RA with CVD.

Here MTX was used against the model group for RA, and Atorvastatin and Telmisartan were used in RA with CVD groups to see the effects on severity of the disease and the drug was proved to reduce the disease severity in the treated models, so the answer of this question was given as **yes *and Yes Partially*** in suitable models.

Justification

Here in the Rheumatoid arthritis model, Methotrexate was taken as a standard drug for comparison, which is the most frequently, used DMARD in the treatment of RA in clinical situations. The pathophysiological events are quite similar as per the result assessment in human and rat RA development; the effective drug MTX in human condition here also proved to reduce the severity in the standard group as compared to the model control animals.

All the criteria mentioned in the source article were met while giving the answer to this question.

5.2 Are ineffective drugs in humans also ineffective in this model?

As per the source literature, the ineffective drug should be listed in the drugs withdrawn from the market.

No such drug was evaluated here in this study, so the answers are ***Unclear*** for the relevant groups.

5.3 Have the drugs with different mechanism of action and acting on different pathway been tasted? If yes, which ones?

Herbal drugs were evaluated in this study, and they were found to have different modes of action as compared to the existing therapies for the disease.

6. Histological Validation

The major Histopathological changes generated in human RA conditions and cardiovascular complications progressed in RA patients were taken as a reference via review literature, and the affected organs like bone, heart and targeted muscles (Vistus medialis and biceps Femoris) were studied to see the changes as per the disease progression and were compared with normal and treatment groups along with standard human treatment drugs to see the changes and recoveries.

7. Endpoint Validation

Some of the methods were similar in estimation of the endpoints like estimation of biochemical markers, which are comparable with clinical methods, and some of the methods were different like paw edema measurement, but the purpose of estimation was similar (i.e. for estimation of inflammation). In this section, the endpoints were validated differently for both the conditions.

The endpoints in the context of similarity of the developed model are compared with the clinical symptoms generated in the selected disease in humans to see whether the disease is translated or not. As per the reference at least one or more than one endpoints similar to clinical relevance should be included in pharmacological validation to compare the model with clinical situations.

In the RA model, these Translatable endpoints were symmetric progression of disease, walking disability, nodule formation, change in X-Ray and histopathological changes. In models with

cardiovascular complications associated with RA, the endpoints were gut infiltration, TLR and NLRP activation, fibre length of Vistus medialis and biceps Femoris muscle and histopathology of heart.

How to score?

The evaluation parameters which were hypothetized were answered as ***Yes Partially*** to avoid the bias due to species difference.

And the biomarkers which are confirmed in humans and seen in the models also were answered as ***Yes complitely***, with the grading according to severity in different models sensitized with different inducing agents.

8. Genetic Validation

Genetic validation is one of the methods which can confirm the disease, its complications, and the root cause of that particular pathophysiological event in the form of genes involved in activation of the disease. Due to limitations in facility, funds, or ethical consideration, the genetic validation is not possible in each type of research. In this study, the genetic aspects of Rheumatoid arthritis as well as RA with its complications were not studied on genetic validation measures; hence, each and every model is answered accordingly.

8.1 Does this species also have orthologous genes and/or proteins involved in the human disease?

In this section, all the models were answered as ***Yes partially.***

Justification

In this particular disease, Wistar rats were used as models, and the literature search suggests that the proteins and the genes are similar in the pathophysiological condition of RA and RA along with associated complications in both the species (rat as well as human).

In this study, the TLR-4 Protein was hypothesized to get activated in the representative models of RA along with co-morbid conditions and the expression was checked by the ELISA methods. The data of ELISA methods were not sufficient to give the gene activation accounts, and there is a scope of proper detection and identification of genes involved in both the conditions in this study with advanced techniques. However, the literature search revealed the mechanisms of primary inducing agents and role of some similar genes and heat shock proteins incorporated in both the species for the selected condition. On this account, this section was answered as ***Yes partially*** in all the developed models.

8.2 If so, are the relevant genetic mutations or alterations also present in the orthologous genes/proteins?

This question was answered as ***Unclear*** in all the model groups.

Justification

The supporting studies which can represent the mutation or alterations in the relevant proteins or gene were not performed here, and so the question was answered as ***Unclear.***

8.3 If so, is the expression of such orthologous genes and/or proteins similar to the human condition?

This question was answered as ***Unclear.***

Despite the involvement of similar proteins and genes, the similarity was not accounted fully similar, and this will be counted as partial as there is a species variation present in rats and humans. Moreover, genetic alteration was not evaluated according *to in-vitro* and *in-vivo* studies suggested by the experts in this area of research, so the question was answered as ***Unclear.***