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Running Title / Short Title: Synovial tissue signatures enhance clinical classification and prognostic/treatment response prediction in early arthritis

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Abstract

Objective: To establish whether synovial pathobiology improves current clinical classification and prognostic algorithms in early inflammatory arthritis and identify predictors of subsequent biologic therapy requirement.

Methods: 200 treatment-naïve early-arthritis patients were classified as fulfilling RA1987 ACR criteria (RA1987) or as undifferentiated arthritis (UA) and UA patients further classified into those fulfilling RA2010 ACR/EULAR criteria. Treatment requirements at 12 months (csDMARDs vs biologics vs no-csDMARDs treatment) was determined. Synovial tissue was retrieved by minimally-invasive, ultrasound-guided biopsy and underwent processing for immunohistochemical (IHC) and molecular characterisation. Samples were analysed for macrophage, plasma-cell and B- and T-cells markers, pathotype classification (lympho-myeloid, diffuse-myeloid or pauciimmune) by IHC and gene expression profiling by Nanostring.

Results: 128/200 patients were classified as RA1987, 25 as RA2010 and 47 as UA. Patients classified as RA1987 criteria had significantly higher levels of disease activity, histological synovitis, degree of immune cell infiltration and differential upregulation of genes involved in B and T cell activation/function compared to RA2010 or UA, which shared similar clinical and pathobiological features. At 12 months follow up, a significantly higher proportion of patients classified as lympho-myeloid pathotype required biologic therapy. Performance of a clinical prediction model for biologic therapy requirement was improved by integration of synovial pathobiological markers from 78.8% to 89-90%.

Conclusion: The capacity to refine early clinical classification criteria through synovial pathobiological markers offers the potential to predict disease outcome and stratify therapeutic intervention to patients most in need.

Keywords (5) Early Arthritis**,** Rheumatoid Arthritis Classification Criteria, Synovium pathotype, Ultrasound-guided biopsy.

INTRODUCTION

The introduction of new classification criteria for Rheumatoid Arthritis (RA) in 2010 [1] has been demonstrated to be clinically useful with enhanced diagnostic sensitivity in early disease compared to 1987 criteria [2]; however, this is balanced by a lower specificity [3,4]. This is of particular importance, as data suggest that approximately 40% of patients with early inflammatory arthritis, not fulfilling 1987 criteria, may spontaneously remit whilst approx. 30% will progress to RA [5]. Critically the mechanisms undelying the transition from undifferentiated arthritis (UA) to RA remain unknown though it has been suggested that qualitative or quantitative difference within synovial tissue may contribute to diverse disease evolution and/or treatment response [6,7]. Thus, pre-treatment stratification of early inflammatory arthritis is important in order to target therapy to poor prognosis patients. Previous data suggest that stratifying early arthritis according to RA2010 vs RA1987 classification criteria reveals significant clinical heterogeneity in diagnosis at 2 year follow up [8] although subsequent analysis of synovial tissue did not suggest that such clinical heterogeneity translated to significant differences in synovial pathobiology [9]. However, recently published data from a cohort of 144 early RA patients has demonstrated that synovial cellular and molecular signatures define prognostic and treatment response phenotypes [10]. Importantly whether clinical heterogeneity associated with the introduction of the 2010 ACR/EULAR criteria can be explained by synovial pathobiological signatures and whether they associate with subsequent disease outcome, up to now, remains unknown.

Therefore, the aim of this study was to investigate whether in patients with early inflammatory arthritis synovial cellular and molecular signatures: (i) segregate according to clinical classification (RA1987 vs RA2010 vs UA) (ii) change depending on symptom duration and, (iii) determine prognosis including subsequent requirement for biologic therapy.

PATIENTS AND METHODS

Patients

200 consecutive inflammatory arthritis patients recruited at Barts Health NHS Trust as part of the multi-centre pathobiology of early arthritis cohort (http://www.peac-mrc.mds.qmul.ac.uk) were included within the study. Patients were treatment naïve (csDMARD and steroid) and had <1 year symptoms.

At baseline patients underwent collection of routine demographic data and were categorised according to the following criteria: (i) RA1987 [2] or (ii) UA. 2010 ACR/EULAR criteria for RA [1] were then applied to further classify patients with UA, resulting in three groups: (i) RA1987 (RA1987+/RA2010+), (ii) RA2010 (RA1987-/RA2010+) and (iii) UA (RA1987-/RA2010-). An ultrasound (US) guided synovial biopsy of a clinically active joint was performed [11]. Patients were then commenced on standard conventional synthetic (cs)DMARD therapy with a treat-to-target approach to treatment escalation (DAS28<3.2). Patients failing csDMARD therapy were commenced on biologic therapy (anti-TNF, Tocilizumab or Rituximab) according to the prevailing UK National Institute for Clinical Excellence (NICE) prescribing algorithm if they continued to have a DAS28>5.1 following 6 months of therapy [12]. At 12 months follow-up patients were categorised as follows: i. self-limiting (SL) disease (DAS28<3.2 and off csDMARD/steroid therapy) vs persistent disease (PD) (DAS28>3.2 and/or csDMARD) and ii. Symptomatic treatment (non-steroidal anti-inflammatories) treatment vs csDMARD therapy vs Biologic+/-csDMARD therapy.

Synovial biopsy collection and processing

A minimum of 6 biopsies per patient were collected for paraffin embedding and if intact lining layer identified underwent histopathological assessment. Synovitis score was determined using a previously validated scoring system [13]. Following immunohistochemical staining of

sequentially cut slides using previously reported protocols for B cells (CD20), T cells (CD3), macrophages (CD68) and plasma cells (CD138) the degree of immune cell infiltration was assessed semi-quantitatively (0-4) [14]. Biopsies were stratified into 1 of 3 synovial pathotypes according to the following criteria: i) Lympho-myeloid presence of grade 2-3 CD20+aggregates, (CD20≥2) and/or CD138>2 ii) diffuse-myeloid CD68 SL≥ 2, CD20≤1 and/or CD3≥1, CD138≤2 and iii) pauciimmune CD68 SL<2 and CD3, CD20, CD138<1

Nanostring analysis

A minimum of 6 synovial samples per patient were immediately immersed in RNA-Later and RNA extraction performed as previously described [10]. RNA samples then underwent profiling for expression of 238 genes preselected based on previous microarray analyses of synovial tissue from patients with established RA [15] and/or relevance to RA pathogenesis. Raw NanoString counts were processed using the NanoStringQCPro package in R 3.2.0. Counts were normalised for RNA content by global gene count normalisation and then log transformed (base 2). The validity of normalisation was then checked via box- and scatter plots of normalised counts. Benjamini-Hochberg method was used to adjust for multiple testing, and genes were considered to be differentially expressed if they demonstrated an FDR-adjusted p-value <0.01.

Statistical analysis

Statistical analyses were run using R.3.0.2. For three way comparisons, Kruskal-Wallis test was used for continuous and Chi-squared or Fisher's exact test used for categorical variables as appropriate. A p-value <0.05 was considered statistically significant. Post hoc comparison tests were performed using Dunn test or Bonferroni correction as appropriate.

Linear regression models: Logistic regression using forward, backward and bidirectional stepwise selection was employed using the glm function in R.

Gene expression predictors were selected by L1 (LASSO) sparse logistic regression using R package glmnet. The penalty parameter λ was optimised using 10-fold cross-validation. λ corresponding to the minimum mean cross-validated error was retained as final penalty parameter in the model.

Predictive performance evaluation: Predictive performance of the final prediction model was assessed by computing the area under the receiver operating characteristic curve (AUC), using both apparent and internal validation with 95% CI. Internal validation using a bootstrap method [16,17] (performed with R package boot version 1.3-18) was employed to correct for over-fitting, to generate unbiased optimism-adjusted estimates of the C statistic (AUC) with low absolute error. Bootstrap estimate of the AUC statistic was computed by random sampling with replacement 500 times to enable estimation of the optimism corrected AUC.

RESULTS

Patient demographics and clinical correlations

200 PEAC patients were included, 128/200 (64%) patients were classified as RA1987 (RA 1987+/RA2010+) and 72/200 (36%) as UA. Of the UA patients, 25 were further classified as RA2010 (RA1987-/RA2010+) (25/200, 12.5%) and 47 remained as UA (RA1987-/RA2010-) (47/200, 23.5%) (Figure 1A). No significant difference in mean age, disease duration or ESR between groups was demonstrated. However, the RA1987 group had significantly higher levels of CRP, TJC, SJC, DAS28, RF, ACPA and VAS and significantly higher numbers of patients sero positive for RF and ACPA compared to either the RA2010 or UA groups (Figure 1B). SJC and ACPA titre were the only clinical parameters with significant differences between the

RA2010 and UA groups, indicating that in terms of clinical measures of disease activity these two groups are relatively homogenous.

Synovial pathotypes distinguish clinical phenotypes regardless of disease duration

Synovial biopsies were obtained predominantly from small joints (81.5%) (Figure 2A). Patients with synovial tissue suitable for histological analysis (166/200) were segregated according to baseline synovial pathotype (Figure 2B) and differences in clinical parameters evaluated. We demonstrated significantly higher mean DAS28 within the lympho-myeloid compared to either the diffuse-myeloid or pauciimmune group (5.82 vs 4.93 vs 4.86, $p<0.001$). Mean CRP was significantly higher in the lympho-myeloid and diffuse-myeloid vs pauciimmune groups (16.86 vs 15.52 vs 9.55, $p<0.001$) and a significantly higher number of patients were sero-positive for either RF ($p=0.012$) or ACPA ($p=0.011$) within the lympho-myeloid group (Figure 2C). To evaluate whether disease duration influenced prevalence of synovial pathotype, patients were stratified into four groups according to disease duration at baseline (1-3m, 4-6m, 7-9m and 10-12m) and frequency of synovial pathotype determined. No significant differences in synovial pathotype frequency at each time point was demonstrated ($p=0.65$) (Figure 2D).

RA1987 patients display significantly higher levels of synovial immune cell infiltration compared to RA2010 and UA patients

Patients were segregated according to pathotype and further into RA1987, RA2010 and UA categories. A higher proportion of patients within the RA1987 group were categorised as lympho-myeloid (vs diffuse-myeloid or pauciimmune) (43.5% vs 33% vs 23.5%) (Figure 3A). We also demonstrated a significantly higher mean synovitis, CD3+ T cell, CD20 +B cell, CD138+ plasma cell and CD68+ SL/L macrophage score between the RA1987 group and both

the RA2010 and UA groups ($p<0.001$) (Figure 3B). We saw no significant differences in synovitis score, mean CD3+T, CD20+B, CD68+ L or SL macrophage or CD138+ plasma cell number between the RA2010 and UA group (Figure 3B), indicating that these two groups are relatively homogenous in terms of tissue pathology.

Synovial genes regulating B cell activation and function are significantly upregulated in RA1987 patients compared to the RA2010/UA groups.

145/200 patients had RNA available for nanostring analysis (95/128 RA1987, 12/25 RA2010 and 38/47 UA patients) and were analysed for differential gene expression (238 genes) between groups.

Comparing RA1987 vs RA2010 groups we demonstrated a significant differential expression of 53 genes (Figure 3C). In line with the histological analysis a number of differentially upregulated genes within the RA1987 cohort were involved in mediating B cell activation/function (e.g. *CD79A*, *CD38*, *IGJ*, *CXCL13*, *IRF4*, *CCL19*, *CD38*, *TNFA*, and *IL6*). When evaluating gene expression between RA1987 and UA groups we found a similar trend with differential upregulation of a number of genes within the RA1987 cohort mediating B cell activation/function although only *CXCL13* remained significant following correction for multiple comparisons (Figure 3D). Conversely when evaluating gene expression between the RA2010 and UA cohorts only 7 genes appeared as significant with a preponderance of differentially upregulated genes within the RA2010 cohort mediating cartilage biology (*COMP*, *DKK3*, *INHBA*) and none remaining significant after correction for multiple comparisons (Figure 3E).

Classification as RA1987 criteria at disease onset predicts persistent disease at 12 months

190/200 patients had 12 month follow up data available, we examined whether baseline synovial pathotype was associated with disease evolution. 119/121 (99%) RA1987 patients and 19/22 (90%) RA2010 had PD (Figure 4A). Within the UA cohort 11/47 (23%) had other diagnoses. Of the remaining 36 patients, 26/36 (72.2%) had PD, and 10/36 (27.8%) SL. Of the UA patients with PD 4/26 (15.3%) progressed to fulfil 2010ACR/EULAR criteria RA at 12 months. Results demonstrated a significantly higher proportion of patients with SL disease in the UA group compared to the RA2010 or RA1987 groups and a significantly higher number of patients within the RA1987 group with PD (Figure 4B). When evaluating the effect of baseline pathotype we demonstrated a higher proportion of patients with a lympho-myeloid vs diffuse-myeloid or pauciimune pathotype (39% vs 32% vs 13%) with PD and a higher number of patients with a diffuse-myeloid vs lympho-myeloid or pauciimmune pathotype (54% vs 18% vs 27%) with SL (Figure 4C).

A baseline lympho-myeloid pathotype significantly associates with 12 month requirement for biologic therapy.

Patients stratified according to diagnostic group or pathotype were further classified according to 12 month treatment requirement: i. symptomatic treatment, ii. csDMARDs or iii. biologics+/-csDMARDs. A significantly higher proportion of RA1987 patients required biologic compared with RA2010 and UA (27.82% vs 20.83% vs 10.63%) ($p<0.001$) (Figure 5A) and importantly, lympho-myeloid (vs diffuse-myeloid or pauciimmune) pathotype significantly associated with 12 month requirement for biologic therapy (57% vs 21% vs 21% $p=0.02$) (Figure 5B).

We then compared expression of the 238 genes in the Nanostring panel between patients requiring biologic therapy (n=34) or not (n=106) and found 119 differentially expressed genes.

Patients requiring biologic therapy had significantly higher differential upregulation of genes regulating B and T cell proliferation, differentiation and activation (e.g. *TNFRSF13C, CD79A, CD2, CD3E and CD38*), genes involved in matrix metallopeptidase production/regulation (e.g. *MMP1* and *TIMP1*), genes involved in cytokine mediated cellular activation (*TNFA, TRAF3IP3, IFNA1*), and osteoclastogenesis inhibition (*DEF6*). Patients who did not require biologic therapy expressed some B and T cell regulation genes and B proliferation markers but mostly markers of fibroblast proliferation and cartilage turnover (Figure 5C).

To determine whether disease duration influenced outcome we segregated patients according to 12 month treatment (biologic therapy or not) and further into disease duration quartiles (Figure 5D) and demonstrated no significant differences in terms of disease duration at diagnosis. Next, we segregated patients treated with biologic therapy (n=39) according to quartiles of disease duration and then synovial pathotype. We found no significant differences in patient number in each quartile (P=0.3) (Figure 5E). These results strongly suggest that synovial pathotype rather than disease duration influences 12 month treatment outcome.

Synovial gene expression signatures enhance the performance of clinical prediction models for biologic requirement

To determine whether baseline clinical and gene expression data could be combined into a model for predicting requirement for biologic therapy, we used 2 complementary approaches: a logistic regression model to identify predictive clinical covariates, and a penalized method based on logistic regression with an L1 regularisation penalty (LASSO) to identify genes improving the clinical model.

9 baseline clinical covariates were considered as candidates in the regression model: disease duration, ESR, CRP, RF, ACPA, TJC, SJC, DAS28, and pathotype (two categories, lympho-myeloid vs pauciimmune/diffuse-myeloid). Logistic regression models using backward

forward and bidirectional stepwise selection resulted in selection of the same set of clinical covariates: DAS28, pathotype, CRP and TJC. The apparent predictive performance of the model evaluated by AUC was 0.78 (95% CI=0.70-0.87).

Genes were selected to improve the clinical model using logistic regression with an L1 regularization penalty (LASSO) applied on the 4 clinical covariates selected by the previous logistic regression and the 119 genes identified as being significantly differentially expressed between the biologic and non-biologic groups. Models in which clinical predictors were penalised or subject to forced inclusion were compared. When all predictors were penalised, 11 predictors were retained in the final model and when the clinical covariates were not penalised, 13 predictors were retained (Figure 6A). In both the penalised and unpenalized clinical model the apparent prediction performance was improved (apparent AUC=0.89, 95% CI=0.83-0.95 and AUC=0.90, 95% CI=0.84-0.95) (Figure 6B). We additionally performed internal validation to correct the AUC performance measure for over-fitting by calculating the optimism of the AUC for each model by boot-strapped sampling with replacement from the original dataset. The optimism corrected AUC was 0.75 for the pure clinical model and 0.81 for the clinical and gene model (LASSO) (Figure 6C and 6D) suggesting that including both clinical covariates and genes in the model results in an improvement of the predictive ability of the model.

DISCUSSION

These results present a number of novel findings: firstly they strongly suggest that early inflammatory arthritis patients not fulfilling RA1987 criteria display similar clinical, synovial histological and molecular features irrespective of further classification according to RA2010 or UA criteria. Secondly these data also suggest that a lympho-myeloid pathotype at disease onset predicts poor outcome with patients subsequently requiring biologic therapy irrespective

of clinical classification, and finally that integration of histological and molecular signatures into a clinical prediction model enhances sensitivity/specificity for predicting whether patients will require biologic therapy.

To the best of our knowledge these results emerge from the largest synovial tissue treatment-naïve early arthritis cohort reported to date and support previous data from early RA cohorts suggesting that a synovial immune cell infiltrate characterised by a predominant infiltrate of B cells associates with more active disease [18] and sero-positivity for RF and ACPA [10]. The results suggest that this effect also extends to patients within the UA cohort. The clinical similarities between RA2010+/RA1987- patients and those with UA has been reported previously [8] and the data presented herein provides a pathophysiological explanation for this with the demonstration of homogeneous synovial cellular and molecular signatures among the two groups. The data show a lower percentage of patients requiring biologic therapy in RA2010+/RA1987- group, in line with the expectation that the ACR/EULAR 2010 criteria enable an earlier diagnosis and thus efficacious treatment. However, it is also possible that this group has a milder pathology from the beginning.

Although synovial pathotypes per se do not appear to distinguish between patients at risk of developing PD rather than SL disease, this is not surprising given the early and treat-to-target approach pursued in the study rather than observing untreated natural disease evolution. However when applying 12 month biologic requirement as a prognostic outcome we demonstrated that patients with a lympho-myeloid pathotype with a dense synovial infiltrate enriched in B cells and significant upregulation of T/B cell genes at disease onset predicted requirement for subsequent biologic therapy and critically that this was independent of disease duration. These results are consistent with recently published data in early RA which reports that the lympho-myeloid pathotype is associated with highly agressive disease and worse radiographic outcomes [10]. The current study reinforces these findings demonstrating that, at

12-months follow-up, a significantly higher proportion of patients classified as lympho-myeloid pathotype required biologic therapy. The study also calls into question the current dogma surrounding "an early window of opportunity" for all patients with RA [19–21], suggesting that pathotype rather than simply disease duration influences outcome and that intensive therapeutic regimens should be targeted to poor prognostic pathotypes. This notion is supported by the demonstration that the integration of synovial histological and molecular markers into a clinical prediction model for biologics use improves sensitivity/specificity from 78.8% to 89-90% independently from disease duration.

Discrepancy with previously reported data suggesting that synovial heterogeneity does not relate to clinical phenotypes [9], maybe explained by the fact that in our study the majority of biopsies were performed on small joints while in that cohort arthroscopic biopsy was restricted to patients with mainly large joint involvement and, thus, a potential selection bias [22]. Additionally, the paired histological and molecular data in the largest biopsy-driven early arthritis cohort reported to date ensured internal validation and high classification accuracy.

Our study does have limitations however, for example the real-life nature of the study did not permit the true evaluation of the natural history of the disease or outcome, as no patients were left untreated and therapy was not actively withdrawn. Also a treat to target approach, treatment escalation and initiation of biologic therapy was determined by treating physicians according to NICE guidelines rather than study protocol.

Within these limitations, our results are robust and suggest that the introduction of the new RA2010 classification criteria brings additional clinical and biological heterogeneity into early patient classification compared to the 1987 criteria with limited ability of RA2010 criteria alone to predict poor outcome. The demonstration that the integration of synovial pathobiological markers into clinical algorithms predicting poor outcome (requirement for biologic therapy) independent of disease duration suggests that the "window of opportunity" is wider than 6

months and early stratification of biologic therapies according to poor prognostic synovial pathobiological subtypes at disease onset may improve the outcome of these patients.

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Competing interest None

Ethics approval The study received local ethical approval (REC 05/Q0703/198) and all patients gave written informed consent.

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Figure Legends

Figure 1. Baseline Patient Demographics.

A. **Baseline classification of patients.** 200 patients were classified into RA1987 vs undifferentiated arthritis (UA). RA 2010 ACR/EULAR Criteria was then applied to UA patients. Final 3 groups obtained showed 47 patients UA (RA 1987-/RA2010-), RA 2010 (RA1987-/RA2010+), RA 1987 (RA1987+/RA2010+).

B. **Demographics according to classification criteria**. Data are presented as mean (SD, standard deviation) for continue variables and frequency and percentages for categorical variables. Baseline characteristics between the 3 groups were compared using Kruskal-Wallis or Fisher's exact test as appropriate. For post hoc comparison, Dunn tests were run and p-value from pairwise comparison reported in the last 3 columns of the table. ESR: Erythrocyte sedimentation rate ; CRP: C-reactive protein; 28TJC: 28 tender joint count; 28SJC: 28 swollen joint count; DAS28: Disease Activity Score 28 joints; RF titre: Rheumatoid factor titre (IU/ml); ACPA Titre: Anti-citrullinated protein antibody titre (IU/L); RF +ve: rheumatoid factor serum positive (>15IU/L); ACPA +ve: Anti-citrullinated protein antibody (>20IU/L).

Figure 2. Patient demographics and disease activity: comparison between pathotypes.

A. **Number of biopsy procedures per joint** MCP (Metacarpophalangeal), MTP (Metatarsophalangeal), PIP (Proximal Inter phalangeal).

B. **Representative images of synovial pathotypes.** H&E: Haematoxylin & Eosin. Sections underwent immunohistochemical staining and semi-quantitative scoring (0-4) to determine the degree of CD20+ B cells, CD3+ T cells, CD68+ lining (l) and sublining (sl) macrophage and CD138+ plasma cell infiltration. Sections were categorised into

three pathotypes: (i) Pauci-iumne (CD68 SL<2 and or CD3, CD20, CD138<1), (ii) Diffuse-Myeloid: (CD68SL>2, CD20<1 and or CD3>1) and (iii) Lympho-Myeloid: (grade 2-3 CD20+ aggregates, CD20>2). Arrow heads indicate positive stain cells. Empty arrows indicate B cell aggregates.

C. **Demographic Analysis by Pathotype.** Data are presented as mean and standard deviation (SD) for numerical variables and frequency and percentage for categorical variables. Baseline characteristics between the 3 pathotypes were compared using a Kruskall-Wallis test and Fisher-test (RF and ACPA positivity) as appropriate. Post hoc analysis for significant differences using Dunn test for multiple comparison. A *P*-value of <0.05 was considered statistically significant**.**

D. **Pathotype according to disease duration (months) at diagnosis.** Absolute values (N) and percentage. A *P*-value of <0.05 was considered statistically significant.

Figure 3. Variation in synovial pathobiology according to clinical classification of patients.

A. **Baseline clinical classification compared with pathotype.** Baseline subgroups (RA 1987, RA2010 and UA) were compared with pathotype. Fisher test used for analysis.

B. **Immune cell infiltration for each clinical subgroup.** Kruskal-Wallis test for comparison between 3 groups. Post hoc analysis for significant differences using Dunn test for multiple comparison.

C. **(C-E) Gene expression analysis for comparison between subgroups.** T-test for comparison and Volcano plot for representative image. Positive values represent upregulation and negative values downregulation. Green circles above green horizontal line represents non-corrected for multiple analysis expressed genes between groups. Red circles above red line represents corrected p-values (Benjamini-Hochberg method)

for multiple analysis. **(C)** Volcano plot RA 1987 vs RA 2010: Difference in gene expression between patient fulfilling RA 1987 ACR criteria and RA 2010 ACR/EULAR Criteria. **(D)** Volcano plot RA 1987 vs UA: Difference in gene expression between patient fulfilling RA 1987 ACR criteria and Undifferentiated Arthritis**. (E)** Volcano plot RA 2010 vs UA: differences in gene expression between patient fulfilling RA 2010 ACR/EULAR criteria and UA.

Figure 4. Disease evolution.

A. **Patient classification after 12 months follow up.** Disease outcome after 12 months of follow up for each of the initial baseline subgroups (RA1987/RA2010/UA). Disease evolution classified as self-limiting or persistent disease. Other diagnosis as described for those who were re-classified after 1 year form UA cohort**.**
B. **Disease evolution by subgroups.** Disease evolution was compared with Baseline subgroups (RA 1987, RA2010 and UA). Fisher test used for analysis.
C. **Disease evolution by pathotype.** Disease evolution was compared with pathotype (Pauci-imune vs Diffuse-Myeloid vs Lympho-Myeloid. Fisher test used for analysis. A *P*-value of <0.05 was considered statistically significant.

Figure 5.

A. Comparison between diagnostic subgroups and treatment outcome at 12month follow up. Treatment required was divided in 3 groups: (i) No treatment; (ii) csDMARDs only, (iii) csDMARDs +/- Biologics. Fisher test for analysis**.**
B. Comparison between pathotype and treatment outcome at 12 months.
C. Gene expression analysis, represented in a Volcano plot comparison between patient requiring Biologics vs non-biologic group. T-test comparison for gene difference

expression between groups. Positive values represents upregulation and negative values downregulation. An adjusted (Benjamini-Hochberg correction for multiple analysis) *P*-value of <0.01 was considered statistically significant, represented as dots above red line. Green dots above green line for gene expression significance when no correction applied for multiple analysis (*P value <0.05).* **D. Treatment outcome according to baseline disease duration.** Fisher test for analysis. **E. Pathotype according to baseline disease duration for Biologic patient cohort.** Fisher test for analysis. A *P*-value of <0.05 was considered statistically significant unless otherwise stated.

Figure 6. Prediction model.

A-B Identification of clinical and gene expression features predictive of biologic therapy use at 1 year. Logistic regression, coupled with backward and stepwise model selection was applied to baseline clinical parameters against a dependent variable of Biologic therapy use or not at 12 months to select which clinical covariate contributed the most to the prediction. Selected covariates (119 genes+4 clinical covariates) were entered simultaneously into a logistic model with an L1 regularization penalty (LASSO) in order to determine the optimal sparse prediction model. A similar predictive performance of the model when clinical was seen when results were penalized (blue dashed line, figure 6A) than when they were not penalized (red dotted line, figure 6A) with a slightly different set of selected covariates (Figure 6B). Figure 6B shows the non-zero weights associated with the final variables selected by the LASSO regression. The grey spaces represent the variables that were not selected by the model.

C-D Lambda training curve from the final glmnet fitted model. The red dots

represent mean binomial deviance using 10-fold cross-validation. The error bars represent standard error of binomial deviance. The vertical dotted lines indicate minimum binomial deviance (λ_{min}) and a more regularized model for which the binomial deviance error is within one standard error of the minimum binomial deviance (λ_{1se}). λ_{min} was selected, corresponding to 11 non-zero coefficients in the final model for the LASSO where clinical were penalized (Figure 6C) and 13 non-zero coefficients in the final model for the LASSO where clinical were not penalized (Figure 6D).

Key messages:

What is already known about this subject?

The introduction of ACR/EULAR RA classification criteria have impacted positively on early diagnosis and treatment RA leading to better outcomes. By the same token, broader criteria have led to the inclusion of patients with milder and more heterogenous disease. This, together with the inability to precisely predict disease prognosis and treatment response at the individual patent levels, emphasise the need to identify patients at risk of accelerated structural damage progression and fast-track aggressive/biologic therapies to patients with poor prognosis.

What does this study add?

This study analyses the largest biopsy-driven early inflammatory arthritis cohort to date (200 patients) and, through a detailed synovial cellular and molecular characterization refines ACR/EULAR disease classification. In addition, the study identifies synovial pathobiological markers associated with with the lympho-myeloid pathotype and the requirement of biologic therapy at 12 months, reinforcing recently published data the indicates that these patients are affected by highly agressive disease and worse radiographic outcome. Notably, these findings are independent from the time of diagnosis within the first 12 months of symptoms initiation, suggesting that the so called "window of opportunity" is wider than 6 months and early stratification of biologic therapies according to poor prognostic synovial pathobiological subtypes at disease onset may improve the outcome of these patients. The integration of synovial pathobiological markers into a logistic regression model improves the prediction accuracy from 78.8% (clinical) to 89-90% (clinical + molecular) and enables the identification at disease onset of patients who subsequently require biologic therapy. Thus, this study

provides support to the notion that biologic therapies should be started early in patients with poor prognosis.

How might this impact on clinical practice or future developments?

The identification at disease onset of patients who are unlikely to respond to csDMARDs, remains a major unmet need. The capacity to refine early clinical classification criteria through application of synovial pathobiological markers and the ability to identify patients who subsequently require biologic therapy at disease onset offers the opportunity to stratify therapeutic intervention to the patients most in need. This present study adds weight to the need to change current therapeutic algorithms and start biologic therapies at disease onset in patients with poor prognosis. This is likely to have a major impact on disease control/remission and long-term disability, as notionally supported by numerous early intervention studies using biologic therapies.

Synovial tissue signatures enhance clinical classification and prognostic/treatment response algorithms in early inflammatory arthritis and predict requirement for subsequent biologic therapy: results from the Pathobiology of Early Arthritis Cohort (PEAC)

Running Title / Short Title: Synovial tissue signatures enhance clinical classification and prognostic/treatment response prediction in early arthritis

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Word count: 3032 ~~2996~~ words

Abstract

Objective: To establish whether synovial pathobiology improves current clinical classification and prognostic algorithms in early inflammatory arthritis and identify predictors of subsequent biologic therapy requirement.

Methods: 200 treatment-naïve early-arthritis patients were classified as fulfilling RA1987 ACR criteria (RA1987) or as undifferentiated arthritis (UA) and UA patients further classified into those fulfilling RA2010 ACR/EULAR criteria. Treatment requirements at 12 months (csDMARDs vs biologics vs no-csDMARDs treatment) was determined. Synovial tissue was retrieved by minimally-invasive, ultrasound-guided biopsy and underwent processing for immunohistochemical (IHC) and molecular characterisation. Samples were analysed for macrophage, plasma-cell and B- and T-cells markers, pathotype classification (lympho-myeloid, diffuse-myeloid or pauciimmune) by IHC and gene expression profiling by Nanostring.

Results: 128/200 patients were classified as RA1987, 25 as RA2010 and 47 as UA. Patients classified as RA1987 criteria had significantly higher levels of disease activity, histological synovitis, degree of immune cell infiltration and differential upregulation of genes involved in B and T cell activation/function compared to RA2010 or UA, which shared similar clinical and pathobiological features. At 12 months follow up, a significantly higher proportion of patients classified as lympho-myeloid pathotype required biologic therapy. Performance of a clinical prediction model for biologic therapy requirement was improved by integration of synovial pathobiological markers from 78.8% to 89-90%.

Conclusion: The capacity to refine early clinical classification criteria through synovial pathobiological markers offers the potential to predict disease outcome and stratify therapeutic intervention to patients most in need.

Keywords (5) Early Arthritis, Rheumatoid Arthritis Classification Criteria, Synovium pathotype, Ultrasound-guided biopsy.

INTRODUCTION

The introduction of new classification criteria for Rheumatoid Arthritis (RA) in 2010 [1] has been demonstrated to be clinically useful with enhanced diagnostic sensitivity in early disease compared to 1987 criteria [2]; however, this is balanced by a lower specificity [3,4]. This is of particular importance, as data suggest that approximately 40% of patients with early inflammatory arthritis, not fulfilling 1987 criteria, may spontaneously remit whilst approx. 30% will progress to RA [5]. Critically the mechanisms undelying the transition from undifferentiated arthritis (UA) to RA remain unknown though it has been suggested that qualitative or quantitative difference within synovial tissue may contribute to diverse disease evolution and/or treatment response [6,7]. Thus, pre-treatment stratification of early inflammatory arthritis is important in order to target therapy to poor prognosis patients. Previous data suggest that stratifying early arthritis according to RA2010 vs RA1987 classification criteria reveals significant clinical heterogeneity in diagnosis at 2 year follow up [8] although subsequent analysis of synovial tissue did not suggest that such clinical heterogeneity translated to significant differences in synovial pathobiology [9]. However, recently published data from a cohort of 144 early RA patients has demonstrated that synovial cellular and molecular signatures define prognostic and treatment response phenotypes [10]. Importantly whether clinical heterogeneity associated with the introduction of the 2010 ACR/EULAR criteria can be explained by synovial pathobiological signatures and whether they associate with subsequent disease outcome, up to now, remains unknown.

Therefore, the aim of this study was to investigate whether in patients with early inflammatory arthritis synovial cellular and molecular signatures: (i) segregate according to clinical classification (RA1987 vs RA2010 vs UA) (ii) change depending on symptom duration and, (iii) determine prognosis including subsequent requirement for biologic therapy.

PATIENTS AND METHODS

Patients

200 consecutive ~~patients with~~ inflammatory arthritis patients recruited at Barts Health NHS Trust as part of the multi-centre pathobiology of early arthritis cohort (http://www.peac-mrc.mds.qmul.ac.uk) were included within the study. P~~All p~~atients were treatment naïve (csDMARD and steroid) and had <1 year symptoms.

At baseline patients underwent collection of routine demographic data and were categorised according to the following criteria: (i) RA1987 [2] or (ii) UA. 2010 ACR/EULAR criteria for RA [1] were then applied to further classify patients with UA, resulting in three ~~final~~ groups: (i) RA1987 (RA1987+/RA2010+), (ii) RA2010 (RA1987-/RA2010+) and (iii) UA (RA1987-/RA2010-). An ultrasound (US) guided synovial biopsy of a clinically active joint was ~~then~~ performed [11]. Patients were then commenced on standard conventional synthetic (cs)DMARD therapy with a treat-to-target approach to treatment escalation (DAS28<3.2). Patients failing csDMARD therapy were commenced on biologic therapy (anti-TNF, Tocilizumab or Rituximab) according to the prevailing UK National Institute for Clinical Excellence (NICE) prescribing algorithm if they continued to have a DAS28>5.1 following 6 months of therapy [12]. At 12 months follow-up patients were categorised as~~according to the~~ follows~~ing~~: i. self-limiting (SL) disease (DAS28<3.2 and off csDMARD/steroid therapy) vs persistent disease (PD) (DAS28>3.2 and/or csDMARD) and ii. Symptomatic treatment (non-steroidal anti-inflammatories) treatment vs csDMARD therapy vs Biologic+/-csDMARD therapy.

Synovial biopsy collection and processing

A minimum of 6 biopsies per patient were collected for paraffin embedding and if intact lining layer identified underwent histopathological assessment. Synovitis score was determined using

a previously validated scoring system [13]. Following immunohistochemical staining of sequentially cut slides using previously reported protocols for B cells (CD20), T cells (CD3), macrophages (CD68) and plasma cells (CD138) the degree of immune cell infiltration was assessed semi-quantitatively (0-4) [14]. Biopsies were ~~then~~ stratified into 1 of 3 synovial pathotypes according to the following criteria: i) Lympho-myeloid presence of grade 2-3 CD20+aggregates, (CD20≥2) and/or CD138>2 ii) diffuse-myeloid CD68 SL≥ 2, CD20≤1 and/or CD3≥1, CD138≤2 and iii) pauciimmune CD68 SL<2 and CD3, CD20, CD138<1

Nanostring analysis

A minimum of 6 synovial samples per patient were immediately immersed in RNA-Later and RNA extraction performed as previously described [10]. RNA samples then underwent profiling for expression of 238 genes preselected based on previous microarray analyses of synovial tissue from patients with established RA [15] and/or relevance to RA pathogenesis. Raw NanoString counts were processed using the NanoStringQCPro package in R 3.2.0. Counts were normalised for RNA content by global gene count normalisation and then log transformed (base 2). The validity of normalisation was then checked via box- and scatter plots of normalised counts. Benjamini-Hochberg method was used to adjust for multiple testing, and genes were considered to be differentially expressed if they demonstrated an FDR-adjusted p-value <0.01.

Statistical analysis

Statistical analyses were run using R.3.0.2. For three way comparisons, Kruskal-Wallis test was used for continuous ~~variables~~ and Chi-squared or Fisher's exact test used for categorical variables as appropriate. A p-value <0.05 was considered statistically significant. Post hoc comparison tests were performed using Dunn test or Bonferroni correction as appropriate.

Linear regression models: Logistic regression using forward, backward and bidirectional stepwise selection was employed using the glm function in R.

Gene expression predictors were selected by L1 (LASSO) sparse logistic regression using R package glmnet. The penalty parameter λ was optimised using 10-fold cross-validation. λ corresponding to the minimum mean cross-validated error was retained as final penalty parameter in the model.

Predictive performance evaluation: P~~The p~~redictive performance of the final prediction model was assessed by computing the area under the receiver operating characteristic curve (AUC), using both apparent and internal validation with 95% CI. Internal validation using a bootstrap method [16,17] (performed with R package boot version 1.3-18) was employed to correct for over-fitting, to generate unbiased optimism-adjusted estimates of the C statistic (AUC) with low absolute error. Bootstrap estimate of the AUC statistic was computed by random sampling with replacement 500 times to enable estimation of the optimism corrected AUC.

RESULTS

Patient demographics and clinical correlations

200 PEAC patients were included, 128/200 (64%) patients were classified as RA1987 (RA 1987+/RA2010+) and 72/200 (36%) as UA. Of the UA patients, 25 were further classified as RA2010 (RA1987-/RA2010+) (25/200, 12.5%) and 47 remained as UA (RA1987-/RA2010-) (47/200, 23.5%) (Figure 1A). No significant difference in mean age, disease duration or ESR ~~level~~ between groups was demonstrated. However, the RA1987 group had significantly higher levels of CRP, TJC, SJC, DAS28, RF, ACPA and VAS and significantly higher numbers of patients sero positive for RF and ACPA compared to either the RA2010 or UA groups (Figure 1B). SJC and ACPA titre were the only clinical parameters with significant differences between

the RA2010 and UA groups, indicating that in terms of clinical measures of disease activity these two groups are relatively homogenous.

Synovial pathotypes distinguish clinical phenotypes regardless of disease duration

Synovial biopsies were obtained predominantly from small joints (81.5%) (Figure 2A). Patients with synovial tissue suitable for histological analysis (166/200) were segregated according to baseline synovial pathotype (Figure 2B) and differences in clinical parameters evaluated. We demonstrated significantly higher mean DAS28 within the lympho-myeloid compared to either the diffuse-myeloid or pauciimmune group (5.82 vs 4.93 vs 4.86, $p<0.001$). Mean CRP was ~~also~~ significantly higher in the lympho-myeloid and diffuse-myeloid vs pauciimmune groups (16.86 vs 15.52 vs 9.55, $p<0.001$) and a significantly higher number of patients were sero-positive for either RF ($p=0.012$) or ACPA ($p=0.011$) within the lympho-myeloid group (Figure 2C). To evaluate whether disease duration influenced prevalence of synovial pathotype, patients were stratified into four groups according to disease duration at baseline (1-3m, 4-6m, 7-9m and 10-12m) and frequency of synovial pathotype determined. No significant differences in synovial pathotype frequency at each time point was demonstrated ($p=0.65$) (Figure 2D).

RA1987 patients display significantly higher levels of synovial immune cell infiltration compared to RA2010 and UA patients

Patients were segregated according to pathotype and further into RA1987, RA2010 and UA categories. A ~~numerically~~ higher proportion of patients within the RA1987 group were categorised as lympho-myeloid (vs diffuse-myeloid or pauciimmune) (43.5% vs 33% vs 23.5%) (Figure 3A). We also demonstrated a significantly higher mean synovitis, CD3+ T cell, CD20 +B cell, CD138+ plasma cell and CD68+ SL/L macrophage score between the RA1987

group and both the RA2010 and UA groups ($p<0.001$) (Figure 3B). WInterestingly we saw no significant differences in synovitis score, mean CD3+T, CD20+B, CD68+ L or SL macrophage or CD138+ plasma cell number between the RA2010 and UA group (Figure 3B), indicating that these two groups are relatively homogenous in terms of tissue pathology.

Synovial genes regulating B cell activation and function are significantly upregulated in RA1987 patients compared to the RA2010/UA groups.

145/200 patients had RNA available for nanostring analysis (95/128 RA1987, 12/25 RA2010 and 38/47 UA patients) and were analysed for differential gene expression (238 genes) between diagnostic groups.

Comparing RA1987 vs RA2010 groups we demonstrated a significant differential expression of 53 genes (Figure 3C). In line with the histological analysis a number of differentially upregulated genes within the RA1987 cohort were involved in mediating B cell activation/function (e.g. *CD79A*, *CD38*, *IGJ*, *CXCL13*, *IRF4*, *CCL19*, *CD38*, *TNFA*, and *IL6*). When evaluating gene expression between the RA1987 and UA groups we found a similar trend with differential upregulation of a number of genes within the RA1987 cohort mediating B cell activation/function although only *CXCL13* remained significant following correction for multiple comparisons (Figure 3D). Conversely when evaluating gene expression between the RA2010 and UA cohorts only 7 genes appeared as significant with a preponderance of differentially upregulated genes within the RA2010 cohort mediating cartilage biology (*COMP*, *DKK3*, *INHBA*) and none remaining significant after correction for multiple comparisons (Figure 3E).

Classification as RA1987 criteria at disease onset predicts persistent disease at 12 months

~~In~~ 190/200 patients had~~with~~ 12 month follow up data available, we examined whether baseline synovial pathotype was associated with disease evolution. 119/121 (99%) RA1987 patients and 19/22 (90%) RA2010 had PD (Figure 4A). Within the UA cohort 11/47 (23%) had other diagnoses ~~(Figure 4A)~~. Of the remaining 36 patients, 26/36 (72.2%) had PD, and 10/36 (27.8%) SL. Of the UA patients with PD 4/26 (15.3%) progressed to fulfil 2010ACR/EULAR criteria RA at 12 months. Results demonstrated a significantly higher proportion of patients with SL disease in the UA group compared to the RA2010 or RA1987 groups and a significantly higher number of patients within the RA1987 group with PD (Figure 4B). When ~~we~~ evaluat~~inged~~ the effect of baseline pathotype we demonstrated ~~a~~a ~~numerically~~ higher proportion of patients with a lympho-myeloid vs diffuse-myeloid or pauciimune pathotype (39% vs 32% vs 13%) with PD and a ~~numerically~~ higher number of patients with a diffuse-myeloid vs lympho-myeloid or pauciimmune pathotype (54% vs 18% vs 27%) with SL (Figure 4C).

A baseline lympho-myeloid pathotype significantly associates with 12 month requirement for biologic therapy.

Patients stratified according to diagnostic group or pathotype were further classified according to 12 month treatment requirement: i. symptomatic treatment, ii. csDMARDs or iii. biologics+/-csDMARDs. A significantly higher proportion of RA1987 patients required biologic compared with RA2010 and UA (27.82% vs 20.83% vs 10.63%) ($p<0.001$) (Figure 5A) and importantly, lympho-myeloid (vs diffuse-myeloid or pauciimmune) pathotype significantly associated with 12 month requirement for biologic therapy ~~when patients were classified as lympho-myeloid vs diffuse-myeloid or pauciimmune~~ (57% vs 21% vs 21% $p=0.02$) (Figure 5B).

We then compared expression of the 238 genes in the Nanostring panel between patients requiring biologic therapy (n=34) or not (n=106) and found 119 differentially expressed genes. Patients requiring biologic therapy had~~, similarly to the RA1987 group,~~ significantly higher differential upregulation of genes regulating B and T cell proliferation, differentiation and activation (e.g. *TNFRSF13C, CD79A, CD2, CD3E and CD38*), genes involved in matrix metallopeptidase production/regulation (e.g. *MMP1* and *TIMP1*), genes involved in cytokine mediated cellular activation (*TNFA, TRAF3IP3, IFNA1*), and osteoclastogenesis inhibition (*DEF6*)~~ (Figure 5C)~~. Patients who did not require biologic therapy expressed some B and T cell regulation genes and B proliferation markers but mostly markers of fibroblast proliferation and cartilage turnover (Figure 5C).

To determine whether disease duration~~ also~~ influenced outcome we segregated patients according to 12 month treatment ~~(with~~ biologic therapy or not~~)~~ and further into ~~quartiles of~~ disease duration quartiles (Figure 5D) and~~, we~~ demonstrated no significant differences ~~between groups~~ in terms of disease duration at diagnosis. Next, we segregated patients treated with biologic therapy (n=39) according to quartiles of disease duration and then~~further into~~ synovial pathotype. We found no significant differences in patient number in each quartile (P=0.3) (Figure 5E). These results strongly suggest that synovial pathotype rather than disease duration influences 12 month treatment outcome.

Synovial gene expression signatures enhance the performance of clinical prediction models for biologic requirement

To determine whether baseline clinical and gene expression data could be combined into a model for predicting requirement for biologic therapy, we used 2 complementary approaches: a logistic regression model to identify ~~the most~~ predictive clinical covariates, and a penalized

method based on logistic regression with an L1 regularisation penalty (LASSO) to identify genes ~~that~~ improvinge ~~the~~ clinical model.

9 baseline clinical covariates were considered as candidates in the regression model: disease duration, ESR, CRP, RF, ACPA, TJC, SJC, DAS28, and pathotype (two categories, lympho-myeloid vs pauciimmune/diffuse-myeloid). Logistic regression models using backward forward and bidirectional stepwise selection resulted in selection of the same set of clinical covariates: DAS28, pathotype, CRP and TJC. The apparent predictive performance of the model evaluated by AUC was 0.78 (95% CI=0.70-0.87).

Genes were selected to improve the clinical model using logistic regression with an L1 regularization penalty (LASSO) applied on the 4 clinical covariates selected by the previous logistic regression and the 119 genes identified as being significantly differentially expressed between the biologic and non-biologic groups. Models in which clinical predictors were penalised or subject to forced inclusion were compared. When all ~~the~~ predictors were penalised, 11 predictors were retained in the final model and when the clinical covariates were not penalised, 13 predictors were retained ~~in the final model~~ (Figure 6A). In both the penalised and unpenalized clinical model the apparent prediction performance was improved (apparent AUC=0.89, 95% CI=0.83-0.95 and AUC=0.90, 95% CI=0.84-0.95) (Figure 6B). We additionally performed internal validation to correct the AUC performance measure for over-fitting by calculating the optimism of the AUC for each model by boot-strapped sampling with replacement from the original dataset. The optimism corrected AUC was 0.75 for the pure clinical model and 0.81 for the clinical and gene model (LASSO) (Figure 6C and 6D) suggesting that including both clinical covariates and genes in the model results in an improvement of the predictive ability of the model.

DISCUSSION

These results present a number of novel findings: firstly they strongly suggest that early inflammatory arthritis patients not fulfilling RA1987 criteria display similar clinical, synovial histological and molecular features irrespective of further classification according to RA2010 or UA criteria. Secondly these data also suggest that a lympho-myeloid pathotype at disease onset predicts poor outcome with patients subsequently requiring biologic therapy irrespective of clinical classification, and finally that integration of histological and molecular signatures into a clinical prediction model enhances sensitivity/specificity for predicting whether patients will require biologic therapy.

To the best of our knowledge these results emerge from the largest synovial tissue treatment-naïve early arthritis cohort reported to date and support previous data from early RA cohorts suggesting that a synovial immune cell infiltrate characterised by a predominant infiltrate of B cells associates with more active disease [18] and sero-positivity for RF and ACPAPA ~~[13]~~ [10]. ~~.~~ The results ~~in this cohort~~ suggest that this effect also extends to patients within the UA cohort. The clinical similarities between RA2010+/RA1987- patients and those with UA has been reported previously [8] and the data presented herein provides a pathophysiological explanation for this with the demonstration of homogeneous synovial cellular and molecular signatures among the two groups. The data show a lower percentage of patients requiring biologic therapy in RA2010+/RA1987- group, in line with the expectation that the ACR/EULAR 2010 criteria enable an earlier diagnosis and thus efficacious treatment. However, it is also possible that this group has a milder pathology from the beginning.

Although synovial pathotypes per se do not appear to distinguish between patients at risk of developing PD rather than SL disease, this is not surprising given the early and treat-to-target approach pursued in the study rather than observing untreated natural disease evolution.

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However when applying 12 month biologic requirement as a prognostic outcome we demonstrated that patients with a lympho-myeloid pathotype with a dense synovial infiltrate enriched in B cells and significant upregulation of T/B cell genes at disease onset predicted requirement for subsequent biologic therapy and critically that this was independent of disease duration. These results are consistent with recently published data in early RA which reports that thea lympho-myeloid pathotype is associateding with both highly agressivective disease and worse radiographic outcomes [10]. The current study presented reinforces these findings demonstrating that, at 12-months follow-up, a significantly higher proportion of patients classified as lympho-myeloid pathotype required biologic therapy. The study ~~through application of an alternative prognostic outcome (requirement for biologic therapy) but also~~~~These results~~ calls into question the current dogma surrounding "an early window of opportunity" for all patients with RA [19–21], suggesting that pathotype rather than simply disease duration influences outcome and that intensive therapeutic regimens should be targeted to poor prognostic pathotypes. This notion is supported

by the demonstration that the integration of synovial histological and molecular markers into a clinical prediction model for biologics use improves sensitivity/specificity from ~~from~~ 78.8% to 89-90% independently from disease duration.

~~The fact that the majority of biopsies were performed on small joints may also explain the differences~~ Discrepancy with previously reported data suggesting that synovial heterogeneity does not relate to clinical phenotypes [9], maybe explained by the fact that in our study the majority of biopsies were performed on small joints while in~~as~~ that cohort arthroscopic biopsy was restricted to patients with mainly large joint involvement ~~risking significant~~and, thus, a potential selection bias [22]. Additionally, the paired histological and molecular data in the largest ~~pathobiological~~ biopsy-driven early arthritis cohort ~~(200 patients)~~ reported to date ensured internal validation and high classification accuracy.

Our study does have limitations however, for example the real-life nature of the study did not permit the true evaluation of the natural history of the disease or outcome, as no patients were left untreated and therapy was not actively withdrawn. Also a treat to target approach, treatment escalation and initiation of biologic therapy was determined by treating physicians according to NICE guidelines rather than study protocol.

Within these limitations, our results are robust and suggest that the introduction of the new RA2010 classification criteria brings additional clinical and biological heterogeneity into early patient classification compared to the 1987 criteria with limited ability of RA2010 criteria alone to predict poor outcome. The demonstration that the integration of synovial pathobiological markers into clinical algorithms predicting poor outcome (requirement for biologic therapy) independent of disease duration suggests that the "window of opportunity" is wider than 6 months and early stratification of biologic therapies according to poor prognostic synovial pathobiological subtypes at disease onset may improve the outcome of these patients.

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Competing interest None

Ethics approval The study received local ethical approval (REC 05/Q0703/198) and all patients gave written informed consent.

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Figure Legends

Figure 1. Baseline Patient Demographics.

A. **Baseline classification of patients.** 200 patients were classified into RA1987 vs undifferentiated arthritis (UA). RA 2010 ACR/EULAR Criteria was then applied to UA patients. Final 3 groups obtained showed 47 patients UA (RA 1987-/RA2010-), RA 2010 (RA1987-/RA2010+), RA 1987 (RA1987+/RA2010+).

B. **Demographics according to classification criteria**. Data are presented as mean (SD, standard deviation) for continue variables and frequency and percentages for categorical variables. Baseline characteristics between the 3 groups were compared using Kruskal-Wallis or Fisher's exact test as appropriate. For post hoc comparison, Dunn tests were run and p-value from pairwise comparison reported in the last 3 columns of the table. ESR: Erythrocyte sedimentation rate ; CRP: C-reactive protein; 28TJC: 28 tender joint count; 28SJC: 28 swollen joint count; DAS28: Disease Activity Score 28 joints; RF titre: Rheumatoid factor titre (IU/ml); ACPA Titre: Anti-citrullinated protein antibody titre (IU/L); RF +ve: rheumatoid factor serum positive (>15IU/L); ACPA +ve: Anti-citrullinated protein antibody (>20IU/L).

Figure 2. Patient demographics and disease activity: comparison between pathotypes.

A. **Number of biopsy procedures per joint** MCP (Metacarpophalangeal), MTP (Metatarsophalangeal), PIP (Proximal Inter phalangeal).

B. **Representative images of synovial pathotypes.** H&E: Haematoxylin & Eosin. Sections underwent immunohistochemical staining and semi-quantitative scoring (0-4) to determine the degree of CD20+ B cells, CD3+ T cells, CD68+ lining (l) and sublining (sl) macrophage and CD138+ plasma cell infiltration. Sections were categorised into three pathotypes: (i) Pauci-iumne (CD68 SL<2 and or CD3, CD20, CD138<1), (ii) Diffuse-Myeloid: (CD68SL>2, CD20<1 and or CD3>1) and (iii) Lympho-Myeloid: (grade 2-3 CD20+ aggregates, CD20>2). Arrow heads indicate positive stain cells. Empty arrows indicate B cell aggregates.

C. **Demographic Analysis by Pathotype.** Data are presented as mean and standard deviation (SD) for numerical variables and frequency and percentage for categorical variables. Baseline characteristics between the 3 pathotypes were compared using a

Kruskall-Wallis test and Fisher-test (RF and ACPA positivity) as appropriate. Post hoc analysis for significant differences using Dunn test for multiple comparison. A *P*-value of <0.05 was considered statistically significant**.**

D. **Pathotype according to disease duration (months) at diagnosis.** Absolute values (N) and percentage. A *P*-value of <0.05 was considered statistically significant.

Figure 3. Variation in synovial pathobiology according to clinical classification of patients.

A. **Baseline clinical classification compared with pathotype.** Baseline subgroups (RA 1987, RA2010 and UA) were compared with pathotype. Fisher test used for analysis.

B. **Immune cell infiltration for each clinical subgroup.** Kruskal-Wallis test for comparison between 3 groups. Post hoc analysis for significant differences using Dunn test for multiple comparison.

C. **(C-E) Gene expression analysis for comparison between subgroups.** T-test for comparison and Volcano plot for representative image. Positive values represent upregulation and negative values downregulation. Green circles above green horizontal line represents non-corrected for multiple analysis expressed genes between groups. Red circles above red line represents corrected p-values (Benjamini-Hochberg method) for multiple analysis. **(C)** Volcano plot RA 1987 vs RA 2010: Difference in gene expression between patient fulfilling RA 1987 ACR criteria and RA 2010 ACR/EULAR Criteria. **(D)** Volcano plot RA 1987 vs UA: Difference in gene expression between patient fulfilling RA 1987 ACR criteria and Undifferentiated Arthritis**. (E)** Volcano plot RA 2010 vs UA: differences in gene expression between patient fulfilling RA 2010 ACR/EULAR criteria and UA.

Figure 4. Disease evolution.

A. **Patient classification after 12 months follow up.** Disease outcome after 12 months of follow up for each of the initial baseline subgroups (RA1987/RA2010/UA). Disease evolution classified as self-limiting or persistent disease. Other diagnosis as described for those who were re-classified after 1 year form UA cohort**.**

B. **Disease evolution by subgroups.** Disease evolution was compared with Baseline subgroups (RA 1987, RA2010 and UA). Fisher test used for analysis.

C. **Disease evolution by pathotype.** Disease evolution was compared with pathotype (Pauci-imune vs Diffuse-Myeloid vs Lympho-Myeloid. Fisher test used for analysis. A *P*-value of <0.05 was considered statistically significant.

Figure 5.

A. Comparison between diagnostic subgroups and treatment outcome at 12month follow up. Treatment required was divided in 3 groups: (i) No treatment; (ii) csDMARDs only, (iii) csDMARDs +/- Biologics. Fisher test for analysis**.**

B. Comparison between pathotype and treatment outcome at 12 months.

C. Gene expression analysis, represented in a Volcano plot comparison between patient requiring Biologics vs non-biologic group. T-test comparison for gene difference expression between groups. Positive values represents upregulation and negative values downregulation. An adjusted (Benjamini-Hochberg correction for multiple analysis) *P*-value of <0.01 was considered statistically significant, represented as dots above red line. Green dots above green line for gene expression significance when no correction applied for multiple analysis (*P value <0.05).* **D. Treatment outcome according to baseline disease duration.** Fisher test for analysis. **E. Pathotype according to baseline disease duration for Biologic patient cohort.** Fisher test for analysis. A *P*-value of <0.05 was considered statistically significant unless otherwise stated.

Figure 6. Prediction model.

A-B Identification of clinical and gene expression features predictive of biologic therapy use at 1 year. Logistic regression, coupled with backward and stepwise model selection was applied to baseline clinical parameters against a dependent variable of Biologic therapy use or not at 12 months to select which clinical covariate contributed the most to the prediction. Selected covariates (119 genes+4 clinical covariates) were entered simultaneously into a logistic model with an L1 regularization penalty (LASSO) in order to determine the optimal sparse prediction model. A similar predictive performance of the model when clinical was seen when results were penalized (blue dashed line, figure 6A) than when they were not penalized (red dotted line, figure 6A) with a slightly different set of selected covariates (Figure 6B). Figure 6B shows the non-zero weights associated with the final variables selected by the LASSO regression. The grey spaces represent the variables that were not selected by the model.

C-D Lambda training curve from the final glmnet fitted model. The red dots represent mean binomial deviance using 10-fold cross-validation. The error bars represent standard error of binomial deviance. The vertical dotted lines indicate minimum binomial deviance (λ_{min}) and a more regularized model for which the binomial deviance error is within one standard error of the minimum binomial deviance (λ_{1se}). λ_{min} was selected, corresponding to 11 non-zero coefficients in the final model for the LASSO where clinical were penalized (Figure 6C) and 13 non-zero coefficients in the final model for the LASSO where clinical were not penalized (Figure 6D).

Key messages:

What is already known about this subject?

The introduction of ACR/EULAR RA classification criteria have impacted positively on early diagnosis and treatment RA leading to better outcomes. By the same token, broader criteria have led to the inclusion of patients with milder and more heterogenous disease. This, together with the inability to precisely predict disease prognosis and treatment response at the individual patent levels, emphasise the need to identify patients at risk of accelerated structural damage

progression and fast-track aggressive/biologic therapies to patients with poor prognosis.

What does this study add?

This study analyses the largest biopsy-driven early inflammatory arthritis cohort to date (200 patients) and, through a detailed synovial cellular and molecular characterization refines ACR/EULAR disease classification. In addition, the study identifies synovial pathobiological markers associated with with the lympho-myeloid pathotype and the requirement of biologic therapy ~~requirement~~ at 12 months, reinforcing recently published data the indicates that these patients are affected by highly agressive disease and worse radiographic outcome. Notably, these findings are independent from the time of diagnosis within the first 12 months of symptoms initiation, suggesting that the so called "window of opportunity" is wider than 6 months and early stratification of biologic therapies according to poor prognostic synovial pathobiological subtypes at disease onset may improve the outcome of these patients. The integration of ~~such~~ synovial pathobiological markers into a logistic regression model improves the prediction accuracy from 78.8% (clinical) to 89-90% (clinical + molecular) and enables the identification at disease onset of patients who subsequently require biologic therapy. Thus, this study provides support to the notion that biologic therapies should be started early in patients with poor prognosis.

How might this impact on clinical practice or future developments?

The identification at disease onset of patients who are unlikely to respond to csDMARDs, remains a major unmet need. The capacity to refine early clinical classification criteria through application of synovial pathobiological markers and the ability to identify patients who

subsequently require biologic therapy at disease onset offers the opportunity to stratify therapeutic intervention to the patients most in need. This present study adds weight to the need to change current therapeutic algorithms and start biologic therapies at disease onset in patients with poor prognosis. This is likely to have a major impact on disease control/remission and long-term disability, as notionally supported by numerous early intervention studies using biologic therapies.

We thank the reviewer for his/her helpful comment and have replied to the specific point below:

Reviewer: 2

Comments to the Author
In the revised version of the paper from Lliso-Ribera et al. the authors have made minor edits to strengthen their main message.

The importance of the paper is unquestionable. However, the authors should point out the additional benefits of this paper as compared to their most recent paper published by Humby et al., (in particular Figure 5).

R: We agree with the reviewer that it is important to point out the additional benefits of this paper as compared to our most recent paper published (Humby et al. ARD 2019). As per the reviewer's specific reference to Figure 5, this shows four important novel aspects compared to our above previous publication: (i) the requirement for bDMARD is significantly greater for early RA when categorised as RA 1987 + / RA 2010 + (27.82%) compared to RA 1987 - / RA 2010 + (20,83%) versus UA (10,63%): $p<0.001$; (ii) the requirement for bDMARD is significantly greater for patients displaying the lympho-myeloid pathotype versus diffuse-myeloid versus pauciimmune: $p<0.02$; (iii) the above findings are independent from the time of diagnosis within the first 12 months of symptoms initiation, suggesting that the so called "window of opportunity" is wider than 6 months and early stratification of biologic therapies according to poor prognostic synovial pathobiological subtypes at disease onset may improve the outcome of these patients; (iv) it reports the identification of genes that improve on clinical prediction models on biologic requirement at 12 months.

The above 4 points have been now further emphasised throughout the manuscript and also we have modified the discussion accordingly, which now reads (page 13-14)

"Although synovial pathotypes per se do not appear to distinguish between patients at risk of developing PD rather than SL disease, this is not surprising given the early and treat-to-target approach pursued in the study rather than observing untreated natural disease evolution. However, when applying 12 month biologic requirement as a prognostic outcome we demonstrated that patients with a lympho-myeloid pathotype with a dense synovial infiltrate enriched in B cells and significant upregulation of T/B cell genes at disease onset predicted requirement for subsequent biologic therapy and critically that this was independent of disease duration. *These results are consistent with recently published data in early RA which reports that the lympho-myeloid pathotype is associated with highly aggressive disease and worse radiographic outcome [10]. The current study reinforces these findings demonstrating that, at 12-months follow-up, a significantly higher proportion of*

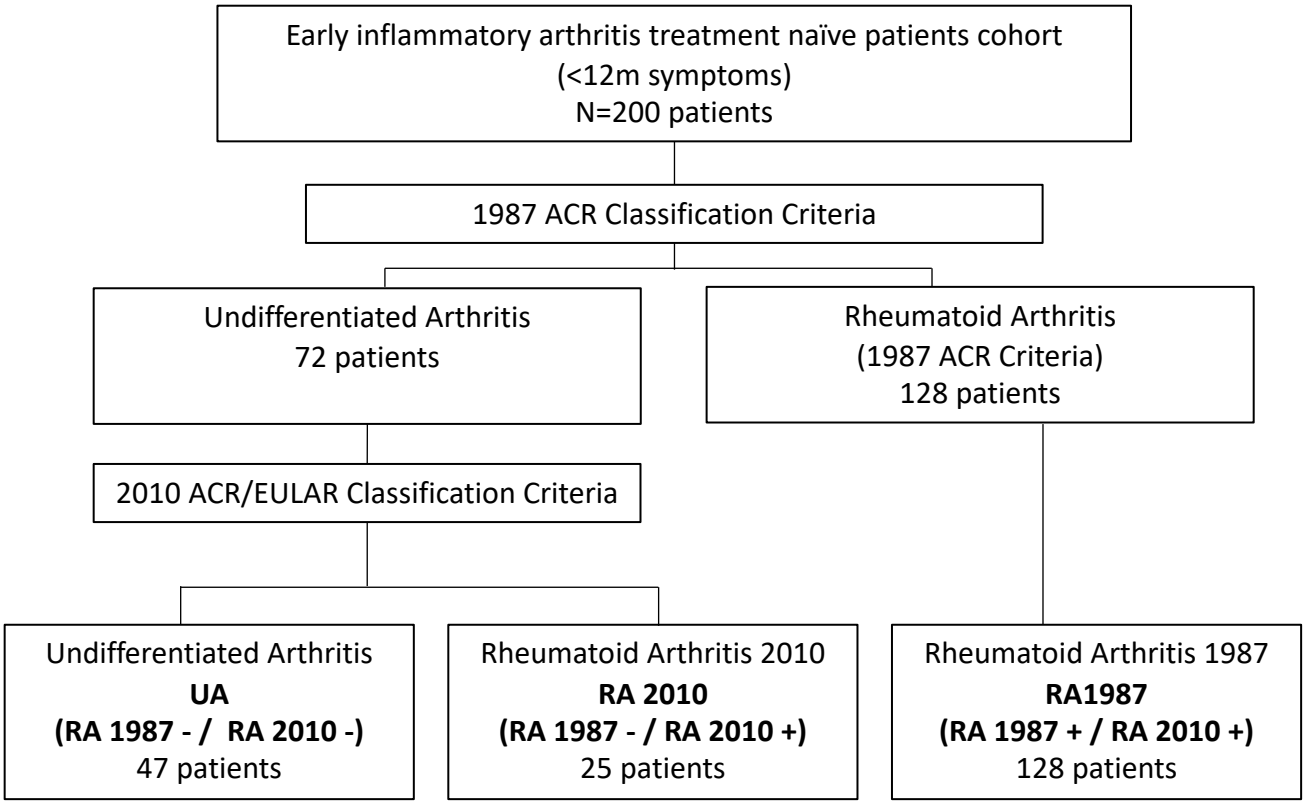
patients classified as lympho-myeloid pathotype required biologic therapy. The study also calls into question the current dogma surrounding "an early window of opportunity" for all patients with RA [18–20], suggesting that pathotype rather than simply disease duration influences outcome and that intensive therapeutic regimens should be targeted to poor prognostic pathotypes. This notion is supported by the demonstration that the integration of synovial histological and molecular markers into a clinical prediction model for biologics use improves sensitivity/specificity."

Moreover, we have emphasised the additional benefits of this paper compared to our above previous publication in the **Key messages: What does this study add?** Page 24 and 25.

Due to the insertion additional text we have also made minor edits throughout the manuscript to remain as close as possible to the 3000-word count (now 3031), which we hope is acceptable.

Figure 1

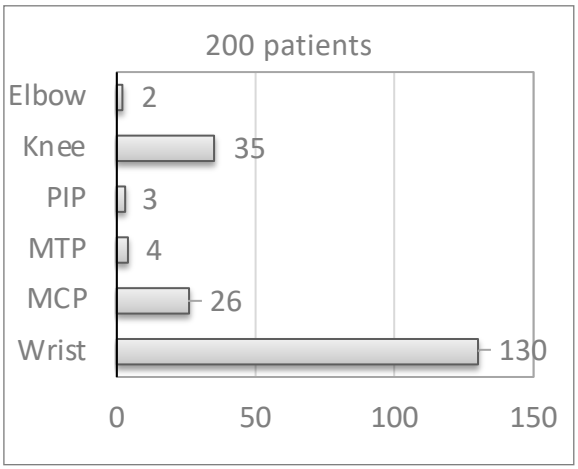
A



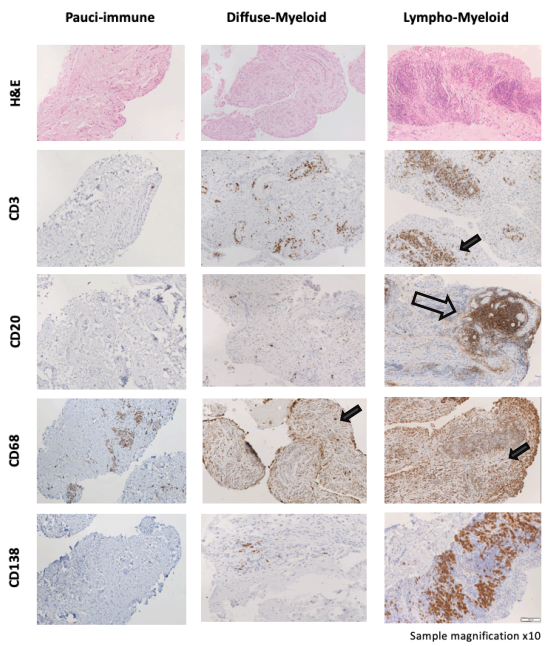
B

N 200	RA 1987 (RA 1987 + / RA 2010 +) N 128	RA 2010 (RA 1987 - / RA 2010 +) N 25	UA (RA1987 - / RA2010-) N 47	p-value	p-value (post-hoc) RA 1987-UA	p-value (post-hoc) RA1987-RA2010	p-value (post-hoc) RA2010-UA
Age (years). Mean (SD)	52.64 (16.02)	52.25 (12.54)	52.76 (15.33)	0.98			
Disease duration (months). Mean (SD)	5.64 (4.48)	10.47 (25.28)	6.11 (3.51)	0.91			
ESR. Mean (SD)	39.05 (19.69)	30.64 (30.06)	10.63 (21.51)	0.56			
CRP. Mean (SD)	17.82 (13.89)	14.6 (20.36)	7.21 (12.35)	0.03 *	<0.001 *	0.12	0.071
28 TJC. Mean (SD)	11.98 (7.29)	6.88 (5.72)	6.80 (6.79)	<0.001 *	<0.001 *	0.0012 *	0.74
28 SJC. Mean (SD)	7.68 (5.62)	5.68 (4.91)	3.10 (2.82)	<0.001 *	<0.001 *	0.042 *	0.031 *
Vas 28. Mean (SD)	5.76 (1.35)	4.73 (1.56)	4.001 (1.51)	<0.001 *	<0.001 *	0.002 *	0.13
Vas global disease activity. Mean (SD)	64.82 (24.80)	45.36 (28.78)	34.55 (29.27)	<0.001 *	<0.001 *	0.0043 *	0.17
RF titre. Mean (SD)	25.53 (22.49)	2.68 (2.95)	1.27 (1.42)	<0.001 *	<0.001 *	<0.001 *	0.21
ACPA titre. Mean (SD)	26.16 (18.42)	75.24 (175.40)	1.68 (10.56)	<0.001 *	<0.001 *	<0.001 *	0.01 *
RF +ve. N (%)	84 (65%)	7 (28%)	1 (2%)	<0.001 *			
ACPA +ve. N (%)	87 (68%)	6 (24%)	2 (4%)	<0.001 *			

A



B



C

N 166	Pauci-immune N 47	Diffuse-Myeloid N 57	Lympho-Myeloid N 62	P value	P-value (post-hoc) Lymphoid-M vs Pauci-immune	P value (post-hoc) Lymphoid-M vs Diff-Myeloid	P value (post-hoc) Diff-Myeloid Vs Pauci-immune
Age (years). Mean (SD)	54.93 (13.37)	52.64 (17.84)	51.90 (16.11)	0.51			
D. Duration (m). Mean (SD)	9.21 (4.90)	9.30 (4.03)	9.54 (4.37)	0.98			
ESR. Mean (SD)	33.04 (21.68)	28.19 (18.49)	36.96 (19.48)	0.12			
CRP. Mean (SD)	9.55 ()13.45)	15.52 (14.68)	16.86 (12.96)	<0.001 *	<0.001 *	0.06	0.013 *
28 TJC Mean (SD)	10.38 (8.08)	8.70 (6.45)	11.22 (7.47)	0.09			
28 SJC. Mean (SD)	5.70 (5.38)	5.96 (4.93)	7.75 (5.73)	0.054			
DAS 28. Mean (SD)	4.86 (1.65)	4.93 (1.49)	5.82 (1.55)	<0.001 *	0.0012 *	0.002*	1
VAS. Mean (SD)	50.29 (26.87)	53.47 (31.33)	61.32 (27.94)	0.08			
RF +ve. N (%)	17 (64%)	27 (53%)	40 (65%)	0.012 *			
ACPA +ve. N (%)	15 (32%)	27 (47%)	43 (70%)	0.011 *			
RF titre. Mean (SD)	10.15 (15.40)	20.94 (23.95)	23.43 (22.74)	0.004 *	0.003 *	0.47	0.04 *
ACPA titre. Mean (SD)	16.16 (28.40)	19.67 (24.31)	43.79 (104.1)	0.002 *	0.007 *	0.06	0.29

D

N 166	1-3m N=54 N (%)	4-6m N=53 N (%)	7-9m N=37 N (%)	10-12m N=22 N (%)	P value
Pauci-immune	19 (34.5%)	22 (38%)	8 (28%)	8 (28.5%)	0.65
Diffuse-Myeloid	22 (40%)	17 (28%)	10 (31%)	5 (18%)	
Lympho-Myeloid	13 (23.5%)	14 (23%)	9 (28%)	9 (32%)	

Figure 3

A

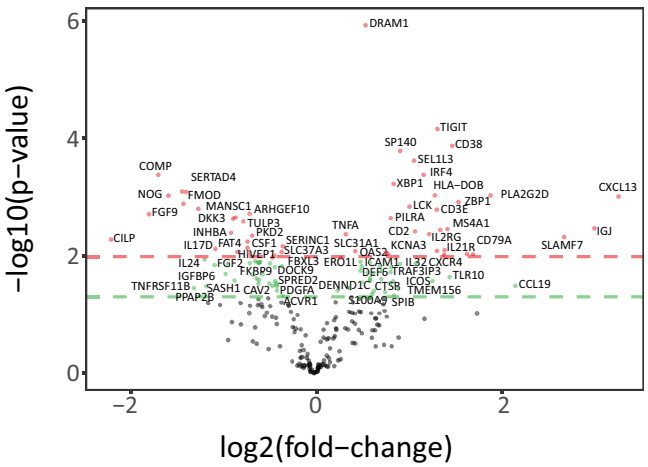
N 166	RA 1987 (RA 1987 + / RA 2010 +) N 155	RA 2010 (RA 1987 - / RA 2010 +) N 23	UA (RA1987 -/ RA2010-) N 40	p-value
Pauci-immune 47 N (%)	27 (23.5%)	6 (37.5%)	14 (40%)	0.10
Diffuse-Myeloid 57 N (%)	38 (33%)	5 (31.2%)	14 (40%)	
Lympho-Myeloid 62 N (%)	50 (43.5%)	5 (31.2%)	7 (20%)	

B

N 166	RA 1987 (RA 1987 + / RA 2010 +) N 155	RA 2010 (RA 1987 - / RA 2010 +) N 23	UA (RA1987 - / RA2010-) N 40	p-value	p-value RA1987 -UA	p-value RA 1987-RA 2010	p-value RA 2010-UA
CD3	3.19	1.21	0.60	<0.001*	<0.001*	<0.001*	0.36
CD20	2.88	0.80	0.75	<0.001*	<0.001*	<0.001*	0.80
CD68L	3.60	1.86	1.34	<0.001*	<0.001*	0.0023*	0.18
CD68SL	3.60	2.18	1.79	0.002*	<0.001*	0.002*	0.24
CD138	2.85	1.06	0.73	<0.001*	<0.001*	<0.001*	0.37
Synovitis Score	6.17	3.26	3.24	<0.001*	<0.001*	0.002*	0.45

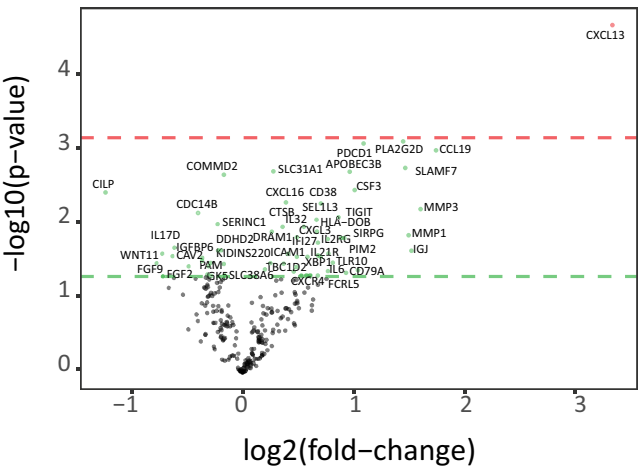
C. RA1987 vs RA2010

RA1987 subjects 95. RA2010 subjects 12
Non corrected significant genes (green): 55
BH corrected significant genes (red): 53



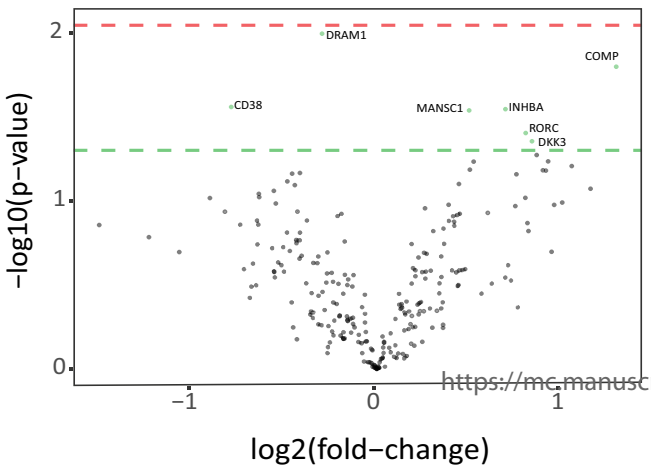
D. RA1987 vs UA

RA1987 subjects 95. UA subjects 38
Non corrected significant genes (green): 62
BH corrected significant genes (red): 1

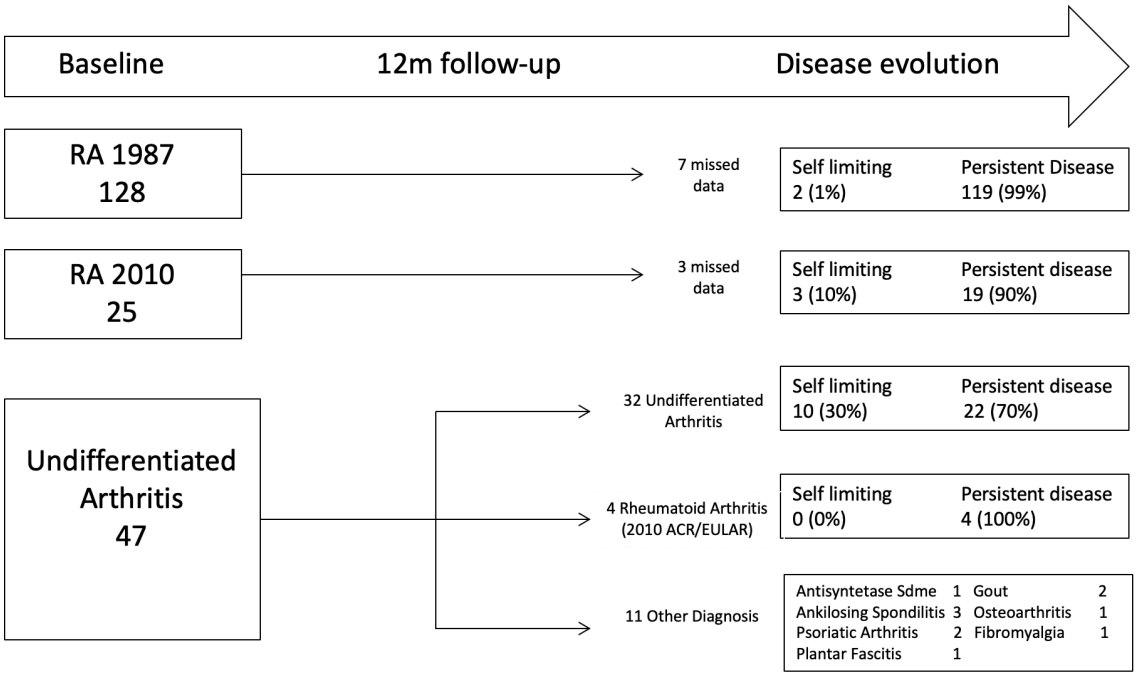


E. RA 2010 vs UA

RA2010 subjects 95. UA subjects 38
Non corrected significant genes (green): 7
BH corrected significant genes (red): 0



A



B

N 179	RA 1987 (RA 1987 + / RA 2010 +) N 121	RA 2010 (RA 1987 - / RA 2010 +) N 22	UA (RA1987 -/ RA2010-) N 36	p-value
Self limiting N 15 (%)	2 (13%)	3 (20%)	10 (64%)	<0.001*
Persistent disease N 164 (%)	119 (72%)	19 (12%)	26 (16%)	

C

N 147	Pauci-immune N 41	Diffuse-Myeloid N 50	Lympho-Myeloid N 56	p-value
Self Limiting N 11 (%)	3 (27%)	6 (54%)	2 (18%)	0.23
Persistent disease N 136 (%)	38 (13%)	44 (32%)	54 (39%)	

Figure 5

A

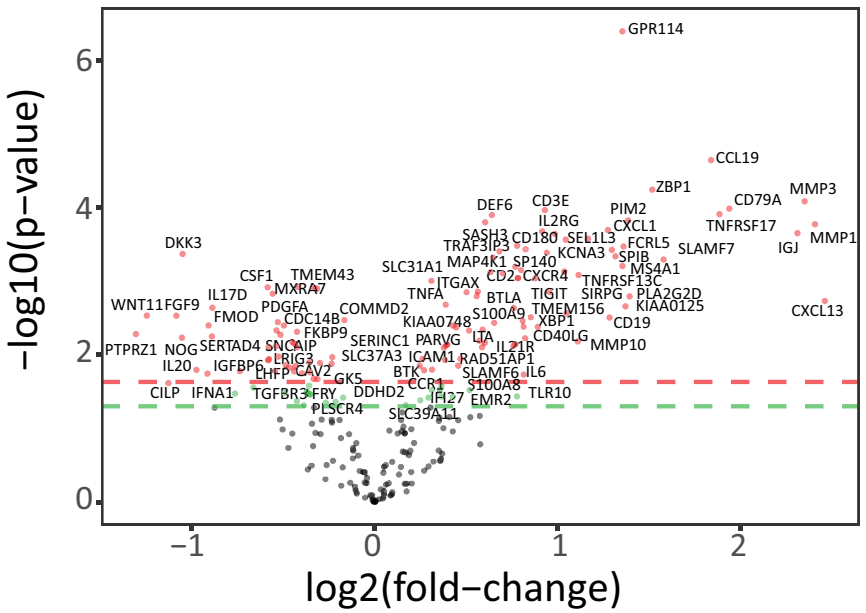
N 186	RA 1987 (RA 1987 + / RA 2010 +) N 115	RA 2010 (RA 1987 - / RA 2010 +) N 24	UA (RA1987 - / RA2010-) N 47	p-value
Symptomatic treatment N 23	2 (1.7%)	4 (16.66%)	17 (36,17%)	<0.001*
csDMARDs N 121	81 (70.43%)	15 (62.50%)	25 (53,19%)	
Biologics +/- csDMARDs N 42	32 (27.82%)	5 (20,83%)	5 (10.63%)	

B

N 153	Pauci-immune N 44	Diffuse-Myeloid N 52	Lympho-Myeloid N 57	p-value
Symptomatic Treatment N 14	6 (42%)	6 (42%)	2 (14%)	<0.02*
csDMARDs N 101	30 (29%)	38 (37%)	33 (33%)	
Biologics +/- csDMARDs N 38	8 (21%)	8 (21%)	22 (57%)	

C. Biologic vs NonBiologic

Biologic subjects 34. NonBiologic subjects 106
Non corrected significant genes (green): 23
BH corrected significant genes (red): 119



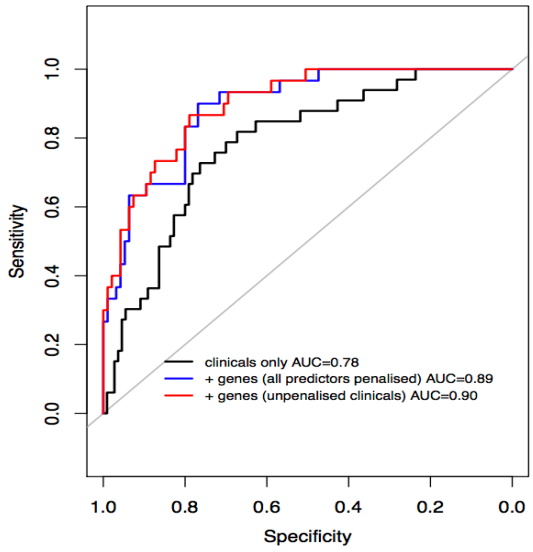
D

N 176	1-3m N 55	4-6m N 61	7-9m N 32	10-12m N 28	p-value
csDMARDs n 137	43 (30%)	43 (30%)	26 (18%)	25 (17%)	0.23
Biologics +/- csDMARDs n 39	12 (29%)	18 (43%)	6 (14%)	3 (7%)	

E

N 39 Biologic cohort	1-3m N 12	4-6m N 18	7-9m N 6	10-12m N 3	p-value
Pauciimmune	1 (58%)	5 (27%)	0 (0%)	2 (66%)	0.30
Diffuse - Myeloid	3 (25%)	1 (5%)	3 (50%)	0 (0%)	
Lympho - Myeloid	7 (58%)	9 (50%)	3 (50%)	1 (33%)	

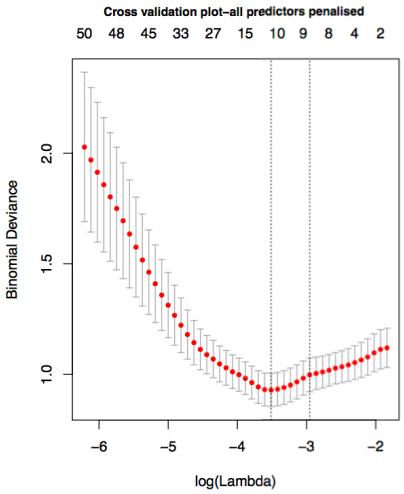
A



B

	All predictors penalised	Unpenalised clinicals
(Intercept)	-0.372	-3.572
Pathotype		-0.324
CRP	-0.015	-0.037
TJC		-0.061
DAS28	0.246	0.88
GPR114	0.242	0.295
IL8	0.26	0.265
CSF1	-0.08	-0.034
MMP3	0.051	0.047
LTB	0.017	
HIVEP1	-0.143	-0.182
IL20	-0.221	-0.239
UBASH3A	0.049	
MMP10	0.149	0.16
NOG		-0.038
IFNB1		-0.023

C



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