Targeted proteomics in extracellular vesicles identifies biomarkers predictive for therapeutic response in sarcoidosis

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There are specific differences in proteins and associated pathways between patients with pulmonary sarcoidosis before initiation of therapy that can be used to predict therapeutic response to prednisone or methotrexate: towards personalised medicine https://bit.ly/3XJ7x9m

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Abstract

Background ∼30% of patients with sarcoidosis, a systemic granulomatous disease of unknown cause, need treatment to alleviate symptoms or prevent organ damage. Prednisone and methotrexate (MTX) are the most commonly used drugs; however, success of treatment varies from patient to patient. In this study, we search for biomarkers and pathways that predict response to treatment with prednisone or MTX in extracellular vesicles (EVs).

Methods A targeted proteomics approach (OLINK Bioscience) was used in which 92 proteins were measured in two baseline EV fractions in 32 patients treated for pulmonary sarcoidosis (eight responders and eight non-responders each for prednisone and MTX). The top three proteins were replicated in 62 prednisone- and 76 MTX-treated patients.

Results We identified 11 differentially expressed proteins (DEPs) between responders and non-responders to prednisone treatment, and 16 DEPs for patients treated with MTX. Reactome pathway analysis showed DEPs in prednisone to be involved in nuclear factor kappa B and interleukin signalling pathways. The DEPs in MTX were involved in transduction of GPI-anchored proteins and MAPK signalling pathway. CHI3L1 for prednisone and CPA1 for MTX were replicated as significant predictors of response.

Conclusion This study is the first to show that in pulmonary sarcoidosis the response to treatment with prednisone or MTX can be predicted at baseline by different EV proteins active in different pathways. Using these markers and associated pathways to identify patients with a high probability of response to therapy will aid personalised treatment choice and improve treatment outcome.

Introduction

Sarcoidosis is a systemic granulomatous disease of unknown cause mainly affecting the lungs, intrathoracic lymph nodes, eyes and skin. Predicting disease course and response to therapy can be challenging when managing patients with sarcoidosis. Pharmacological treatment of sarcoidosis is mostly initiated to either prevent further specific organ damage or alleviate symptoms, and often includes corticosteroids or disease-modifying anti-rheumatic drugs like methotrexate (MTX) [1].

Glucocorticosteroids are currently the first choice of therapy in pulmonary sarcoidosis and are mainly known for their anti-inflammatory capacities [2, 3]. Glucocorticosteroids can diffuse through the cell membrane and subsequently bind to the glucocorticoid receptor (GR) in the cytoplasm. Binding of the GR results in a conformational change, which inhibits multiple steps in the inflammatory pathway. This results in inhibition of transcription factors controlling synthesis of pro-inflammatory mediators, like macrophages and T-cells [4–6]. Additional important effects of glucocorticosteroids are the inhibition of numerous

inflammatory mediators, pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), and various interleukins, such as IL-1 and IL-2 [4, 7].

MTX is most frequently prescribed as second-line therapy in pulmonary sarcoidosis, in case prednisone is either lacking efficacy or not tolerated due to side-effects [8]. MTX is a folate antagonist inhibiting DNA synthesis with immunomodulatory capacities when dosed <30 mg·week^{-1} [9, 10]. After absorption, MTX is transported into the cell where it is polyglutamated by the enzyme folylpolyglutamate synthase (FPGS) which adds glutamate residues creating MTX polyglutamates (MTX-PG), the active metabolites of MTX [11–13]. MTX-PG$_{1–5}$ have an inhibitory effect on cellular proliferation [11]. MTX reduces pro-inflammatory cytokine production by T-helper 1 (Th1) cells and monocytes/macrophages, thereby suppressing production of TNF-α and nuclear factor kappa B (NF-κB) and inhibiting upregulation of several interleukins [10, 14]. Moreover, MTX increases production of anti-inflammatory cytokines in Th2 cells and increases reactive oxygen species synthesis in T-cells and monocytes resulting in apoptosis in these cells [10].

There is an unmet clinical need for biomarkers that predict disease course and that guide therapeutic management. For example, the ability to predict whether patients will respond to prednisone or MTX would guide initiation of therapy and save valuable time, organ function, patient health and resources [15, 16].

In the search for such predictive biomarkers, extracellular vesicles (EVs) have shown to be a new promising potential source of biomarkers for sarcoidosis [17]. EV is an umbrella term for all vesicles found in body fluids, including exosomes, microvesicles and apoptotic bodies, and are present in large amounts [18]. In blood, ∼10^{10}–10^{11} EVs per millilitre can be found, making it possible to detect small amounts of key proteins in blood [19]. EVs share several functions with cytokines as mediators of intercellular communication and are involved in several homeostatic processes [20]. When shedding from the donating cell, EVs can modulate cell functions of other cells by delivering intercellular signals both from their surface proteins as well as through inter-vesicle content (*e.g.* proteins, DNA and RNA) [20]. Furthermore, cytokines can also associate with EVs as either internal or external content. With this, EVs can signal a pro- or anti-inflammatory modulating effect on surrounding cells, activating differentiation, recruitment or various other functions of immune cells [20]. In recent work from our group, additional value was demonstrated in measuring proteins specifically in EV, even if concentrations of these proteins in serum did not have predictive value on disease course or response to therapy [21]. In the current study, we used a targeted proteomics approach on EV isolated fractions to further elucidate proteins and pathways involved in prednisone or MTX treatment response in patients with pulmonary sarcoidosis.

Materials and methods

Study patients and ethics

Patients treated with either prednisone or MTX for pulmonary sarcoidosis were included in this study. Patients were selected from the St. Antonius ILD biobank based on treatment indication, duration of treatment and availability of samples at baseline. The diagnosis of sarcoidosis was made according to the criteria of the American Thoracic Society/European Respiratory Society [22], and duration of treatment was at least 6 months. All included patients had a pulmonary treatment indication and had not had any treatment for at least 6 months prior to blood withdrawal and start of prednisone or MTX monotherapy. The following baseline characteristics were retrieved from medical records: age, sex, comorbidities, Scadding stage and extrapulmonary involvement. Lung function values (forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (D_{LCO})) were retrieved from baseline up to 6 months after treatment initiation.

The study was approved by the Medical Research Ethics Committees United (MEC-U) of the St. Antonius Hospital (R05-08A), and written consent was obtained from all patients.

The workflow for OLINK biomarker discovery analysis and validation is summarised in figure 1.

Assessment of treatment effect

Response to treatment was defined by improvement in pulmonary function after 24 weeks of monotherapy with either prednisone or MTX. Patients who started add-on treatment before completing 6months of monotherapy were excluded from the analysis. Classification of response was based on the results of the recently reported SARCORT trial [23] as well as the sarcoidosis treatment score [24]. Patients were classified as "responders" in case of an improvement of ⩾5% for FVC % pred or ⩾10% for D_{LCO} % pred after 24±2 weeks compared to baseline (before treatment). Patients were classified as "non-responder" when FVC or D_{LCO} had a decrease ⩾5% or ⩾10% respectively after 24±2 weeks compared to baseline.

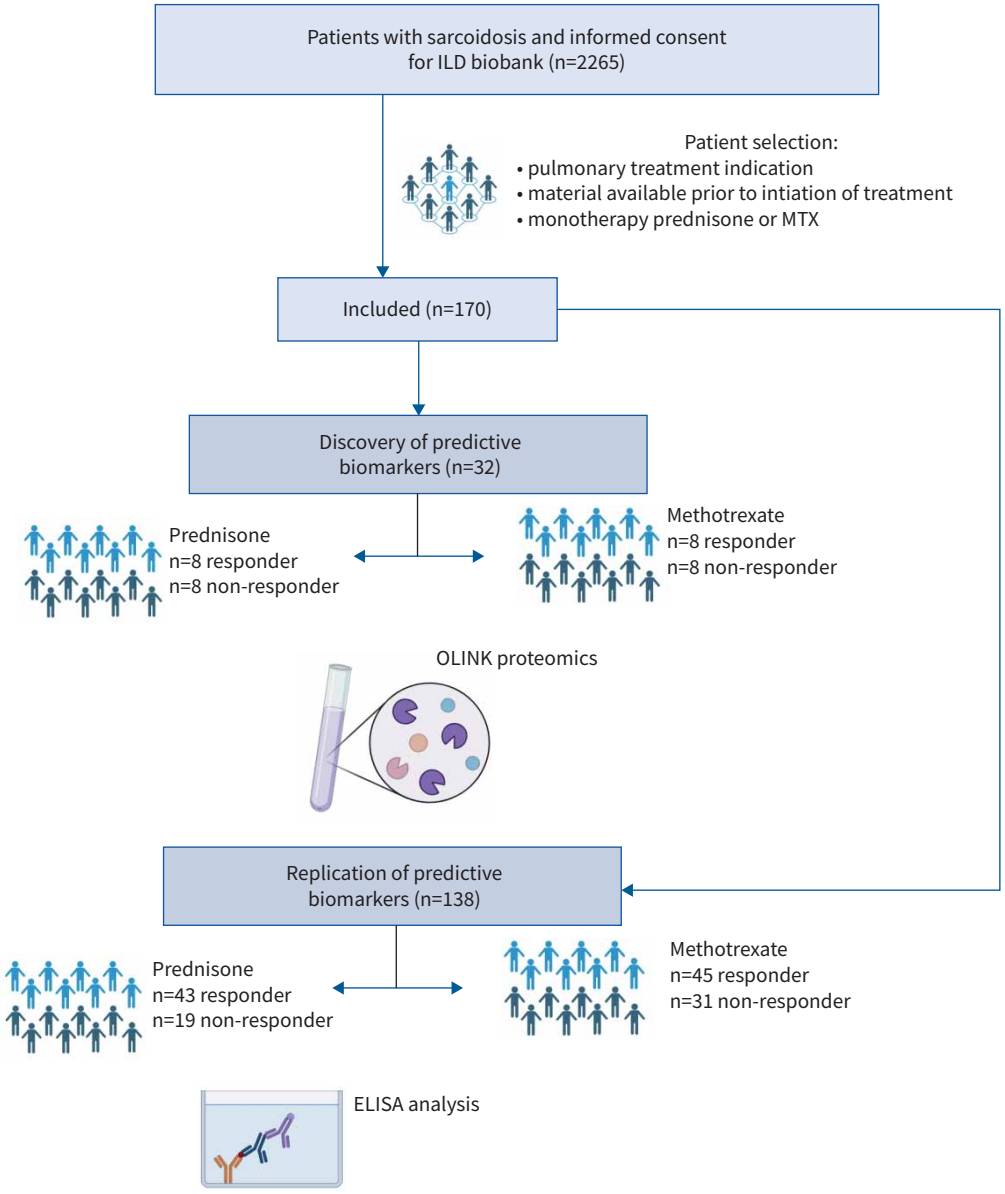

FIGURE 1 Schematic overview of workflow. First an OLINK proteomics approach was performed on a discovery cohort (n=32) to search for potential predictive biomarkers for response to treatment with either prednisone or methotrexate (MTX). The top three predictive proteins for each treatment group were replicated in a second cohort (n=138) by ELISA. ILD: interstitial lung disease.

Patients who did not meet criteria either for responder or for non-responder were classified as having stable disease and were not included in this study.

Proteomic analyses

Proteomic analysis was performed in 32 patients: 16 patients treated with prednisone (eight responders and eight non-responders) and 16 patients treated with MTX (eight responders and eight non-responders). Both groups were matched for age, sex, ethnicity and smoking history. Serum samples were collected before start of treatment, centrifuged and stored at −80°C for future analyses. EVs were isolated from serum in two subfractions: low-density lipid particles (LDL) and high-density lipid particles (HDL), as described before [21]. EV proteins were profiled using the commercially available OLINK Target 96 Cardiovascular disease (CVD) III immunoassay panel (OLINK Proteomics, Uppsala, Sweden), which simultaneously measured 92 proteins and four control proteins. Selection of this OLINK panel was based on previous descriptions of the proteins in relation to either prednisone or MTX and sarcoidosis. Of the available

OLINK panels, the CVD III panel contained the most proteins related to the therapies and sarcoidosis. OLINK data are converted to normalised protein expression (NPX), which is expressed in a log2 scale. The NPX values cannot be converted into absolute protein concentrations. All information regarding detection limits, assay performance and validation are available on the manufacturer's website (www.olink.com). The results of the measurements were considered reliable when detected levels were above the limit of detection in at least 75% of the samples. The abbreviations and full names of measured proteins are described in supplementary tables S1 and S2. Differentially expressed proteins (DEPs) were further evaluated if p-values were <0.10 and log2FC >1 or <−1.

The three proteins with the most significant difference between responders and non-responders for prednisone and the top three for MTX were measured in an independent second cohort consisting of EV samples from 57 patients treated with prednisone (45 responders and 12 non-responders) and 84 patients treated with MTX (52 responders and 32 non-responders). To quantify the changes in concentrations of these candidate proteins, enzyme-linked immunosorbent assay (ELISA) was used according to manufacturers' instructions. Absorbance measurements were recorded at 450 nm on 800 TS Absorbance reader (BioTek, Winooski, VT, USA). The selected proteins for ELISA are listed in supplementary table S3.

Bioinformatics and statistical analysis

To detect significant changes in protein abundance between responders and non-responders to the prescribed medication, IBM SPSS statistics version 26, Rstudio version 4.4.0 and Graphpad Prism 9 were used. Non-parametric tests were used for non-normally distributed data (Mann–Whitney U-test). The fold change (FC) was calculated by dividing mean protein level of responders by the average protein level of non-responders. A p-value <0.10 for the difference between responders and non-responders in the discovery cohort was considered of interest for further analysis. In the second cohort a p-value <0.05 was considered significant. Reactome pathway analysis (www.reactome.org/) "PADOG analysis method" was used to discover if different pathways are involved in prednisone and MTX responders. Discrete normalisation function trimmed mean of M values (TMM) and maximal missing values was set to 0.5. To investigate the relationship between the protein levels and response to therapy, a logistic regression model with the outcome "improvement in lung function" was used. Receiver operating characteristic analysis was used to derive area under the curve (AUC) values to evaluate the predictive value of the proteins. Protein concentrations were log2-transformed. Multiple logistic regression analysis was applied using proteins as predictors and responder as outcome. Additionally, proteins were combined to calculate their predictive probability for the response to treatment with either prednisone or MTX. From these logistic regression analyses the AUC values were evaluated, including sensitivity and specificity using the pROC package in Rstudio.

Results

Baseline characteristics of the discovery cohort are reported in table 1. In both treatment groups 62.5% were male and 25.0% had a history of smoking. There was no significant difference in Scadding stage or extrapulmonary involvement between responders and non-responders. At baseline, FVC did not differ between groups (p=0.248 and p=0.156, respectively). A significant difference in D_{LCO} was found between responders and non-responders in the patients treated with MTX (p=0.010), but not for patients treated with prednisone (p=0.355).

Targeted proteomics analysis

With the OLINK panel, 92 proteins were measured in both LDL and HDL EV fractions of sarcoidosis patients treated with prednisone or MTX. Proteins that were above the limit of detection were considered reliable. For the prednisone cohort, 67 proteins in the LDL faction and 20 proteins in the HDL fraction were reliably detected, and for the MTX cohort these numbers were 67 proteins in the LDL fraction and 32 proteins in the HDL fraction (supplementary figure S1). Detected proteins were then further analysed for differences in expression between responders and non-responders. In total, 27 DEPs were present in EV fractions, including 11 for prednisone and 16 for MTX (p-value <0.10; supplementary figure S2). There was no overlap in the DEPs between the two treatment groups. Table 2 lists the DEPs with p-value and log2FC for each protein.

Pathway enrichment analysis

To further investigate the role of the proteins in relation to response to therapy with either prednisone or MTX a Reactome pathway analysis was used. The pathways that met the threshold of −log10 (p-value) ⩾1.0 are shown in the bubble plots of figure 2. For prednisone, proteins were mainly enriched in NF-κB and mitogen-activated protein kinase (MAPK) pathways, and interleukin mediated signalling, while for MTX the results were mainly enriched in processes involving glycosylphosphatidylinositol (GPI) anchors and protein

TABLE 1 Baseline characteristics of the sarcoidosis patients with a pulmonary treatment indication used for the exploratory proteomic analysis

Parameter	Prednisone-treated group		Methotrexate-treated group	
	Responder	Non-responder	Responder	Non-responder
Patients, n	8	8	8	8
Age years[#]	37.4±8.5	43.3±6.7	52.6±13.0	48.3±7.0
European descent	8 (100)	8 (100)	8 (100)	8 (100)
Disease duration years[¶]	0.2±0.2	1.3±1.6	2.7±2.1	0.7±0.4
Male sex	5 (62.5)	5 (62.5)	5 (62.5)	5 (62.5)
Ever-smoker	2 (25.0)	2 (25.0)	2 (25.0)	2 (25.0)
Scadding stage 0/I/II/III/IV[+]	0/0/3/3/2 (0/0/37/37/25)	0/0/3/2/3 (0/0/37/25/37)	0/1/4/1/2 (0/12/50/12/25)	0/1/5/1/2 (0/0/62/12/25)
Extrapulmonary involvement	5 (62.5)	7 (87.5)	5 (62.5)	5 (62.5)
Lung function[+]				
FVC %	81.8±24.2	94.5±28.5	89.0±21.8	102.7±11.6
D_{LCO} %	77.7±15.3	88.8±13.5	55.5±13.1*	86.1±12.6*

Data are presented as mean±SD or n (%), unless otherwise stated. FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide. [#]: age at time of blood withdrawal; [¶]: disease duration is calculated as the time (years) between disease diagnosis and start of treatment; [+]: lung function and Scadding stage were measured before start of treatment. *: p<0.05.

modification, but also in MAPK and BRAF signalling. Furthermore, we analysed the correlation between the DEPs based on the NPX values. For prednisone, eight DEPs were higher in responders and two were lower in responders compared with non-responders. In the prednisone group, the strongest correlations were found between CD163 and TNFSF13B (R=0.83; p<0.001), IL18BP and ALCAM (R=0.83; p<0.001) and between IL2RA and TNFSF13B (R=0.81; p<0.001). For MTX all DEPs were lower in the responders than in the non-responders. The strongest correlations in the MTX group were found between TIMP4 and CNTN (R=0.83; p<0.001), u-PAR and uPA (R=0.81; p<0.001) and between TIMP4 and vWF (R=0.73; p=0.001).

Replication of top DEPs

For replication of the results, we examined three proteins that were most significantly different between responders and non-responders in both the prednisone-treated and in the MTX-treated discovery cohort, respectively (table 2). ELISA analysis was performed on isolated EV samples of the second cohort consisting of 62 patients treated with prednisone and 76 patients treated with MTX. Baseline clinical patient characteristics are shown in table 3; there were no significant differences between responders and non-responders.

TABLE 2 Differentially expressed proteins between responders and non-responders in the discovery cohort

Patients receiving treatment with prednisone				Patients receiving treatment with methotrexate			
Gene symbol	EV fraction	Log2 FC	p-value	Gene symbol	EV fraction	Log2 FC	p-value
CHI3L1	LDL	1.552	0.003	**MMP2**	LDL	−1.438	0.002
CD163	HDL	1.215	0.016	**CPA1**	HDL	−1.602	0.012
CXCL16	LDL	−1.204	0.021	**uPA**	LDL	−1.270	0.012
IL2RA	LDL	1.227	0.036	vWF	LDL	−1.167	0.016
IL18BP	HDL	1.183	0.036	TLT2	LDL	−1.114	0.016
TNFSF13B	LDL	1.096	0.046	TFPI	HDL	−1.139	0.016
PDGFA	LDL	−1.287	0.046	CNTN1	LDL	−1.092	0.027
CHIT1	HDL	2.138	0.046	IL1RT2	LDL	−1.134	0.046
ALCAM	HDL	1.188	0.059	PRTN3	LDL	−1.330	0.046
CHIT1	LDL	1.510	0.059	U-PAR	LDL	−1.141	0.046
CD163	LDL	1.116	0.093	LDLR	LDL	−1.227	0.046
				PGLYRP1	HDL	−1.193	0.059
				TRAP	LDL	−1.256	0.059
				TIMP4	LDL	−1.197	0.074
				IL6RA	LDL	−1.086	0.074
				ST2	HDL	−1.216	0.093

Proteins marked in bold were analysed in the replication cohort. Proteins are ordered by p-value. FC: fold change, protein level of responder divided by protein level of non-responder; EV: extracellular vesicle.

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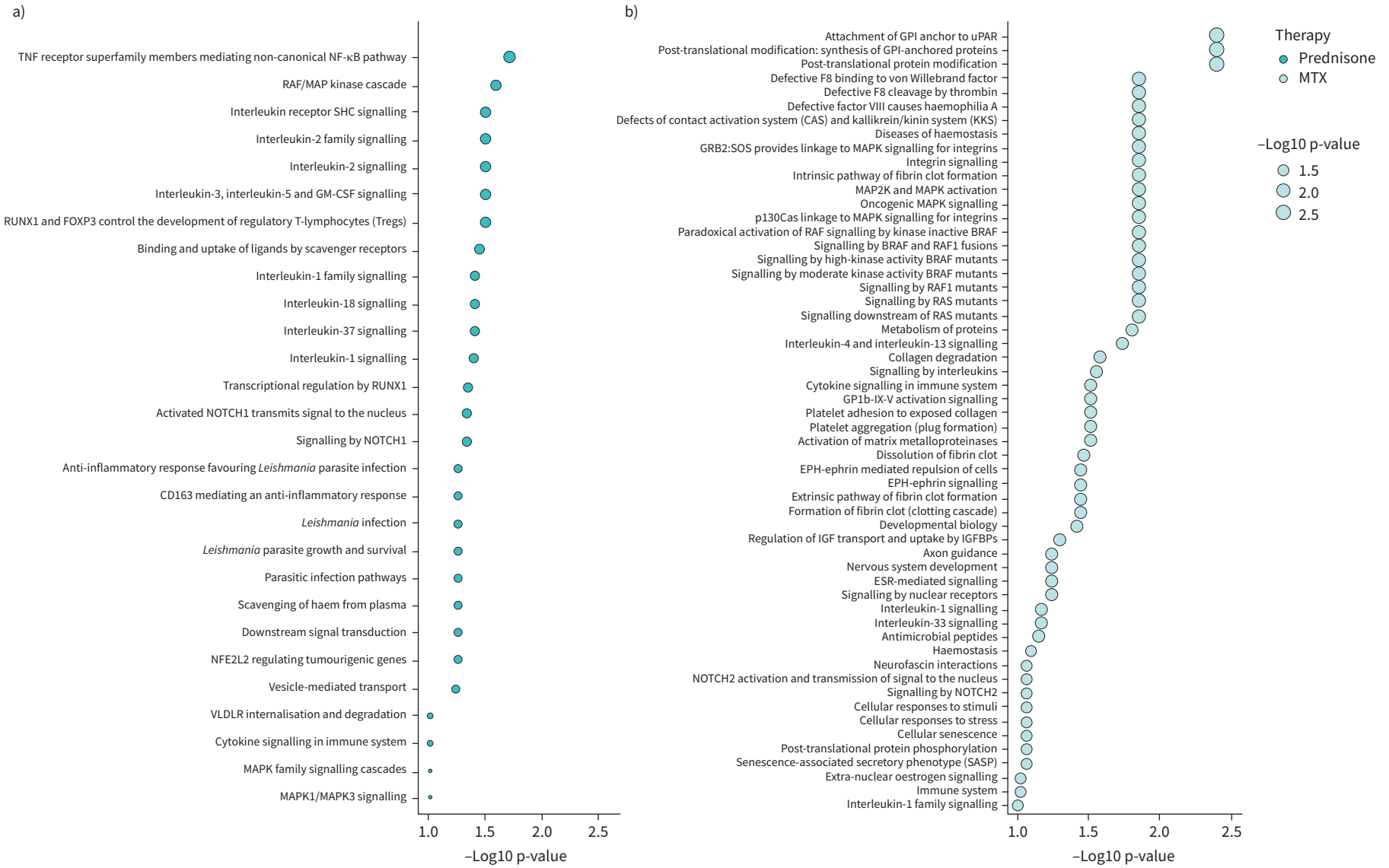

FIGURE 2 The bubble plot of Reactome pathway analysis of proteins differently expressed between responders and non-responders in the discovery cohort treated with a) prednisone and b) methotrexate. GM-CSF: granulocyte-macrophage colony-stimulating factor; GPI: glycosylphosphatidylinositol-anchored glycoproteins; IGFBP: insulin-like growth factor binding protein; MAPK: mitogen-activated protein kinase kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B-cells; NFE2L2: nuclear factor erythroid-derived 2-like 2; VLDLR: very-low-density-lipoprotein receptor.

TABLE 3 Baseline characteristics of the second cohort of sarcoidosis patients with a pulmonary treatment indication; this cohort was used for the replication of the top three proteins that were most significantly different between responders and non-responders

Parameter	Prednisone-treated group		Methotrexate-treated group	
	Responders	Non-responders	Responders	Non-responders
Patients, n	43	19	45	31
Age years[#]	43.3±10.1	48±8.0	47.8±10.9	49.1±10.9
European descent	38 (90.5)	19 (100)	42 (93.3)	28 (90.3)
Disease duration years[¶]	2.0±3.5	4.6±5.6	3.1±3.8	2.3±3.0
Male sex	28 (65.1)	13 (68.4)	24 (53.3)	23 (71.9)
Ever-smoker	14 (40.0)	6 (37.5)	14 (40.0)	16 (59.3)
Scadding stage[+]	0/10/20/4/6	0/0/7/1/3	0/4/19/7/10	0/4/14/3/11
0/I/II/III/IV	(0/25/50/10/15)	(0/0/64/1/27)	(0/10/48/18/25)	(0/13/44/9/34)
Extrapulmonary involvement	31 (73.2)	17 (88.2)	39 (86.0)	25 (82.6)
Lung function[+]				
FVC %	86.7±22.8	90.74± 21.6	93.0±23.8	97.3±18.8
D_{LCO} %	68.1±15.5	72.5±21.6	67.1±15.7	76.0±14.6

Data are presented as mean±SD or n (%), unless otherwise stated. FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide. [#]: age at time of blood withdrawal; [¶]: disease duration is the time (years) between disease diagnosis and start of treatment; [+]: lung function and Scadding stage were measured before start of treatment.

Of the six proteins that were measured, CHI3L1 remained significantly different between responders and non-responders for prednisone (p=0.007) and CPA1 for MTX (p=0.010) respectively (figure 3). CHI3L1 correlated with change in FVC (R=0.32; p=0.032), and both CHI3L1 and CD163 had a significant odds ratio (OR) (p=0.032 and p=0.028, respectively) for improvement in FVC after treatment with prednisone. For patients treated with MTX MMP2 correlated with change in FVC after 24 weeks of treatment (R= −0.40; p=0.012) and also showed a significant OR (p=0.015) for change in FVC after 24 weeks of treatment (table 4).

Protein concentrations of the replication cohort were log2-transformed to determine their sensitivity and specificity in a receiver operating characteristic analysis. The highest AUC values were found for CHI3L1 for the prednisone-treated group and CPA1 for the MTX-treated group and were 0.79 and 0.79, respectively (supplementary figure S3). Sensitivity and specificity of CHI3L1 were 0.72 and 0.70, and the cut-off value was 0.69. For CPA1 sensitivity was 0.78 and specificity 0.70 with a cut-off value of 0.67. Results for combinations of the selected proteins are shown in supplementary table S4. For the patient group treated with prednisone, none of the combinations resulted in an increased AUC compared to the AUC of CHI3L1. For the patient group treated with MTX, CPA1 in combination with uPA showed an increase in the AUC value from 0.79 to 0.80.

Discussion

In this study, we explored the involvement of proteins present in EVs related to response to treatment with either prednisone or MTX. In the discovery cohort of this study, we found 27 EV proteins differently expressed between responders and non-responders to treatment at start of therapy. Interestingly, there was no overlap between DEPs found in the prednisone- and the MTX-treated groups. This suggests that within the sarcoidosis patient population there are already biological differences that correlate with outcome of specific anti-inflammatory treatments. Specifically, levels of CHI3L1 for prednisone and CPA1 for MTX were higher in responders to therapy in both the discovery and replication cohort. Moreover, both proteins showed an AUC of 0.79, which suggests that these proteins possess distinct properties for predicting therapy response in sarcoidosis patients.

Pathway evaluation was utilised to obtain understanding of the biological significance of the proteins in relation to response to treatment. For patients receiving treatment with prednisone, the pathway involved in mediating NF-κB was most significant. This cytokine is related to the MAPK signalling pathway and the RAF/MAP kinase cascade also shown to be involved in the prednisone cohort. Chronic activation of the MAPK signalling pathway was previously shown to result in uncontrolled inflammation and chronic inflammatory diseases, including sarcoidosis [25]. Activation of NF-κB and MAPKs is crucial for synthesis of Th1 cytokines, such as TNF-α and IL-6, which have been reported to be elevated in patients with sarcoidosis [26]. Furthermore, previous studies have already shown that corticosteroids can inhibit

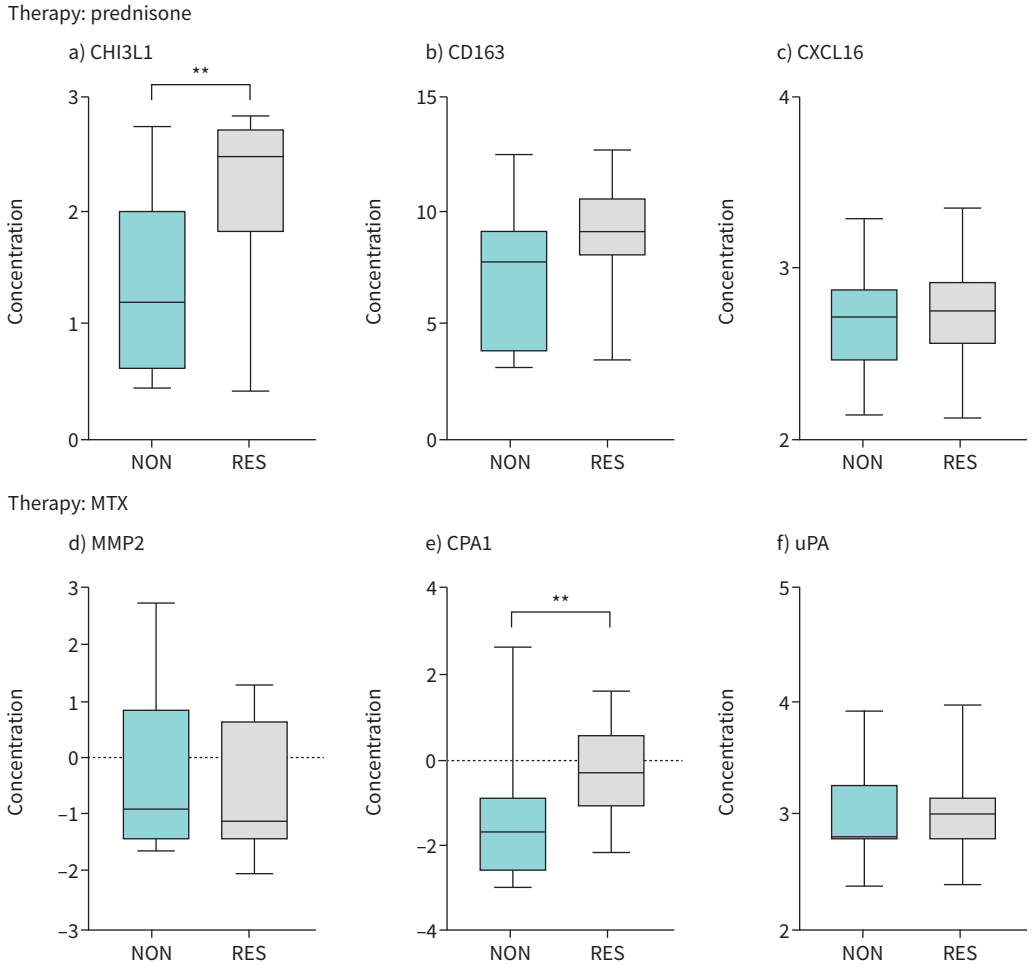

FIGURE 3 Box-plots of protein concentrations in the replication cohort of sarcoidosis patients treated with a–c) prednisone and d–f) methotrexate (MTX). Proteins measured are a) CHI3L1 in LDL, b) CD163 in HDL, c) CXCL16 in LDL, d) MMP2 in LDL, e) CPA1 in HDL and f) uPA in LDL. Concentration is shown in log2 scale with boxes representing interquartile range and whiskers the minimum and maximum for non-responders (NON) and responders (RES) to treatment. Concentrations of CHI3L1 and CPA1 are significantly different between responders and non-responders treated with prednisone and methotrexate. LDL: low-density lipid particles; HDL: high-density lipid particles. **: p<0.01.

activity of the MAPK signalling [27, 28]. Inhibition of the MAPK signalling pathway would lead to inhibition of the pro-inflammatory cytokine release specific for sarcoidosis, resulting in a favourable outcome of this treatment.

TABLE 4 Logistic regression of extracellular vesicle protein levels and change in forced vital capacity after 24 weeks of treatment

Prednisone-treated group			Methotrexate-treated group		
Protein	OR (95% CI)	p-value	Protein	OR (95% CI)	p-value
CHI3L1	**3.6 (1.1–11.8)**	**0.032**	**MMP2**	**0.5 (0.3–0.9)**	**0.015**
CD163	**1.2 (1.0–1.5)**	**0.028**	CPA1	0.9 (0.6–1.2)	0.451
CXCL16	1.3 (0.2–8.4)	0.806	uPA	0.7 (0.1–4.1)	0.718

Bold type denotes statistical significance for improvement of FVC after 24 weeks of treatment with prednisone or methotrexate.

CHI3L1 was previously found to relate to cancer cell survival, transformation, migration and invasion by regulating the PI3K/AKT/mTOR and Ras/Raf/MAPK signalling pathways [29–31]. Moreover, studies showed that MAPK pathway phosphorylation was strongly induced by CHI3L1 in asthma and that CHI3L1 increased IL-8 production in bronchial epithelial cells *via* MAPK and NF-κB pathways [29, 32]. Concentrations of CHI3L1 remained significantly higher for responders compared to non-responders treated with prednisone in the replication cohort, and consistent with the finding, its baseline concentration was shown to relate to improvement in lung function after 24 weeks of treatment.

The precise cellular function of CHI3L1 has not yet been described; however, this protein is produced by a number of cells from the innate immune system, including macrophages and neutrophils [33]. In sarcoidosis, CHI3L1 plays a role in both tissue inflammation as well as development of fibrosis [34]. Furthermore, elevated levels of CHI3L1 were reported in patients with active disease. However, its use as a diagnostic biomarker is limited due to low specificity and association with various other diseases [34, 35]. The influence of treatment on levels of CHI3L1 have not been described, but previous research reported no difference in serum levels of CHI3L1 between patients receiving treatment and patients who did not [34]. Our study adds that relatively high levels of CHI3L1 in EVs point towards the ability to respond well to treatment with prednisone. As a potential biomarker, based on low CHI3L1 levels in baseline blood samples, 70% of patients that did not respond to subsequent prednisone therapy could be identified.

For the patients receiving treatment with MTX, the GPI-anchored proteins were shown to be most significantly different between responders and non-responders. GPI-anchored proteins are often receptors that mediate cell activation or ligand uptake. One GPI-anchored protein that has been studied thoroughly in sarcoidosis is CD14. CD14 is a myeloid differentiation antigen mainly expressed on monocytes and macrophages and is required for recognition by toll-like receptors. CD14 is present as a GPI-anchored membrane protein in the myeloid cell lineage and as a soluble serum protein [36–38]. Previous research on EV proteins in sarcoidosis has already reported levels of CD14 to be upregulated in the process of granuloma formation in patients with sarcoidosis [17]. GPI-anchored proteins and their specific sorting potentials are important for the regulated uptake of folates into the cell [39]. Folate antagonists, such as MTX, modulate expression of CD14. Studies of rheumatoid arthritis (RA) reported that levels of CD14 in serum diminish in RA patients responding to MTX treatment [40]. This supports the anti-inflammatory role of MTX.

Of the three proteins that were replicated in the MTX cohort, CPA1 showed a significant difference in concentration between responders and non-responders. CPA1 (carboxypeptidase A1) is a member of the carboxypeptidase A family of zinc metalloproteases and is produced in the pancreatic acinar cells. Mutations in CPA1 have been linked to chronic pancreatitis and pancreatic cancer [41]. CPA1 plays a role in the SPINK1 pancreatic cancer pathway, which was previously mentioned as one of the canonical pathways differently expressed between sarcoidosis and control subjects in bronchoalveolar lavage cells [42]. Moreover, CPA1 has been described to be a potential prodrug-activating enzyme of MTX, capable of hydrolysing stable prodrugs of MTX [43]. We showed a higher concentration of CPA1 in EVs in patients responding to treatment with MTX, suggesting that for these patients CPA1 possibly helps as an activating enzyme to transport MTX into the cell for polyglutamation.

Our study comes with some limitations. Most importantly, because of the retrospective design of the study, not all information was available, resulting in some missing data on clinical characteristics. Selection bias may be present as patients who did not have lung function data around 24 weeks after start of treatment could not be included in the study. A strength of the study is that protein levels were measured in EV fractions using an assay-specific unit but replicated with an ELISA assay. We intentionally measured proteins at baseline, to determine their predictive characteristics and processes involved in responsiveness.

Conclusion

Because of the heterogeneous disease course of sarcoidosis, there is a need for specific biomarkers that will help predict clinical outcome and guide therapeutic management. In this study, we were able to identify specific proteins predictive for response to treatment with either prednisone or MTX. Moreover, we found that there was no overlap in DEPs between responders and non-responders of patients treated with prednisone and patients treated with MTX, supporting the notion that responsive patients may be identified before treatment initiation. These results are promising and warrant replication in a prospective cohort for future development of a clinical assay predictive of response to prednisone or MTX. A predictive biomarker for selecting the most optimal therapy would significantly decrease non-responsiveness, drug switching, side-effects and loss of organ function, and pave the way towards personalised sarcoidosis care.

Provenance: Submitted article, peer reviewed.

Ethics statement: This study was performed in accordance with the Declaration of Helsinki and GCP guidelines. The study was approved by the Medical Research Ethics Committees United (MEC-U) (R14.023), the local institutional review board (Z.19.004) of the St Antonius Hospital.

Author contributions: R. Kraaijvanger contributed to the design of the study, data acquisition and analysis, statistical analysis, drafting, and editing of the manuscript. M. Janssen Bonás participated in data interpretation and editing the manuscript. I. Paspali contributed to data acquisition. J.C. Grutters supervised the project and edited the manuscript. M. Veltkamp participated in the design of the study, data interpretation, supervision and editing the manuscript. D.P.V. de Kleijn supervised part of the project and edited the manuscript. C.H.M. van Moorsel designed the study, supervised the project and edited the manuscript.

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