Early Prediction of Clinical Response to Anti-TNF Treatment using Multi-omics and Machine Learning in Rheumatoid Arthritis

Niyaz Yoosuf (niyaz.yoosuf@ki.se)
Karolinska Institutet, Department of Medicine, Solna  https://orcid.org/0000-0002-3792-5766

Mateusz Maciejewski
Pfizer Inc

Daniel Ziemek
Pfizer Inc

Scott A. Jelinsky
Pfizer Inc

Lasse Folkersen
Danish National Genome Center, Copenhagen, Denmark

Malin Müller
Karolinska Institutet, Department of Medicine, Solna

Peter Sahlström
Karolinska Institutet, Department of Medicine, Solna

Nancy Vivar
Karolinska Institutet, Department of Medicine, Solna

Anca Catrina
Karolinska Institutet, Department of Medicine, Solna

Louise Berg
Karolinska Institutet, Department of Medicine, Solna

Lars Klareskog
Karolinska Institutet, Department of Medicine, Solna

Leonid Padyukov
Karolinska Institutet, Department of Medicine, Solna

Boel Brynedal
Karolinska Institutet, Department of Medicine, Solna

Research Article

Keywords: Rheumatoid arthritis, anti-TNF, Methotrexate, treatment response, inflammation, peripheral blood mononuclear cells
Abstract

Objectives

Advances in immunotherapy by blocking TNF have remarkably improved treatment outcomes for rheumatoid arthritis patients. Although treatment specifically targets TNF, the downstream mechanisms of immune suppression are not completely understood. The present study was aimed to detect biomarkers and expression signatures of treatment response to TNF inhibition.

Methods

Peripheral blood mononuclear cells were obtained from 39 patients collected before anti-TNF treatment initiation (day 0) and after three months. Response to treatment was defined based on the EULAR criteria and classified 23 patients as responders and 16 as non-responders. We investigated differences in gene expression in peripheral blood mononuclear cells, the proportion of cell types and cell phenotypes in peripheral blood using flow cytometry, and the level of proteins in plasma. Finally, using biological measurements, we run machine learning models to predict non-response.

Results

The gene expression analysis in baseline samples revealed notably a higher expression of the gene EPPK1 in future responders. We also detected the suppression of genes and proteins following treatment, including suppression of expression of the gene, T-cell inhibitor CHI3L1, and its protein YKL-40. The gene expression results were replicated in an independent cohort. Finally, machine learning models mainly based on transcriptomics data showed high predictive utility (ROC AUC ± SEM: 0.81 ± 0.17) in classifying non-response to anti-TNF treatment in RA.

Conclusions

Our integrative multi-omics analyses identified new biomarkers for prediction of response, found pathways influenced by treatment and suggested new predictive models of anti-TNF treatment in RA patients.

Rheumatology Key Messages

- Expression of the CHI3L1 gene and its protein YKL-40 that regulates T cell activation was suppressed in responders following anti-TNF treatment.
- Machine learning models using transcriptomics data at baseline showed high predictive utility in classifying response to future anti-TNF treatment.

Background
Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation in symmetric joints that leads to pain and eventually bone destruction. RA is one of the most common autoimmune diseases which affects approximately 0.5 -1% of the world’s population\(^1\). Currently, there is no cure for RA, but several disease-modifying anti-rheumatic drugs (DMARDs) are used to treat patients with the disease. During a successful treatment course, inflammation in the joints decreases, resulting in disease remission or low disease activity\(^2,3\). Methotrexate (MTX) is recommended as the first-line treatment of early RA, however, at least 30 % of patients do not respond to the treatment where significant disease activity remains\(^4,5\). The patients who do not respond to first-line treatment are recommended for additional treatment, in most cases with drugs that inhibit tumor necrosis factor (TNFi). TNF is a pro-inflammatory cytokine secreted mainly by monocytes and macrophages, but also by other immune and non-immune cells, including fibroblasts and endothelial cells, involved in systemic inflammation.

The anti-TNF therapy has been used to treat RA for two decades, but one-third of treated patients do not respond or have poor responses\(^6\). Hence the prediction of the efficacy of treatments before initiating treatment would help patients to start effective treatment and decrease the number of adverse effects.

In the present study, we selected a well-characterized cohort that allowed us to investigate gene expression differences in peripheral blood mononuclear cells (PBMCs), the proportion of different cell types and cell phenotypes in peripheral blood measured using flow cytometry and the level of several proteins in serum. In addition, we developed models using machine learning techniques to predict non-response for the anti-TNF treatment.

**Materials And Methods**

**Patient Cohort**

In this study, patient samples were obtained from the COMBINE cohort that includes individuals treated with methotrexate (89 patients), treated with anti-TNF drugs (59 patients), or treated with a second biologic agent (31 patients) and healthy controls 60 individuals\(^7\). Healthy controls were recruited from the Swedish Blood Centre service in Uppsala closely matched with age and sex as closely matched with patient groups. The patients who did not respond adequately to MTX treatment (based on local rheumatologist’s judgment) were prescribed anti-TNF treatment in combination with MTX. Patients included in this study donated blood at the clinic before starting anti-TNF therapy and at the follow-up visit after approximately three months. Clinical assessments and routine blood sampling were made at both visits, and were used to calculate the disease activity score DAS28 CRP\(^8\). Of the 59 patients who started anti-TNF therapy, three patients dropped out before the scheduled three-month visit and therefore lacked clinical assessment. For RNA-seq analysis, we did not include patient samples with low quality or low quantity RNA samples. Due to the low number of male non-responders in our study cohort we, therefore decided to include only female patients (n=39) in our study (Supplementary Fig. S1). The clinical and demographic variables at baseline are shown in Table 1.
Table 1: Baseline demographic characteristics of female rheumatoid arthritis patients treated with anti-TNF. HAQ = Health Assessment questionnaire disability index; ACPA = anti-citrullinated peptide antibody; DAS28 = Disease Activity Score in 28 joints; CRP = C-reactive protein.

Response measures

We used the European League Against Rheumatism (EULAR) response criteria to classify patient response to treatment (9,10). In our analysis, we considered Good and Moderate EULAR responders as “responders”, and compared these to the EULAR “non-responders”.

RNA sequencing

The RNA was extracted from PBMCs, freshly isolated using CPT tubes (BD Biosciences), and sequenced as previously described (7). Of the 39 female RA patients, we obtained high-quality RNA-seq data from 28 patients at baseline (responders = 10; moderate-responders = 9; non-responders = 9) and 32 patients after treatment (responders = 11; moderate-responders = 9; non-responders = 12) that leads to a total of 25 patients with paired RNA-seq samples (both anti-TNF naïve and treated) (Supplementary Fig. S1). The sequencing reads were trimmed using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and then mapped to the GRCh38 human reference genome and subsequently gene counts were generated using Star v.2.5.3a (11).
**Results**

Gene expression signatures of response to anti-TNF treatment at baseline

The gene expression analysis identified 59 differentially expressed genes between future responders and non-responders, but most of these genes achieved significance (FDR ≤ 0.1) due to a single outlier sample showing different gene expression profile (Supplementary Fig. S2A-B). Therefore, we decided to exclude this patient sample (both anti-TNF naïve and treated) and a subsequent analysis (without the outlier sample) yielded 192 differentially expressed genes (FDR ≤ 0.1), including 103 genes with higher expression, and 89 genes with lower expression, in future responders. The top differentially expressed genes are represented in Fig. 1A (also see Table 2 and Supplementary Table. S1A).

To assess possible heterogeneity and detect consistent differential gene expression, we employed a leave-one-out (LOO) approach, where one sample is removed in each iteration and the association analysis was repeated. The genes are considered if they meet statistical significance (FDR < 0.1) in each iteration. The LOO approach yielded two genes, Epiplakin (EPPK1) and FosB Proto-Oncogene, AP-1 Transcription Factor Subunit (FOSB) with higher expression in responders in all 28 iterations (Table 2). An additional five genes, EGR1, EGR2, BCL6-AS1, IGLV10-54, and IGKV1D-39 showed significance (FDR < 0.1) in 27 LOO iterations. The genes EGR1, EGR2, and BCL6-AS1 were expressed higher in future responders, whereas immunoglobulin light chain genes IGLV10-54 and IKV1D-39 had lower expression in future responders. The genes EPPK1, BCL6-AS1 and CDC20 showed a clear expression difference between responders and non-responders (Fig. 1B). We also noticed 6 immunoglobulin light chain genes (IGLV10-54, IGKV1D-39, IGKV3-20, IGLV3-1, IGKV1-17, IGKV2-24) and 1 heavy chain gene (IGHV5-10-1) expressed lower in responders compared to non-responders, however these genes did not pass the LOO analysis criteria (Fig. 1A).
Table 2: Differentially expressed genes in future responders and non-responders before anti-TNF treatment. The iteration count is the number of leave-one-out iterations where the gene remained significant.

The gene set enrichment analysis based on ranking of differentially expressed genes (log2fold change) identified a total of 127 regulated pathways (Supplementary Table. S1B) between response groups. The pathway analysis reveal that responders were preferentially characterized by higher expression of genes involved in graft versus host disease, antigen processing and presentation, and AP-1 transcription factor network, whereas non-responders were characterized by higher expression of genes involved in cell cycle pathways, mainly - cell cycle mitotic activity, cell cycle checkpoints. The top 15 most upregulated and downregulated pathways are represented in Supplementary Fig. S3A; Supplementary Table. S1B).

Effect on gene expression in PBMCs during anti-TNF treatment

Since biological and technical confounders between individuals may significantly affect downstream analyses, using paired samples before and after treatment is the most preferable approach to address changes related to the treatment. We analyzed treatment effects on gene expression using 25 paired RA
patients without considering the response. The analysis identified 25 differentially expressed genes, of which 14 genes were suppressed whereas 11 genes showed a slight increase in expression (Supplementary Table. S1C). The genes BHLHE40 that controls cytokine production by T cells and CHI3L1, Chitinase-3-like protein were suppressed during treatment whereas the B cell novel protein 1 (alias FAM129C) and TTC21A were induced by treatment (Fig. 2A). The pathway analysis of differentially expressed genes did not show any enrichment of gene sets, but when using a less conservative threshold of FDR<0.1, we detected 114 gene sets regulated during the treatment (Supplementary Table. S1D). Induced genes are significantly enriched for genes involved in the RNA and protein metabolism, and interferon signaling, whereas suppressed genes are predominantly enriched for genes involved in hemostasis and signaling by GPCR (Fig. 2B).

Distinct gene expression signatures for response during anti-TNF treatment

The trajectory of gene expression changes may correlate with measured clinical outcomes. Therefore, we investigated the transcriptional changes in paired samples of responders (n=17) and non-responders (n=8) to anti-TNF treatment separately. Our analysis identified five genes regulated in responders, whereas no significant regulation was identified in non-responders. Of the five regulated genes in responders, C-X-C Motif Chemokine Receptor 2 (CXCR2), Myeloperoxidase (MPO), Myeloid Associated Differentiation Marker (MYADM), TNF Alpha Induced Protein 6 (TNFAIP6) were suppressed by treatment, whereas gene Low-Affinity Immunoglobulin Gamma Fc Region Receptor II-B (FCGR2B) was induced during anti-TNF treatment. The gene expression plots for all five genes using normalized counts show a clear difference in responders before and after anti-TNF treatment (Fig. 3A). Interestingly, for these genes we observed a similar trend of regulation in non-responders (same directionality), but it does not reach statistical significance (Supplementary Fig. S4). The gene set enrichment analysis of differentially expressed genes ranked based on foldchange identified 78 regulated pathways in responders (Supplementary Table. S1E). We noticed induction of pathways involved in the regulation of cell cycle mitotic and protein metabolism, whereas genes involved in the extracellular matrix organization, signaling by GPCR and Toll-like receptor cascades were downregulated in responders (Fig. 3B; Supplementary Table. S1E).

Replication of gene expression associations at baseline

To validate our study, we sought to replicate our findings in an independent cohort (BiOCURA) of gene expression on PBMCs with 80 RA patients before they began adalimumab or etanercept anti-TNF treatment (12). From the replication cohort, we selected only female patients gene expression data for the analysis. At baseline, we replicate the gene CDC20 that showed lower expression in responders compared to non-responders (p-value<0.087). None of the remaining 20 genes (Table 2) achieved statistical significance with p-value<0.1. In the study cohort, we reported the suppression of the gene, CHI3L1 upon anti-TNF treatment in responders, we observed similar regulation in replication cohort with lower expression in responders compared to non-responders (p-value<0.096) at baseline.

Changes in cell phenotypes and protein blood plasma during anti-TNF treatment
We studied the changes in 422 immune phenotypes measured by flow cytometry during anti-TNF treatment using paired samples. When analyzing the effect of treatment in responders and non-responders, we observed differences in seven cell phenotypes in responders and no significant differences in non-responders (Supplementary Table. S1F). With treatment, responders showed a strong suppression of the proportion of granulocytes among leukocytes (defined as CD45+ cells), and the overall concentration of neutrophils in whole blood. The proportion of T cells (defined as CD3+ cells) and B cells (CD3-CD19+) among leukocytes and the proportion of NKG2A+ NKp44+ NK cells out of all NKp44+ NK cells were instead up-regulated (Fig. 3C).

The longitudinal analysis using paired samples identified regulation of 12 proteins in plasma in responders and one protein in non-responders. The proteins were predominantly downregulated by anti-TNF treatment in responders, including CRP, IL-6, MMP-1, MMP-3, SAA, TNF-RI, VEGF, YKL-40, MIG, and MIP-1 beta, whereas TNFR2 is upregulated (Fig. 3D). Moreover, we identified the downregulation of protein matrix metalloproteinase-3 (MMP-3) measured using two different methods (Supplementary Data S1), whereas protein adiponectin is induced during anti-TNF treatment in non-responders, while showing a trend for induction in responders (FDR: 0.11). The list of proteins that were regulated during anti-TNF treatment is shown in Supplementary Table. S1G.

Further, our association analysis of immune phenotypes and plasma protein levels to clinically defined response to anti-TNF treatment did not show any significant association between responders and non-responders before treatment.

Classifier performance for anti-TNF response data

We investigated the utility of machine learning methods to predict anti-TNF response using clinical data, flow cytometry measurements, protein measurements, and transcriptomic data. At baseline, the linear model predicted response with high accuracy for transcriptomics data (ROC AUC ± SEM: 0.81 ± 0.17) (Fig. 4) whereas the kernel based method predicted response with highest accuracy for clinical data (ROC AUC: 0.73 ± 0.17), for flow cytometry (ROC AUC: 0.72 ± 0.18), and for protein data (ROC AUC: 0.72 ± 0.15) (Fig. 4). We further studied the classifier performance for the anti-TNF treated data and observed limited classifier utility for models based on flow cytometry, protein as well as transcriptomics data. In contrast, as expected, the linear model based on clinical data showed high classifier performance with ROC AUC: 0.85 ± 0.15. For the FACS data, we found maximum classifier utility of ROC AUC: 0.68 ± 0.17 while using linear model, whereas the non-linear models based on proteins and transcriptomics data showed maximum classifier utility with ROC AUC: 0.73 ± 0.15 and ROC AUC: 0.72 ± 0.18 respectively.

Discussion

This study represents a comprehensive analysis of transcriptomics, proteomics, and flow cytometry data analysis of female RA patients treated with anti-TNF. Our analysis was directed towards finding transcriptional and translational regulations during anti-TNF treatment and predict response using blood
samples collected at two-time points. We identified genes that are differentially regulated between responders and non-responders at baseline and reported changes induced by treatment in gene expression, in protein measurements, and in cell phenotypes. Finally, using biological measurements, we employed machine learning models to predict non-response to anti-TNF treatment. Our integration of multi-omics data revealed the gene CHI3L1 and subsequent protein product YKL-40 expressions were suppressed upon anti-TNF treatment. We also observed an increase in the proportion of B cells, T cells, and NK cells in responders during treatment, whereas the proportion of granulocytes was strongly suppressed.

The identification of potential biomarkers for RA with prognostic value for response to a given therapy is challenging as RA is a heterogeneous disease by its clinical characteristics and pathological processes. This also may be due to the high clinical heterogeneity in RA patient samples and strong confounding (covariates) effects, for example, variation in cell subsets is one of the strong covariates of gene expression (13). Also, RNA sequencing experiments can often generate outlier read counts in one or few RNA samples and this considerably limits the power of differential testing. Therefore we performed extensive transcriptomics analysis using leave-one-out approach to find genes with stable association to response.

The gene expression results at baseline highlighted cell adhesion gene Epiplakin1, EPPK1 showed strong association with response and was expressed higher in future responders compared to non-responders. Importantly, the difference we observed in the gene, EPPK1 (log2 fold change:1.95, FDR:5.5E-03) showed similar regulation following the adjustment to CRP measurements in the gene expression model suggesting that EPPK1 is not a surrogate marker for inflammation in patients. Previous studies have reported the mechanism of infiltration of inflammatory cells into the synovial lining due to deregulation of several adhesion receptors, therefore association of response to corresponding genes suggested its role in the pathogenesis of rheumatoid arthritis and hemophilic arthropathy (14,15). When findings from the discovery analysis were investigated in an independent cohort, we replicate the lower expression of the gene cell division cycle 20 gene (CDC20) in future responders compared to non-responders (p-value, p = 0.087). Interestingly, non-responders at baseline showed an increase in cell cycle pathways mainly related to mitotic activity and cell cycle checkpoints. This may partly correspond to the hyperproliferation of cells that leads to the accumulation of pro-inflammatory cytokines in the RA inflamed joints (16,17). Next, we extended our transcriptomics analysis using paired patient’s samples to study the changes induced by treatment with two different approaches: i) changes in gene expression patterns in samples from all RA patients not considering response status; ii) treatment effects in responders and non-responders separately to understand differences in gene expression regulation. Our first approach identified twenty-five genes differentially expressed in RA after treatment, whereas in the second approach yielded five genes differentially expressed in responders and no significant changes in non-responders. Interestingly, both approaches found four common genes CXCR2, MPO, and MYADM that were downregulated and gene, FCGR2B that was upregulated upon treatment. Also, we observed TNFAIP6 gene (TNF Alpha Induced Protein 6, also called tumor necrosis factor-stimulated gene 6, TSG-6) was suppressed in responders upon treatment. Previous studies have shown that the expression of TSG-6 has a strong
correlation with disease severity and is a potential biomarker of inflammation (18–20). The gene TSG-6 plays a key role in the remodeling of the extracellular matrix, regulation of leukocyte migration, and stimulation of cell proliferation during inflammation (21,22). These findings correspond well with the pathway analysis that showed downregulation of genes involved in extracellular matrix organization and signaling by interleukins, and an upregulation of genes mitotic cell cycle regulation. These results substantially extend our understanding of the transcriptomic profile of responders to anti-TNF treatment.

Our longitudinal studies on cell phenotypes revealed significant changes in seven cell populations in responders, indicating a marked change in major cell type proportions during treatment (Fig. 3C). The proportions of B cells and T cells among leukocytes were increased and the proportions of granulocytes were suppressed, upon treatment in responders. There was also a decrease in the overall concentration of neutrophils in whole blood in responders as well as in non-responders. The reduction of neutrophils seen by flow cytometry was also associated with a significant reduction in peripheral blood neutrophil count seen by routine blood analysis, leading to 19% of patients becoming neutropenic by clinical judgement. This finding corroborates a recent study that showed that patients treated with anti-TNF in combination with methotrexate decreased the blood neutrophil counts regardless of their clinical response to therapy (23,24). Additionally, our results also corroborate with earlier studies showing induction of B cells and NK cells in whole blood following anti-TNF treatment (25).

Similarly, the longitudinal analysis of protein data showed changes in plasma protein levels in responders. The expression of the protein YKL-40 was suppressed upon treatment (beta: -0.26) and the gene encoding for YKL-40, CHI3L1, was also down-regulated (Fig. 2A, Fig. 3D). The model including only responders showed similar suppression of CHI3L1 (log2 fold change: -1.07) with a p-value <1.73E-04 and FDR <0.21. Interestingly, we also observed that the gene CHI3L1 was expressed lower in responders compared to non-responders (p-value<0.096) at baseline in the replication cohort. The CHI3L1 gene may negatively regulate T cell activation and may also inhibit Th1 differentiation via the IFNγ-STAT1 pathway (26). We also showed that treatment with TNF inhibitors leads to a larger proportion of T cells among responders (beta: 8.16).

Interestingly, two proteins, soluble TNFR2, and adiponectin showed induced plasma levels in responders and non-responders during treatment. The result of our study replicates previously shown similar induction of adiponectin after treatment with anti-IL-6R, Tocilizumab and also highlights its multifaceted role in RA with both pro-inflammatory and anti-inflammatory functions (27,28). Similarly, an increase in TNFR2 signaling leads to the activation and proliferation of Tregs and promotes tissue regeneration in RA patients (29–31).

Our machine learning models showed high predictive utility in classifying non-response prior to anti-TNF treatment. The linear model based on transcriptomics data at baseline found suggestive good predictability using the presently applied gene expression classifier. To our surprise, the models with transcriptomics data predict response with higher accuracy compared to models with clinical data. Interestingly, we also found clinical variables add less further utility to the transcriptomics based
predictive models at baseline (data not shown). Previously, a similar outcome was observed in a study where models using transcriptomics data alone predicted fibrous cap thickness response to statin treatment with better accuracy compared to the models with clinical data or transcriptomics plus clinical variables (32). Our prediction model should be used with caution and these findings need to be validated with a larger patient independent cohort, before its potential use in clinical settings.

There are some limitations to this study. This study addressed multi-omics profiling of female RA patients treated with anti-TNF and with relatively few patients included. Also, we observed genes that are regulated upon treatment in responders showed similar direction of regulation in non-responders with low significance. This might be partly explained due to the low number of non-responders included in the current study. Extended future studies with a larger patient cohort with similar frequency of responders and non-responders are thus warranted to validate these results.

In summary, the integrative multi-omics study expanded our growing knowledge of biology of anti-TNF treatment in RA patients by finding a number of changes in gene expression, protein and cell phenotypes during treatment in anti-TNF responders. We reported genes that showed distinct expression in responders and non-responders before treatment initiation. Our analyses demonstrate that treatment causes a major regulation of cell subsets, which are also mirrored in the protein analysis. Also, our study highlights the machine learning predictive utility of transcriptomics data at baseline in stratifying patients and/or predicted response before anti-TNF treatment initiation. We envision that in the future machine learning models based on multi-omics data could predict response to anti-TNF treatment and support rheumatologist’s decision towards personalized treatment.

**Abbreviations**

ACPA: Anti-citrullinated peptide

AUC: Area under curve

CRP: C-reactive protein

DAS28: Disease activity score in 28 joints

DMARDs: Disease modifying anti-rheumatic drugs

EULAR: European League Against Rheumatism

FDR: False discovery rate

GSEA: Gene set enrichment analysis

HAQ: Health Assessment questionnaire disability index

LOO: Leave one out approach
Acknowledgements

We wish to thank all the participating patients and healthy controls. We acknowledge support and resources provided by SNIC through Uppsala Multidisciplinary Centre for Advanced Computational Science (UPPMAX) and NGI Sweden.

Funding

The parts of the study were supported by Novo Nordisk A/S and Pfizer Inc. LP was supported by the Swedish Council grant.

Conflicts of interest

The authors have declared no conflicts of interest

Author information

Affiliations

Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet and Karolinska University Hospital, Stockholm, Sweden

Niyaz Yoosuf, Malin Müller, Peter Sahlström, Nancy Vivar, Anca Catrina, Louise Berg, Lars Klareskog, Leonid Padyukov
Translational Epidemiology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

Niyaz Yoosuf, Boel Brynedal

Pfizer, Cambridge, Massachusetts, USA

Mateusz Maciejewski, Daniel Ziemek, Scott Jelinsky

Danish National Genome Center, Copenhagen, Denmark

Lasse Folkersen

Authors contributions

NY, BB, AC, LB, LP and LK conceived the study. MMU, PS and NV performed wet lab experiments. NY, BB, AC, LF and LP contributed to the data preparation. NY, BB and MM performed the data analysis. NY, BB, MM, DZ, LP and LK interpreted the data. NY prepared figures. NY, BB, MM, SJ LB, LP, LK wrote the manuscript. All authors read and approved the final version of the manuscript.

Corresponding author

Correspondence to Niyaz Yoosuf.

Ethics declarations

Ethics approval and consent to participate

The COMBINE biobank was generated after written informed consent from all participants had been obtained according to the declaration of Helsinki and with approval by the Stockholm (number 2010-351-31-2) and Uppsala (2009-013) Regional Ethics Committees.

References


