GENOMIC STRATIFICATION BY HLA-DRB4 EXPRESSION IDENTIFIES INNATE AND ADAPTIVE IMMUNE PATTERNS AS DIFFERENTIAL PREDICTORS OF RESPONSE TO METHOTREXATE IN RHEUMATOID ARTHRITIS – A STRATEGY TO DETECT PREDICTORS OF METHOTREXATE RESPONSE IN EARLY RHEUMATOID ARTHRITIS

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Background and objective:
Variability of clinical course and response to therapy in Rheumatoid Arthritis (RA) suggests differences in molecular mechanisms depending on stage and sub-entity of the disease. Most dominant molecular discriminators are HLA-DR4 shared epitope (SE), and the status of rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) development, all indicating sub-entities with influence on severity of disease and therapeutic outcome. Methotrexate (MTX) is one of the most prescribed long-term effective disease-modifying anti-rheumatic drugs (DMARDs) and therefore considered as the standard medication for the treatment of RA. Nonetheless, about 40-50% of the patients does not show adequate improvement under MTX therapy, while some patients even suffer from adverse and toxic side effects like pulmonary, hepatic, renal or bone marrow abnormalities. Identification of cellular & molecular processes (CMP) would be an option to minimize such effects, to determine the most promising therapy for each individual patient subgroups, and last but not least to reduce socio-economic costs. Early RA prior to MTX treatment was investigated to exclude major therapeutic influence. By comparing whole blood transcriptomes between subsequent responders and non-responders we aimed to unravel molecular mecha-nisms with influence on MTX outcome of RA. This study aimed at defining CMPs, as well as to identify predict- tive mRNA and miRNA candidate genes in PAXgene whole blood samples of early RA patients for the response to future treatment with the anchor drug MTX.

Materials and Methods:
Whole blood PAXgene samples from 52 patients with early RA were obtained in the HITHARD and in the ArthroMark study. Exclusively, patients who had received no or only low doses of steroids were analyzed. Clinical characteristics assessed at baseline and after 16 weeks included RF, ACPA, DAS28 and EULAR response or classification in good (GR), moderate (MR) and non-responders (NR). Total RNA was globin reduced and processed according to standard protocols for hybridization to Affymetrix Hg. U133 Plus 2.0 microarrays. Biostatistical methods included MAS5.0 and RMA algorithms with limma, lasso and Wilcoxon tests. For functional interpretation, expression of candidate genes was tested in various blood cell types and stimulation conditions using own and GEO reference transcriptomes. Marker selection was validated by qPCR with commercial primers and four different housekeeping genes (β-actin, γ-actin, GAPDH, HPRP0).

Results:
Assuming impact by immunological or genetic characteristics on pathomechanisms and treatment outcome, patients were grouped either by gender, RF, ACPA, SE or the haplotype-specific HLA transcripts DRB4 (209728_at) and DQA1 (203290_at). In each subgroup, responders and non-responders were compared and genes ranked by frequency of change call and fold change. For each subgroup comparison, the best 100 genes increased and 100 decreased in responders were selected. Scoring differential expression of all 200 genes for each HLA-DRB4 patient revealed a high correlation with clinical response. Moderate responders represented independent samples and were
located between responders and non-responders. Validation of HLA-DRB4\(^{-}\) candidate genes by “leave-one-out” excluded each patient at least once and generated 12 different comparisons based on MAS5.0/BioRetis algorithms. Each validation gene set overlapped with the complete group comparison set by 66-80\% for increased and 67-82\% for decreased in responders. Of the complete group comparison set, 96.5\% (range: 91-100\%) were ranked within the top 250 transcripts of any validation comparison. Overall, validation comparisons revealed correct classification of responders in 96\% (range: 83%-100\%) and non-responders in 73\% (range: 57%-86\%). Different biostatistical approaches favored MAS5.0 algorithms marker for marker Comparison between good responders and non-responders in unselected patients revealed insufficient discriminatory power. Generating different subgroups defined by gender, status of RF, ACPA, SE, HLA-DRB4 or DQA1 identified molecular patterns with a strong discrimination between GR and NR in HLA-DRB4\(^{-}\) patients. All other subgroups did not improve pattern selection. In HLA-DRB4\(^{-}\) patients, genes increased in GR were related to phagocytes and bone marrow activation (innate), whereas genes increased in NR were associated with lymphocyte (adaptive) activity. In HLA-DRB4\(^{-}\) patients, patterns of adaptive immunity were again related to non-response but frequently combined also with innate patterns. After separation of the patients into HLA-DRB4\(^{+}\) (n=29) and HLA-DRB4\(^{-}\) (n=23) subgroups 2 predictive gene sets (n=16 each) were identified. Hierarchical cluster analyses using these marker gene panels resulted in a clear discrimination of R and NR and revealed sensitivity and specificity rates of 100\% in the HLA-DRB4\(^{-}\) patient subgroup and sensitivity of 92.9\% and 100\% in the DRB4\(^{+}\) RA patient subgroup. Integration of corresponding mRNA expression data from MR to the hierarchical cluster analyses confirmed these results. In the HLA-DRB4\(^{-}\) patient subgroup sensitivity and specificity rates of 83.3\% and 100\% were obtained, the separation of R, MR, and NR in the HLA-DRB4\(^{-}\) RA patient subgroup resulted in a 100\% sensitivity and specificity. In validation of MTX predictor genes using qPCR ‘Receiver-Operating-Characteristics’ (ROC) with the top 20 markers in HLA-DRB4\(^{-}\) and DRB4\(^{+}\) subgroups revealed ‘Area Under the Curve’ (AUC) <0.96 and AUC<0.91 for response and AUC<0.98 and AUC<0.99 for non-response associated genes, respectively. Technical validation by qPCR with the 16 top candidates confirmed AUC-values up to 0.97 for prediction in the HLA-DRB4\(^{-}\) group and reached AUC up to 0.89 in HLA-DRB4\(^{-}\) patients (data not shown).

Conclusions:
An early outcome prediction of successful therapy, in particular with MTX as anchor drug in RA even after initiation of the disease, provides the opportunity for individually customized medications and may therefore reduce costs and prevent serious side effects of current RA treatment strategies. Identification of specific predictive response marker genes such as CD11c for anti-TNF monotherapy (2, 3) leads to a substantial interest to define predictive biomarkers for MTX monotherapy (4), as well. Furthermore, the combination of those markers is an approach to calculate the outcome of the standard combination therapy with both drugs. Combined genetic, genomic cellular stratification revealed transcriptional patterns that indicate different molecular pathomechanisms in RA depending on response to MTX therapy. Interestingly, the group responding to MTX was dominated by phagocyte but not lymphocyte activity, which may indicate an important contribution of innate triggers to the pathomechanisms in RA.