Explorative analyses of protein biomarkers in patients with early rheumatoid arthritis achieving sustained drug-free remission after treatment with tocilizumab- or methotrexate-based strategies: from transcriptomics to proteomics

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Abstract

Objective

Previously, we identified networks of co-expressed genes related to achieving sustained drug-free remission (sDFR). The aim of the present exploratory analysis was to identify inflammatory proteins associated with achieving sDFR and their enriched biological pathways, and compare these pathways with those found in the previous transcriptomic analyses.

Methods

Serum samples were used from 60 patients who participated in the U-Act-Early trial and were treated-to-target with tocilizumab plus methotrexate, or tocilizumab or methotrexate; 37 achieved sDFR (≥3 months drug-free) and 23 did not (controls). Luminex® multi-analyte profiling (xMAP)® was used to measure 85 proteins. Partial least square discriminant analyses (PLSDA) identified proteins associated with achieving sDFR within each strategy arm, which were thereafter used for pathway analyses.

Results

PLSDA identified 9, 14 and 13 relevant proteins in the tocilizumab plus methotrexate, tocilizumab and methotrexate arm, respectively and pathway analyses thereafter identified respectively 49, 88 and 117 significantly enriched gene ontology (GO) terms. When comparing these terms with those previously found in the transcriptomic analyses, corresponding pathways were related in the tocilizumab arm to activity of leukocytes; in the methotrexate arm to response of stimuli and regulation of the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway. In the tocilizumab plus methotrexate arm, no corresponding enriched pathways were found.

Conclusion

Multiple proteins were associated with achieving sDFR and several biological pathways corresponded, mainly in the methotrexate arm, with our previous transcriptomic findings potentially providing further insights into gene expression and protein translation in newly diagnosed RA patients.

Key words

rheumatoid arthritis, tocilizumab, methotrexate, drug-free remission, proteomic

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Introduction

Rheumatoid arthritis (RA) is an inflammatory disease affecting synovial joints, which may eventually lead to irreversible joint damage (1-3). Although the pathogenesis of the disease is not completely understood, recent advances in '-omics' technologies have enabled exploration of certain disease pathways, potentially facilitating early diagnosis and treatment (4-6). Genomic studies for example demonstrated a role of certain human leucocyte antigen (HLA) class II alleles and non-HLA (*e.g.* protein tyrosine phosphatase, non-receptor type 22) genes in the development of RA (7-9). However, this has to date not led to reliable prediction of the disease course. In the treatment of newly diagnosed RA patients, methotrexate is considered an anchor drug although biological disease modifying anti-rheumatic drugs (bDMARDs), (*e.g.* tocilizumab, an interleukin (IL)-6 receptor-antibody) have proven to be more efficacious (10-18). Starting a bDMARD-based treatment strategy right after diagnosis in all patients would however lead to considerable increases in health care cost and unnecessary exposure to adverse events in those for whom methotrexate would have provided adequate disease control (19-21). So, predictors are needed for treatment response to methotrexate, and to identify RA patients who would clinically benefit from initiating a bDMARD from start, which then could subsequently be tapered and finally discontinued, if persistent remission has been achieved. We previously demonstrated in DMARD-naïve patients with early RA who participated in the U-Act-Early trial an association between networks of co-expressed genes and achieving sustained drug-free remission (sDFR) after therapy. In these transcriptomic analyses, several predisposing signature genes were identified by high-throughput sequencing of isolated messenger ribonucleic acid (mRNA) obtained from positive cluster of differentiation 4 ($CD4^+$) cells (22). The aim of this explorative study among the same patients as who were included in the transcriptomic analyses was to identify, inflammatory proteins associated with achieving sDFR and to investigate the potentially associated biological pathways. Subsequently, we compared these pathways with those found previously in the transcriptomic analyses (22) for a better understanding of complex processes involved with the transcription and translation of mRNA into proteins. By exploring such techniques, hopefully it will eventually become possible to identify predictors, enabling more personalised treatment strategies for individual RA patients.

Methods

Design

This analysis included patients who were diagnosed with very early RA (1987/2010 classification criteria) (23, 24) and participated in the two-year, multi-centre, double-blind, placebo-controlled, randomised U-Act-Early strategy trial (ClinicalTrials.gov identifier: NCT01034137). In this trial, DMARD-naïve patients initiated tocilizumab plus methotrexate, or tocilizumab plus placebo or methotrexate plus placebo and were treated to the target of sustained remission (defined as disease activity score assessing 28 joints (DAS28) <2.6 with ≤4 swollen joints for ≥24 weeks). The study design has been described previously (10). Briefly, tocilizumab (8mg/kg) was given intravenously every 4 weeks and step-up methotrexate (orally) was started at 10mg/week and increased to 30mg/week in steps of 5mg/4 weeks until remission or the maximum tolerable dose was reached. If no remission occurred, hydroxychloroquine (200mg twice/day) was added as part of the initial treatment strategy. Hereafter, if remission still was not achieved after the addition of hydroxychloroquine, patients switched to a subsequent treatment regimen, in which patients who started with tocilizumab or methotrexate therapy switched to tocilizumab plus methotrexate combination therapy; those who started with this treatment switched to the standard of care (*i.e.* methotrexate plus tumour necrosis factor (TNF) inhibitor). When sustained remission was achieved, medication was tapered and finally discontinued if remission persisted. First, methotrexate was tapered with 5 mg/4 weeks until 10

Table I. Baseline characteristics of the patients included in the analyses.

	Tocilizumab plus methotrexate		Tocilizumab		Methotrexate	
	sDFR (n=14)	Controls (n=5)	sDFR (n=13)	Controls (n=11)	sDFR (n=10)	Controls (n=7)
Female gender, n (%)	6 (43)	4 (80)	9 (69)	8 (73)	8 (80)	6 (86)
Age (years)	53 (16)	64 (10)	58 (14)	51 (13)	50 (14)	46 (17)
BMI (kg/m^2)	25 (4)	27 (4)	25 (2)	25 (5)	29 (4)	26 (3)
Caucasian, n (%)	13 (93)	4 (80)	13 (100)	10 (91)	10 (100)	7 (100)
Current smokers, n (%)	3 (21)	1 (20)	2 (15)	3 (27)	1 (10)	1 (14)
Symptom duration (days), median (IQR)	22 (21-40)	19 (14-55)	24 (18-39)	21 (16-25)	30 (13-40)	31 (20-45)
RF positive, n (%)	5 (34)	3 (60)	8 (62)	6 (55)	9 (90)	5 (71)
Anti-CCP positive, n (%)	5 (34)	3 (60)	8 (62)	7 (64)	7 (70)	6 (86)
CRP (mg/L), median (IQR)	5 (2-13)	5 (4-9)	15 (4-27)	14 (4-30)	11 (5-18)	5 (4-12)
ESR (mm/h), median (IQR)	18 (12-39)	25 (23-29)	26 (14-28)	20 (9-39)	25 (13-47)	16 (13-25)
DAS28 (range 0-9.4, 9.4-maximum)	4.7 (1.2)	5.1 (0.9)	5.0 (1.1)	5.3 (1.3)	4.6 (1.2)	4.8 (0.9)
HAQ (range 0-3, 3=worst function)	0.8 (0.5)	1.5 (0.9)	1.0 (0.6)	1.4 (0.7)	0.9 (0.6)	1.0 (0.5)
Sharp/van der Heijde score, median (IQR)	0 (0-0)	0 (0-0)	0 (0-3)	0 (0-2)	0 (0-1)	0 (0-0)

Continuous data presented as mean (SD) unless otherwise indicated. SD: standard deviation; IQR: interquartile range; sDFR: sustained drug-free remission; BMI: body mass index; RF: rheumatoid factor; CCP: cyclic citrullinated peptide; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28: disease activity assessing 28 joints; HAQ: health assessment questionnaire.

mg/week and then stopped; thereafter the dose of tocilizumab was decreased to 4 mg/kg and finally discontinued after three months. At baseline, before the first dose of medication, whole blood samples were collected of which serum was extracted and stored at -80°C. In the present study, we analysed the serum of those achieving sDFR (defined as being drug-free for ≥3 months and remained drug-free until the end of the study period, whereas one visit with low disease activity (DAS28 ≤3.2) was allowed). As controls, we selected patients who never achieved a drug-free status during the study period. The serum samples were measured using Luminex® multi-analyte profiling (xMAP)® technology simultaneously detecting multiple proteins using protein-specific colour-coded beads, which were analysed using flow cytometry (25). A pre-defined selection of 85 inflammatory proteins, based on clinical relevance and availability, was measured (Supplementary Table I).

Statistical analyses

Baseline characteristics are described as mean (standard deviation, SD), median (interquartile range, IQR) or proportions (%); between-group differences (sDFR *vs*. controls) were tested within each strategy arm using independent *t*, Mann Whitney U or Pearson Chi-square tests, respectively. Before analysing the protein concentrations, we imputed values that exceeded the limit of detection (LOD) of Luminex® and therefore could not be quantified. Values <LOD were imputed by the value 0.5, so the value just in between lower LOD and zero, assuming this is a more valid imputation than imputing these values by LOD, which would lead to an overestimation. Likewise, values >LOD were imputed by multiplying the highest actually measured value by 1.5, trying to avoid the underestimation. Furthermore, TNF-α values were assessed with a high sensitivity immunoassay (human TNF-α Quantikine® HS ELISA, R&D Systems, Inc., Minneapolis, MN, USA), as with Luminex® for 97% of sample values were <LOD. Data was then normalised (natural log transformed), because of its skewed distribution, and standardised (z-score) before performing partial least square discriminant analyses (PLSDA) to identify relevant proteins within each strategy arm. The number of components in the PLSDA was determined for each model separately using leave-one-out cross validation; the variable importance on projection (VIP) score was then calculated for each protein. VIP-scores reflect the variables that best explain the outcome variance in a multidimensional dataset and accumulate the importance of each variable across the components (26). As the squared sum of all VIP-scores is "1", which thus equals the average VIP, proteins with VIP ≥1 were considered as important and selected for further analyses. As this selection procedure yielded many potential relevant proteins in the strategy arms, we performed PLSDA again, analysing only the proteins with VIP ≥1 and re-calculated the VIP-scores. Proteins with VIP ≥1 in the second analyses were tested for significance in each strategy arm using logistic regression analyses with achieving sDFR (yes *vs*. no) as dependent variable and the protein score as independent variable. We calculated Pearson's correlation coefficients (PCC) for all pairs of proteins within each strategy arm and constructed an adjacency matrix, and depicted it in cross-correlation heatmaps, in which negative correlations ("<0") are denoted in *black* and positive correlations (">0") in *grey;* also differences in concentrations of the selected proteins between the sDFR and controls are visualised. To study the biological pathways that are involved in the proteins that were identified for each strategy arm, we performed Genes Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using the STRING database (27). Furthermore, to gain more insight into the systems biology of early RA patients achieving sDFR or not, we compared the currently enriched pathways in the proteins with the relevant pathways previously found in high-throughput sequenced mRNA (22). In addition, we performed integrative analyses using the transcriptomic

Table II. Overview of proteins related to achieving sustained drug-free remission.

Protein	VIP score	β	*p*-value
Tocilizumab plus methotrexate			
CCL18	1.48	-3.31	0.047
IL-2Rα	1.37	1.40	0.039
CCL20	1.29	-1.24	0.035
I-CAM1¶	1.27	-1.85	0.051
CCL5	1.25	-6.83	0.10
RBP4	1.14	-1.39	0.07
Leptin¶	1.08	-0.81	0.18
MMP-8	1.06	1.79	0.07
TNF-R1¶	1.06	13.41	0.99
Tocilizumab			
CCL22	1.49	-0.87	0.08
TPO	1.41	1.06	0.07
CCL3	1.37	-0.56	0.25
Resistin	1.24	-0.71	0.14
IL-23	1.19	0.41	0.36
NGF	1.18	-0.48	0.29
IL-22¶	1.16	7.50	0.99
TSLP	1.14	-0.39	0.37
TNF-α	1.13	-0.71	0.17
IL-10	1.13	0.74	0.16
IL-9	1.11	0.79	0.14
IL-6	1.09	0.60	0.19
LIGHT	1.05	0.55	0.22
TIMP-1	1.02	1.22	0.25
Methotrexate			
PAI-1	1.62	-3.06	0.18
I-CAM1¶	1.50	-0.79	0.18
PD-1	1.30	-1.04	0.12
IFN-α	1.27	16.08	0.99
CCL2	1.17	0.85	0.20
TNF-R1¶	1.13	0.76	0.17
G-CSF	1.11	1.03	0.16
GM-CSF	1.08	-0.36	0.48
Leptin¶	1.02	-0.90	0.18
IL-22¶	1.03	0.72	0.30
IL-29	1.02	8.15	0.99
IL-25	1.01	0.68	0.25
IL-11	1.01	0.74	0.23

¶ These proteins were identified as relevant (*i.e.* VIP ≥1) in both the tocilizumab plus methotrexate arm and the methotrexate arm (I-CAM1, Leptin, TNF-R1) and in the tocilizumab arm and methotrexate arm (IL-22). Effect estimates (β) presented in z-scores. VIP: variable importance on projection; CCL: C-C motif chemokine ligand; I-CAM1: intercellular adhesion molecule 1; IL-2Rα: soluble IL-2 receptor alpha; MMP-8: matrix metalloproteinase-8; TNF-R1 = tumour necrosis factor 1; RBP4: retinol binding protein 4; TPO: thyroid peroxidase; IL: interleukin; NGF: nerve growth factor; TSLP: thymic stromal lymphopoietin; TIMP-1: TIMP metallopeptidase inhibitor 1; TNF-α: tumour necrosis factor alpha; PAI-1: plasminogen activator inhibitor-1; PD-1: programmed cell death protein 1; IFN-α: interferon alpha; G-CSF: granulocyte-colony stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor.

and proteomic biomarkers and calculated significant transcript-proteomic correlations. For all analyses, a *p*-value <0.05 at two-tailed testing was considered statistically significant; the statistical programme R v. 3.4.1 was used for data analyses.

Results

The patients' baseline clinical characteristics are shown in Table I. From the tocilizumab plus methotrexate arm, 19 patients (n=14 achieved sDFR, n=5 controls) were included; from the tocilizumab arm 24 patients (n=13 achieved sDFR, n=11 controls) and from the methotrexate arm 17 patients (n=10 achieved sDFR, n=7 controls). The majority was female (68%) and Caucasian (95%); mean (SD) age was 53 (14) years. Median (IQR) symptom duration was 23 (18-40) days and 60% was positive for rheumatoid factor and 60% for anti-cyclic citrullinated peptide. Mean (SD) DAS28 at baseline was 4.9 (1.1) and median (IQR) C-reactive protein and erythrocyte sedimentation rates were 9 mg/L (3-18) and 20 mm/h (12-32), respectively. When comparing the characteristics of those achieving sDFR *vs*. controls within the strategy arms, no statistically significant differences ($p \geq 0.07$) were found. With PLSDA, we found 26, 28 and 33 proteins with VIP≥1 in the tocilizumab plus methotrexate arm, in the tocilizumab arm and in the methotrexate arm, respectively. These proteins were then used to re-perform PLSDA, which yielded 9 proteins with VIP ≥1 in the tocilizumab plus methotrexate arm, 14 in the tocilizumab arm and 13 in the methotrexate arm (Table II). In the tocilizumab plus methotrexate arm, 6/9 (n/N) proteins were associated with a decreased chance (*i.e.* negative effect estimate) of achieving sDFR and in the tocilizumab and methotrexate arms these numbers were 6/14 (n/N) and 5/13 (n/N), respectively. Intercellular adhesion molecule 1 (I-CAM1), Leptin and TNF receptor 1 (TNF-R1) were found relevant in both the tocilizumab plus methotrexate and methotrexate arms and IL-22 was found relevant in the tocilizumab and methotrexate arms, but all others proteins differed between the groups. Fig. 1 shows the PLSDA score plots, using the first two components, depicting a clear group separation between those achieving sDFR *vs*. controls within the three strategy arms when using the selected proteins. The protein with the highest VIP-score, and therefore considered as most important, in the tocilizumab plus methotrexate arm was chemokine (C-C motif) ligand 18 (CCL18, VIP 1.48); in the tocilizumab arm it was the CCL20 (VIP 1.49) protein, and in the methotrexate arm, the protein plasminogen activator inhibitor-1 (PAI-1, VIP 1.62).

Proteomic biomarkers

Correlations between the proteins within the three strategy arms are shown in Fig. 2. Most significant correlations were positive (PCC ≥0.42), indicating a similar expression between the proteins. A significant negative correlation could only be demonstrated in the tocilizumab plus methotrexate arm be-

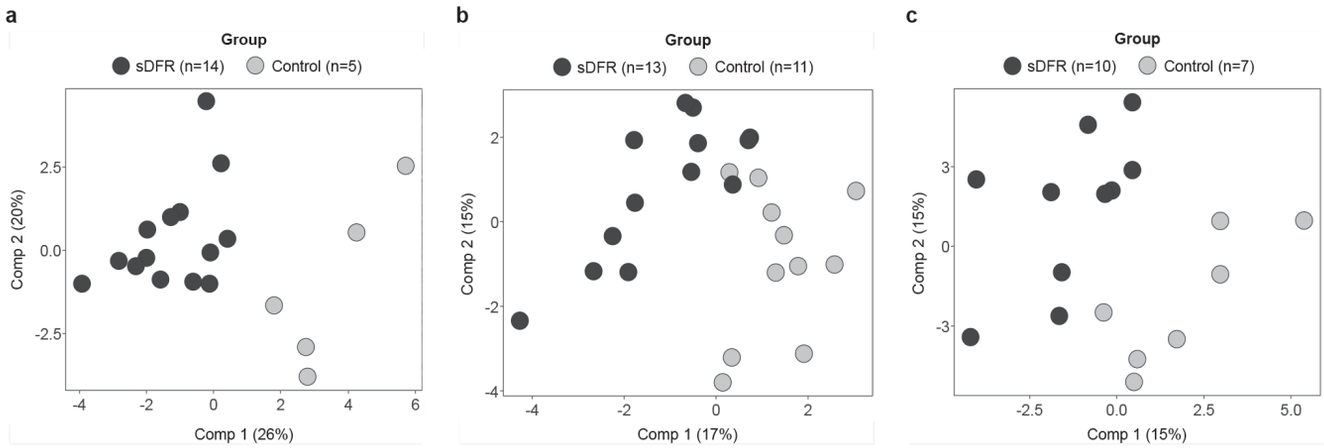

Fig. 1. Score plots of the final PLSDA of the (**a**) tocilizumab plus methotrexate, (**b**) tocilizumab, and (**c**) methotrexate strategy arms depicting group separation between those achieving sDFR *vs*. controls. Percentage of components indicates the explained variance. PLSDA: partial least square discriminant analyses; sDFR: sustained drug-free remission

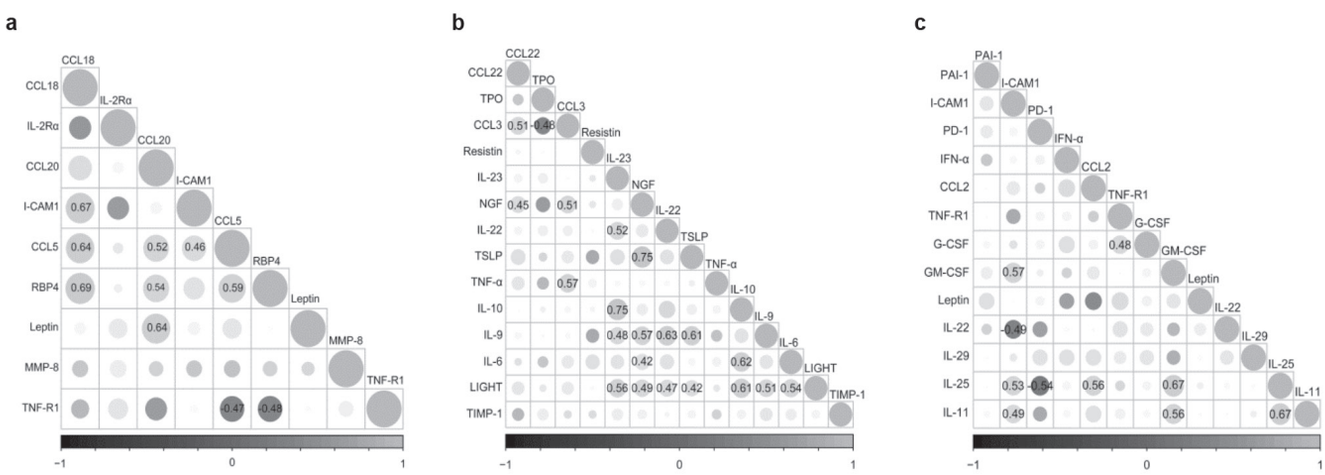

Fig. 2. Cross-correlation heatmaps, ranked at VIP score, of proteins identified by PLSDA in the (**a**) tocilizumab plus methotrexate, (**b**) tocilizumab, and (**c**) methotrexate strategy arms. Pearson's correlation coefficients only shown when $p<0.05$. *Black* colour depicts a negative correlation ("- 1") and *grey* colour depicts a positive correlation ("1") whereas the size of the circle is related to the correlation coefficient.

tween CCL5 *vs*. TNF-R1 (PCC -0.47, $p=0.042$) and retinol binding protein 4 (RBP4) *vs*. TNF-R1 (PCC -0.48, $p=0.039$); in the tocilizumab arm between thyroid peroxidase (TPO) *vs*. CCL3 (PCC -0.48, $p=0.019$) and in the methotrexate arm between I-CAM *vs*. IL-22 (PCC -0.49, $p=0.030$) and programmed cell death protein 1 (PD-1) *vs*. IL-25 (PCC -0.54, $p=0.025$). Fig. 3 depicts the differences in concentrations of the selected proteins between those achieving sDFR *vs*. controls. To evaluate if protein levels statistically significantly differed between both groups, we performed logistic regression analyses in which a negative effect estimate (β) indicates a lower protein level in the sDFR group (Table II). In the tocilizumab plus methotrexate arm, CCL18 (β=-3.31, $p=0.047$), CCL20 (β=-1.24, $p=0.035$) and soluble IL-2 receptor alpha (sIL-2Rα; β=1.40, $p=0.039$) were significantly associated with achieving sDFR; in the tocilizumab and methotrexate arm no significant associations ($p\geq0.07$) were found.

Pathway analyses

In the tocilizumab plus methotrexate arm, 49 significantly enriched GO terms were identified; in the tocilizumab arm 88, and in the methotrexate arm 117. The top five significant GO terms with the highest number of proteins included in the pathway are shown in Supplementary Table II. The pathways "extracellular space" (GO:0005615) and "extracellular region" (GO:0005576) were significantly enriched ($p\leq1.06^{E-02}$) within all treatment arms. We performed in addition pathway analyses in the KEGG database; 9 significant pathways were found in the tocilizumab plus methotrexate arm; 34 in the tocilizumab arm; and 14 in the methotrexate arm. Important KEGG pathways significantly enriched within all three strategy arms were: "rheumatoid arthritis" ($p\leq5.46^{E-04}$), "nuclear factor-kappa B signalling" (NF-κB, $p\leq3.05^{E-02}$), "cytokine-cytokine receptor interaction" ($p\leq9.29^{E-08}$) and "TNF signalling pathway" ($p\leq2.30^{E-02}$).

From transcriptomics to proteomics

We previously demonstrated within networks of co-expressed genes, based on sequenced mRNA isolated from $CD4^+$ cells, that several pathways were

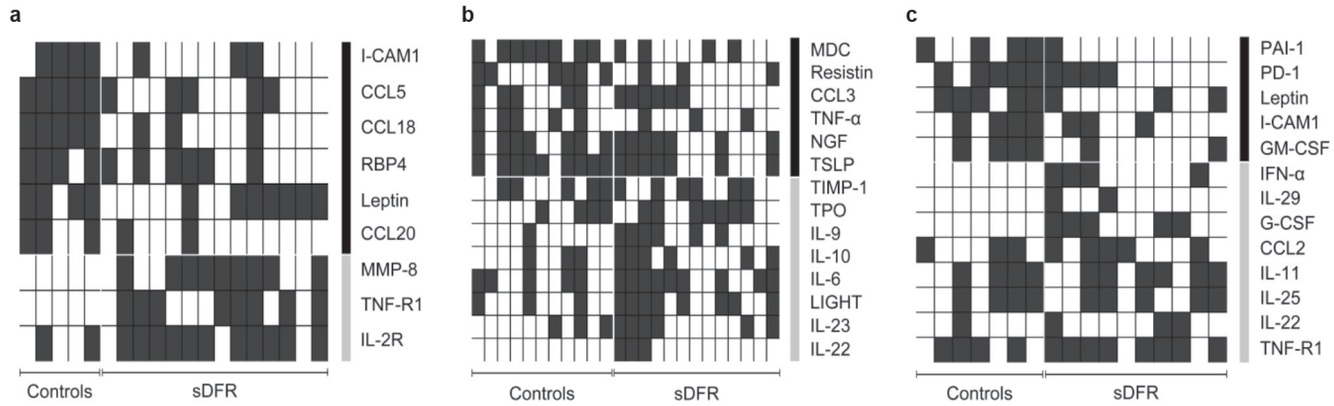

Fig. 3. Clustered heatmap of the relevant proteins in the (**a**) tocilizumab plus methotrexate, (**b**) tocilizumab, and (**c**) methotrexate strategy arms. *White* colour depicts a negative z-score (*i.e.* lower concentration) and *black* colour depicts a positive z-score (*i.e.* higher concentration). Proteins in the *black* cluster have on average level lower concentration (*i.e.* effect estimate <0) in the sDFR group and those in the *grey* cluster a higher concentration (*i.e.* effect estimate >0).

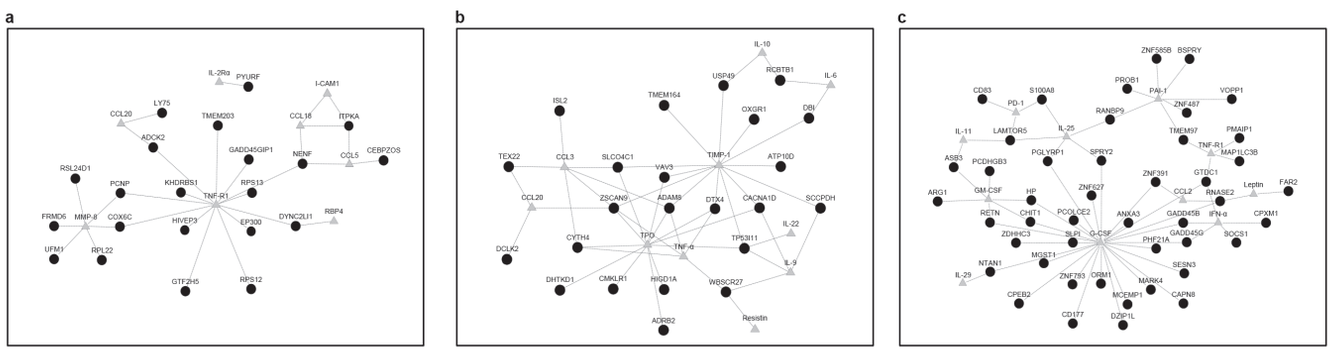

Fig. 4. Network visualisation of significant transcript-protein correlations in the (**a**) tocilizumab plus methotrexate, (**b**) tocilizumab, and (**c**) methotrexate strategy arms. Circular nodes depict transcripts and triangular nodes depict proteins. Significant transcript-transcript and protein-protein correlations are not displayed and also proteins without significant correlations with transcripts.

related to achieving sDFR after initiating tocilizumab plus methotrexate, or tocilizumab or methotrexate therapy (22). We now, in the present explorative study, compared the significantly enriched pathways found in the transcriptomic analyses with those in the present study when analysing relevant proteins. In the tocilizumab plus methotrexate arm, no corresponding significant enriched GO terms were found; in the tocilizumab arm 5, of which "positive regulation of leukocyte migration" (GO:0002687) and "leukocyte chemotaxis" (GO:0030595), were found in both biological systems ($p \leq 3.41^{E-02}$ and $p \leq 9.49^{E-03}$, respectively); in the methotrexate arm 33, of which important corresponding pathways are related to response of several stimuli or Janus kinase signal transducer and activator of transcription (JAK-STAT) activity (GO:0007259, Supplementary Table III). When comparing the significantly enriched pathways in the KEGG database, we found in the methotrexate arm one pathway ("JAK-STAT signalling pathway") that was significantly enriched in both the transcriptional and proteomic pathways ($p \leq 2.22^{E-04}$). The only identified significant pathway ("Ribosome") in the co-expressed genes of the tocilizumab plus methotrexate arm was not significantly enriched in the proteins; no KEGG pathways were found in both biological systems in the tocilizumab arm. Fig. 4 shows the significant transcript-protein correlations within the three strategy arms. In the tocilizumab plus methotrexate arm, the TNF-R1 (13 correlations) protein showed most correlations with transcripts; in the tocilizumab arm it was TIMP metallopeptidase inhibitor 1 (12 correlations) and in the methotrexate arm granulocyte-colony stimulating factor (27 correlations).

Discussion

By performing multi-analyte profiling in pre-treatment serum of DMARD-naïve patients with early RA, we identified several potentially relevant proteins associated with achieving sDFR after treatment. Between the treatment strategy arms, we mostly found different inflammatory proteins, indicating that achieving sDFR might be dependent of both the pre-treatment concentrations of specific proteins and the therapy that is initiated. Although the concentration levels of most proteins individually were not statistically different for the group achieving sDFR *vs.* controls, analyses of the networks showed clear group separation.

To better understand the complex processes involved in the systems biology of newly diagnosed RA patients, we integrated the results of earlier analyses of the transcription of genetic information (transcriptome) with those of their translation into proteins (proteome). While the genome remains almost static over time, both the transcriptome and proteome are prone to modifications resulting in variations across the multiple layers of gene regulation (28). Most

reports only show a weak correlation between transcriptomic and proteomic analyses, probably due to the regulatory mechanisms affecting the expression of both the RNA as the protein (29). We therefore chose to evaluate the biological pathways found enriched in the proteomic analyses and compare these with the pathways previously found in the transcriptomic analyses of the same patients (22). Our findings could not be validated as U-Act-Early is to date the only study in which previously untreated early RA patients were treated-to-target with tocilizumab and/or methotrexate and in which medication thereafter was tapered and finally discontinued, if remission persisted. However, our present comparison of the findings of the analyses of two different biological systems, measured within different tissues with different techniques, probably minimises the risk of false positive findings.

In the tocilizumab plus methotrexate arm, important transcriptional pathways were related to processes associated with translation of mRNA (22) but in this study these pathways could not be verified when analysing the pathways related to the network of proteins identified. In total, 325 significantly enriched GO terms were found in the mRNA and 49 GO terms in the protein, none of which overlapped. Furthermore, the only significant enriched KEGG pathway ("Ribosome") in the mRNA could not be verified either when performing analyses of the relevant proteins. One of the causes might be a Type I error; the biomarkers have not been externally validated. Another explanation could be related to the pathways found in the mRNA, of which nonsense-mediated decay (NMD) was the most important GO term (GO:0000184). NMD is a surveillance pathway involved in several biological processes, such as controlling gene expression of natural occurring transcripts, but it is also important for eliminating premature stop codons preventing the production of truncated proteins (30, 31). Furthermore, in the proteomic analyses, signalling of NF-κB proteins was a significantly enriched KEGG pathway, which is important for transcription of deoxyribonucleic acid (DNA) and post-translational modifications, and therefore for cytokine production (32-34). In the tocilizumab arm, 88 significantly enriched GO terms were found in the relevant proteins; in the previous mRNA analyses, we identified 304 significant GO terms; 5 terms overlapped, of which "positive regulation of leukocyte migration" (GO:0002687) and "leukocyte chemotaxis" (GO:0030595) were significantly enriched in both the proteomic and transcriptomic analyses. In the mRNA, network analyses of co-expressed genes in sequenced monocytes ($CD14^+$) did not resulted in any relevant findings. Thus, although leukocyte activity is not further specified in the GO database, for achieving sDFR activation of lymphocytes seems more important than activation of monocytes, warranting future research. In the methotrexate arm, the most important GO terms in the mRNA were all related to "Response to stimulus" (GO:0050896); they thus seem to mirror the response to therapy (22). "Response to stimulus" was included in the 33 overlapping GO terms, and 16 other terms of the remaining 32 were also related to this term. Methotrexate in RA likely acts through targeting the production of multiple cytokines (*e.g.* TNF-α and interferon-γ) (35-37). *In vitro* methotrexate suppresses JAK-STAT signalling by several ligands (38), including pro-inflammatory cytokines (39, 40). In the pathway analyses of both the mRNA as proteins within the methotrexate arm, in the KEGG database "JAK-STAT signalling" (map04630) was significantly expressed and in the GO database "JAK-STAT cascade" (GO:0007259). Also the GO term "Positive regulation of tyrosine phosphorylation of STAT protein" (GO:0042531), important for signal transduction and enzymatic activity in the JAK-STAT signalling pathway, was found significantly enriched in both biological systems. These results provide further evidence for the inflammation-reducing role of methotrexate via JAK-STAT. To our knowledge, this is the first study reporting on analyses of both transcriptomics as proteomics in newly diagnosed RA patients. Only one study has to date reported on predictive proteomic biomarkers for treatment response to tocilizumab in biologic DMARD naïve patients when analysing multiple proteins simultaneously in serum, but it did not report on transcriptomics (41).

There are however several limitations to our study that should be considered when interpreting the results. First, the numbers of samples analysed are relatively small, possibly impairing the validity of the results. Second, the proteins that were measured were selected based on relevance and availability. It cannot be excluded that other (non-measured) inflammatory proteins such as MMP-3 are also of importance for achieving sDFR or not, after treatment with bDMARDs (*e.g.* tocilizumab).(42).

Conclusion

In DMARD-naïve early RA patients, pre-treatment concentrations of several inflammatory proteins were associated with achieving sDFR, but achieving it also seems dependent on the initiated treatment strategy. Several enriched biological pathways within the protein biomarkers were also previously identified pathways in the co-expressed genes in the tocilizumab- (related to leukocyte activity) and methotrexate-based (related to JAK-STAT activity) strategies, in contrast to in the tocilizumab plus methotrexate-based strategy. These findings may lead to a better understanding of gene expression and translation of inflammatory proteins in newly diagnosed RA patients. Ultimately, these kind of analyses might identify predictors, enabling more personalised treatment strategies for RA.

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