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# Interaction between rheumatoid arthritis and pregnancy: correlation of molecular data with clinical disease activity measures

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**Objective.** The factors that induce remission of RA during pregnancy and the relapse occurring after delivery remain an enigma. In a previous study, we investigated gene-expression profiles of peripheral blood mononuclear cells (PBMC) in patients with RA and healthy women in late pregnancy and postpartum. Profiles of samples from both groups were similar in late pregnancy with elevated monocyte and decreased lymphocyte signatures. Postpartum, in RA PBMC the high level of monocyte transcripts persisted. Further increase was observed in adhesion, migration and signalling processes related to monocytes but also in lymphocytes despite similar clinical activity due to intensified drug treatment. This prompted us to investigate correlations between clinical parameters of disease activity and gene profiles.

**Methods.** Transcriptome data were correlated with RADAI, CRP, monocyte and lymphocyte counts. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations, monocytes and lymphocytes signatures were used as reference information.

**Results.** Comparative analysis of PBMC expression profiles from RA patients during and after pregnancy with RADAI and CRP revealed a correlation of these disease activity parameters predominantly with monocyte transcripts. Genes related to cellular programs of adhesion, migration and response to infections were upregulated. Comparing clinically active and not-active RA patients postpartum revealed a cluster of 19 genes that could also identify active disease during pregnancy.

**Conclusion.** The data suggest that an increase of the RADAI and an elevation of CRP is a consequence of molecular activation of monocytes. Furthermore, they indicate that molecular activation of T lymphocytes may remain clinically unrecognized postpartum. It is conceivable that a set of 19 genes may qualify as molecular disease activity marker.

**Key words:** Rheumatoid arthritis, Pregnancy, Gene expression, Correlation analysis, Molecular pathways, Biomarkers, Monocyte activation, Postpartum flare.

## Introduction

The remission of rheumatoid arthritis (RA) during pregnancy occurring in the majority of patients remains an enigma. Improvement of signs and symptoms during pregnancy is followed by a relapse after delivery [1].

Recently we have studied gene expression of peripheral blood mononuclear cells (PBMC) in the third trimester and 24 weeks after delivery in six patients with RA and in eight age-matched healthy women (Häupl *et al*., submitted). Gene-expression profiles performed by affymetrix analysis revealed an elevated monocyte and a decreased lymphocyte transcriptome during pregnancy in healthy women. Postpartum this proportion was inverted. This may reflect a suppression of the adaptive immune response as part of the tolerance mechanisms towards the semiallogenic fetus. The complementary increase of phagocytes during pregnancy also found by Crocker *et al*. [2] suggests compensatory mechanisms provided by innate immune functions. In RA patients, the PBMC transcriptome during pregnancy was comparable to the one in healthy pregnant women, but it became activated postpartum with a rise in the level of monocyte and a concomitant increase in lymphocyte transcripts (Häupl *et al*., submitted).

Traditionally, disease activity in RA is defined by clinical symptoms. The only objective variable of the commonly used Disease Activity Score (DAS) is the erythrocyte sedimentation rate or the acute phase reactant CRP. Recent data clearly showed that behind improvement of clinical symptoms subclinical disease activity—for example, progressive joint destruction—may persist [3, 4]. This constellation prompted a search for correlations between clinical parameters of RA and expressed genes and the comparison of the setting during and after pregnancy.

## Patients and methods

### *Patients and controls*

Six patients with RA (fulfilling the ACR criteria [5]) and eight age-matched healthy women were studied at gestational week 32–34 and 24 weeks postpartum. The study was performed at the Department of Rheumatology and Clinical Immunology/Allergology of the University Hospital of Bern after approval by the institutional review board of Bern and informed patient consent was obtained. Mean age of pregnant patients was 31 (range 21–38), and of pregnant healthy women 33 (21–40). Disease activity by the RA Disease Activity Index (RADAI) was 1.7 (range 0–8.6). Tender and swollen joint count were 3.8 (range 0–10) and 2.7 (range 0–6), respectively. Mean level of CRP was 20.1 mg/l (range 9–52), monocyte counts 0.63 cells/pl (range 0.41–0.88) and lymphocyte counts 1.91 cells/pl (range 1.28–3.31). During pregnancy the following medications were allowed: NSAIDs until week 32 of gestation, prednisone maximum 10 mg/day throughout pregnancy, antimalarials and sulfasalazine. Flares postpartum were treated according to standard protocols.

### *RNA isolation and Affymetrix analysis*

Gene-expression profiles were determined in PBMC derived from the patients and the healthy women (ND) during the third trimester of pregnancy and 24 weeks after delivery and processed as described (Häupl *et al*., submitted). Reference data from different highly purified cell types were used to estimate cell-type association of each individual gene as described elsewhere

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(Häupl *et al.*, submitted). In brief, after extraction by Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany), 3 $\mu$g of total RNA were used for *in vitro* transcription and biotin-labeling of cRNA (ENZO Biochem, New York, NY, USA). Total 50 $\mu$g/ml cRNA was hybridized on HG-U133A GeneChips (Affymetrix, Santa Clara, CA, USA) for 16 h at 45°C, subsequently washed, stained (fluidic station) and scanned (Hewlett Packard Genearray Scanner, Affymetrix).

### *Statistical analysis*

Spearman rank correlation was performed for all RA patients during and after pregnancy between clinical parameters and expression levels for all genes on the microarray. Cell-type association scores for individual genes were calculated as previously defined (Häupl *et al.*, submitted), and compared for the top 100 genes with best correlation to RADAI, CRP, monocytes and lymphocytes. Functional association with the 34 KEGG pathways on 'cell communication', 'immune system', 'infectious diseases', 'signal transduction', 'signaling molecules and interaction' as well as 'cell growth and death' was estimated by the percentage of genes that revealed a correlation coefficient of $R \geq 0.5$. For hierarchical clustering, the Genesis software tool was applied [6].

## Results and discussion

### *Correlation of molecular data with clinical criteria of disease activity*

Disease activity of RA was absent or very low in the third trimester in all but one patient. It increased in most patients after delivery and required immunosuppressive treatment. Spearman rank correlation was performed for all RA patients during and after pregnancy comparing clinical parameters (RADAI, CRP, lymphocyte and monocyte counts) and expression levels for all genes on the microarray. Each of the top 100 genes identified for correlation either with RADAI, CRP, monocyte or lymphocyte blood count revealed coefficients between $R = 0.99$ and 0.63. Scores for association of these genes with the major blood cell types were calculated based on comparative analysis of reference signatures for each cell type as described elsewhere (Häupl *et al.*, submitted). RADAI- and CRP-related gene sets both displayed a dominant role of monocyte transcripts (mean score $\pm$ S.E.M; RADAI: $0.16 \pm 0.02$; CRP: $0.16 \pm 0.02$) compared to CD4 T-cell (RADAI: $-0.11 \pm 0.02$; CRP: $-0.10 \pm 0.02$) or CD19 B-cell transcripts (RADAI: $-0.08 \pm 0.02$; CRP: $-0.07 \pm 0.02$).

The analysis of the data demonstrated for the first time a correlation between RADAI as well as CRP with monocyte but not with lymphocyte profiles. Of note, the correlation could be found despite treatment of patients with different immunosuppressive drugs. One might argue that this correlation could be expected as drugs targeting primarily monocytic functions (e.g. TNF blocking agents [7], tocilizumab [8]) show a decrease in CRP levels and clinical signs of inflammation much faster and more potently than drugs targeting lymphocytes or lymphocyte functions (rituximab, cyclosporin, abatacept). Despite differences in kinetics lymphocyte inhibition is also effective to repress disease progression in RA [9–11]. With our current findings of a dominant monocyte profile in active disease but also a broad panel of expression changes related to lymphocytes during postpartum disease reactivation (Häupl *et al.*, submitted), one is tempted to speculate that a defined molecular profile would perform better in the prediction of therapeutic efficacy of a given drug than clinical symptoms.

### *Correlation with genes of molecular pathways*

For functional interpretation, 1822 genes of six relevant pathway groups with 34 pathways defined in the KEGG database were investigated. The portion of genes per pathway was determined that correlated with RADAI, CRP or the calculated PBMC lymphocyte or monocyte fraction by a coefficient of $R \geq 0.5$. Based on this portion, pathways were ranked all together or for each of the six groups separately (Table 1).

Genes correlating with the RADAI were associated with adhesion, leucocyte endothelial migration, infection-related processes including TLR signaling and cholera-associated responses as well as signal transduction in the Notch-, phosphatidylinositol- and mTOR-signalling pathway. Correlation with CRP revealed genes also associated with cell adhesion and communication, responses in infections (cholera, *H. pylori*) and Notch signalling as well as genes associated with the hematopoietic lineage, the complement and coagulation cascade, B-cell receptor and ErbB signalling.

The monocyte fraction correlated with genes predominantly associated with infection-related processes, with migration, ECM interaction, hematopoietic lineage, antigen processing, Calcium and Notch signalling. The lymphocyte fraction correlated with genes of the T-cell and B-cell receptor signalling pathway, with cell growth and death-related gene activity and with the phosphatidylinositol-, mTOR-, ErbB- and VEGF-signalling pathways.

Taken together, correlation with functional processes revealed differences between the four parameters. While RADAI, CRP and monocyte fraction-related processes focused on adhesion, migration, response to infections and Notch-signalling, lymphocyte fractions were predominantly associated with typical lymphoid receptor signalling pathways, different pathways of the signalling cascade and cell growth.

As lymphocytes are suppressed during pregnancy (Häupl *et al.*, submitted) [12] and increase postpartum, one can speculate that lymphocytes are unleashed after pregnancy, proliferate and differentiate, in order to upregulate adaptive immune functions. In light of the discrepancy that RA patients benefit from pregnancy with its dominance of phagocytes but flare postpartum when lymphocyte function is recurring [12] by further increase of the monocyte dominance, a role of lymphocytes in this process seems obvious. While expression of lymphocyte marker genes were not or even negatively correlated with markers of disease activity, phosphatidylinositol- and mTOR-signalling were correlating with both, PBMC lymphocyte fractions and disease activity and not with the PBMC monocyte fraction. This suggests that both signalling processes reflect lymphocyte activation. Targeting lymphocytes in RA via the mTOR pathway has been discussed earlier [13]. Genes of the phosphatidylinositol pathway are involved in many different cellular processes. In our study, especially expression of phosphoinositide 3-kinases was correlating with the lymphocyte fraction, kinases that have been implicated in inflammatory processes of RA including T cells but also other cell types [14].

### *Genes differentially expressed in active disease*

In a third step of analysis, we addressed the question whether a discrete panel of genes might serve as molecular disease activity marker set. A pattern consisting of 19 genes was identified that reflected disease activity in this cohort of patients. It was derived by comparative analysis of RA patients postpartum with high and low disease activity as defined by increased levels of CRP and/or RADAI. With this gene selection we identified also one patient with active disease throughout pregnancy and excluded all other patients as well as all normal controls during and after pregnancy (Fig. 1). No relevant overlap existed

TABLE 1. Correlation of pathway with criteria of disease acitivity and fractions of lympocytes and monocytes

| | | Genes per pathway[a] | Lymphocyte fraction in PBMC | | | Monocyte fraction in PBMC | | | CRP | | | RADAI | | | Mean[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 3314 | | | 617 | | | 629 | | | 365 | | | |
| | | | | 370 | | | 111 | | | 124 | | | 62 | | | |
| | Genes with correlation coefficient ≥0.5[b] | | | Rank | Rank | | Rank | Rank | | Rank | Rank | | Rank | Rank | |
| | Thereof identified in pathways[c] | | %[d] | group | all | % | group | all | % | group | all | % | group | all | % |
| Cell communication | Adherens junction | 75 | 21.3 | 4 | 23 | 6.7 | 1 | 11 | 12.0 | 1 | 4 | 8.0 | 1 | 1 | 5.8 |
| | Tight junction | 106 | 22.6 | 3 | 21 | 5.7 | 3 | 17 | 9.4 | 2 | 7 | 5.7 | 2 | 6 | |
| | Focal adhesion | 185 | 23.2 | 2 | 19 | 6.5 | 2 | 14 | 6.5 | 4 | 19 | 4.9 | 3 | 11 | |
| | Gap junction | 89 | 25.8 | 1 | 16 | 4.5 | 4 | 24 | 7.9 | 3 | 13 | 4.5 | 4 | 14 | |
| Infectious diseases | Cholera | 38 | 15.8 | 3 | 28 | 15.8 | 1 | 1 | 26.3 | 1 | 1 | 5.3 | 1 | 8 | 4.2 |
| | Pathogenic *E. coli* infection | 48 | 18.8 | 2 | 27 | 8.3 | 2 | 6 | 6.3 | 3 | 22 | 4.2 | 2 | 16 | |
| | Epithelial cell signalling in *H. pylori* infection | 63 | 20.6 | 1 | 25 | 7.9 | 3 | 7 | 14.3 | 2 | 3 | 3.2 | 3 | 24 | |
| Immune system | Leucocyte transendothelial migration | 108 | 21.3 | 7 | 24 | 9.3 | 2 | 3 | 6.5 | 6 | 20 | 7.4 | 1 | 3 | 3.9 |
| | Toll-like receptor signalling pathway | 87 | 27.6 | 5 | 14 | 3.4 | 7 | 25 | 6.9 | 5 | 17 | 4.6 | 2 | 12 | |
| | Complement and coagulation cascades | 67 | 11.9 | 9 | 33 | 6.0 | 4 | 15 | 11.9 | 2 | 5 | 4.5 | 3 | 15 | |
| | Fc epsilon RI signalling pathway | 74 | 36.5 | 3 | 6 | 2.7 | 8 | 28 | 8.1 | 4 | 11 | 4.1 | 4 | 17 | |
| | Antigen processing and presentation | 85 | 18.8 | 8 | 26 | 7.1 | 3 | 9 | 5.9 | 7 | 24 | 3.5 | 5 | 18 | |
| | Haematopoietic cell lineage | 86 | 24.4 | 6 | 17 | 14.0 | 1 | 2 | 17.4 | 1 | 2 | 3.5 | 6 | 19 | |
| | B-cell receptor signalling pathway | 60 | 40.0 | 2 | 2 | 5.0 | 5 | 19 | 8.3 | 3 | 9 | 3.3 | 7 | 21 | |
| | Natural killer cell-mediated cytotoxicity | 123 | 29.3 | 4 | 11 | 4.9 | 6 | 21 | 5.7 | 8 | 26 | 3.3 | 8 | 23 | |
| | T-cell receptor signalling pathway | 90 | 48.9 | 1 | 1 | 1.1 | 9 | 33 | 4.4 | 9 | 29 | 1.1 | 9 | 31 | |
| Signal transduction | Notch signalling pathway | 39 | 28.2 | 6 | 12 | 7.7 | 2 | 8 | 10.3 | 1 | 6 | 7.7 | 1 | 2 | 3.9 |
| | Phosphatidylinositol signalling system | 72 | 37.5 | 1 | 4 | 5.6 | 5 | 18 | 6.9 | 5 | 15 | 6.9 | 2 | 4 | |
| | mTOR signalling pathway | 47 | 36.2 | 2 | 7 | 2.1 | 10 | 31 | 6.4 | 6 | 21 | 6.4 | 3 | 5 | |
| | Calcium signalling pathway | 164 | 14.6 | 11 | 29 | 8.5 | 1 | 4 | 5.5 | 8 | 27 | 5.5 | 4 | 7 | |
| | Jak-STAT signalling pathway | 139 | 23.0 | 9 | 20 | 5.8 | 4 | 16 | 7.9 | 3 | 12 | 5.0 | 5 | 9 | |
| | VEGF signalling pathway | 66 | 31.8 | 4 | 9 | 3.0 | 7 | 27 | 7.6 | 4 | 14 | 4.5 | 6 | 13 | |
| | Wnt signalling pathway | 132 | 31.1 | 5 | 10 | 6.8 | 3 | 10 | 4.5 | 9 | 28 | 3.0 | 7 | 25 | |
| | ErbB signalling pathway | 84 | 32.1 | 3 | 8 | 4.8 | 6 | 22 | 8.3 | 2 | 9 | 2.4 | 8 | 28 | |
| | TGF-$\beta$ signalling pathway | 79 | 21.5 | 10 | 22 | 1.3 | 11 | 32 | 3.8 | 10 | 30 | 1.3 | 9 | 30 | |
| | MAPK signalling pathway | 256 | 27.7 | 7 | 13 | 2.3 | 8 | 29 | 5.9 | 7 | 25 | 0.4 | 10 | 32 | |
| | Hedgehog signalling pathway | 46 | 23.9 | 8 | 18 | 2.2 | 9 | 30 | 0.0 | 11 | 34 | 0.0 | 11 | 33 | |
| Signalling molecules and interaction | Cell adhesion molecules (CAMs) | 121 | 14.0 | 3 | 32 | 6.6 | 3 | 13 | 9.1 | 1 | 8 | 5.0 | 1 | 10 | 3.3 |
| | Cytokine–cytokine receptor interaction | 231 | 14.3 | 2 | 31 | 4.8 | 4 | 22 | 6.9 | 2 | 16 | 3.5 | 2 | 20 | |
| | ECM–receptor interaction | 82 | 14.6 | 1 | 29 | 8.5 | 1 | 4 | 6.1 | 3 | 23 | 2.4 | 3 | 27 | |
| | Neuroactive ligand–receptor interaction | 270 | 6.7 | 4 | 34 | 6.7 | 2 | 11 | 2.6 | 4 | 33 | 2.2 | 4 | 29 | |
| Cell growth and death | p53 signalling pathway | 61 | 26.2 | 3 | 15 | 3.3 | 2 | 26 | 6.6 | 1 | 18 | 3.3 | 1 | 22 | 1.9 |
| | Apoptosis | 81 | 37.0 | 2 | 5 | 4.9 | 1 | 20 | 3.7 | 2 | 31 | 2.5 | 2 | 26 | |
| | Cell cycle | 105 | 38.1 | 1 | 3 | 0.0 | 3 | 34 | 2.9 | 3 | 32 | 0.0 | 3 | 33 | |

A total of 1822 genes of 6 relevant pathway groups with 34 pathways defined in the KEGG database were correlated with RADAI, CRP and the calculated PBMC lymphocyte or monocyte fraction. [a]The table indicates the numbers of genes that were annotated to each pathway, [b]that correlated by $R \geq 0.5$ [c]and that were identified in all 34 pathways. [d]Association with each pathway was calculated as the percentage of genes per pathway that correlated by $R \geq 0.5$. Dominance of association was Ranked based on the percentages either within each group of pathways or for all pathways together. [e]Pathway groups were sorted by the mean percentages of each group.

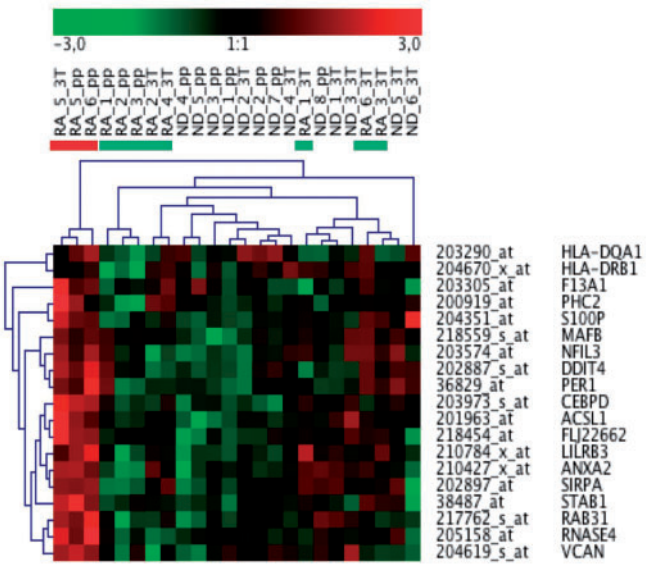


FIG. 1. Genes were selected based on differential expression between clinically active and not-active RA patients postpartum (≥80% of all pairwise comparisons increased in active disease). This identified a cluster of 19 genes which separated by hierarchical clustering not only RA patients post partum but also RA patients during pregnancy into active and not-active RA patient. Not-active RA patients co-clustered with healthy women.

with genes identified by others except from members of the HLA class II and the S100 family [15–18]. The increase of S100 family members in our study was concordant with results reported by others [15, 18]. In contrast, HLA expression was found decreased in RA compared to healthy controls by Bovin *et al.* [15] and van der Pouw Kraan *et al.* [17] using *PBMC* and *PAX* gene, respectively. However, analyses by Lequerré *et al.* [16] support our findings of increased expression of HLA class II which they found elevated in non-responders towards infliximab.

## Conclusion

Taken together this study provides new insights into the correlation between gene-expression profiles of PBMC and disease activity in RA. In our previous analysis (Häupl *et al.*, submitted) we observed an interesting and unexpected finding. There was concordance between molecular and clinical disease activity of RA in the third trimester. However, in spite of low disease activity under therapy with immunosuppressive drugs postpartum, we found a consistent difference of gene expression between all RA patients and all healthy women after delivery. In our correlation analysis we could identify particular sets of marker genes that can indicate clinical disease activity, not only postpartum but also during pregnancy. Nevertheless, prospective studies will have to prove whether these sets of genes identified as markers of active RA qualify for quantification of disease activity and eventually may help to predict disease outcome.

## Rheumatology key messages

- Postpartal flares in RA correlated with monocyte activation.
- Molecular programs related to adhesion, migration and response to infection were activated.
- A biomarker pattern is suggested that indicated disease activity.

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