Received: 18 March 2022 | Revised: 15 September 2022 | Accepted: 17 September 2022

DOI: 10.1111/cts.13432

ARTICLE

Proteomic study on the lymphocytes from pregnant Wistar rat females treated with immunosuppressive regimen

Bartosz Wojciuk[1] | **Aleksandra Bogucka**[2] | **Paulina Czaplewska**[3] | **Patrycja Okulewicz**[1] | **Iwona Wojciechowska-Koszko**[1] | **Kazimierz Ciechanowski**[4] | **Joanna Kabat-Koperska**[4]

[1]Department of Diagnostic Immunology, Pomeranian Medical University in Szczecin, Szczecin, Poland

[2]Laboratory of Biopolymers Structure, Intercollegiate Faculty of Biotechnology, Medical University of Gdańsk and University of Gdańsk, Gdańsk, Poland

[3]Laboratory of Mass Spectrometry, Intercollegiate Faculty of Biotechnology, Medical University of Gdańsk and University of Gdańsk, Gdańsk, Poland

[4]Clinic of Internal Medicine, Nephrology and Transplantation, Pomeranian Medical University in Szczecin, Szczecin, Poland

Correspondence
Bartosz Wojciuk, Department of Diagnostic Immunology, Pomeranian Medical University in Szczecin, ul. Powstańców Wielkopolskich 72, 70-123 Szczecin, Poland.
Email: bartosz.wojciuk@pum.edu.pl

Abstract

Kidney transplantation remains the therapeutic option for patients with end-stage kidney disease. Current immunosuppressive regimens are efficient in combating acute kidney rejection. However, insights into chronic kidney allograft injury remains limited. Simultaneously, pregnancy is more common after kidney transplantation than during dialysis treatment. Due to ethical issues, comprehensive studies on the impact of immunosuppressive regimens on pregnancy are challenging. The study aimed to investigate the proteomic status of lymphocytes obtained from pregnant female rats under immunosuppressive treatment. The experiment involved a group of 10 female, pregnant Wistar rats, five of which were treated with tacrolimus, mofetil mycophenolate, and glucocorticosteroids; five were used as control. The lymphocytes were obtained and analyzed with mass spectrometry. Measurements were processed by a database search in the ProteinPilot software with a cutoff of 1% false discovery rate. The outcomes were verified statistically by a *t*-test (p value <0.05) regarding proteins up- and downregulation. A total of 2082 proteins were identified in all experiments. Eight hundred five proteins were quantified in an absolute manner in a data-independent acquisition-total protein approach analysis. Ninety-five proteins were recognized as present at different concentrations in analyzed groups and were annotated to intracellular pathways. The proteins involved in nonsense-mediated decay and L13a-mediated translational silencing of ceruloplasmin expression were recognized as downregulated. The set of proteins clinically identified as acute phase proteins was upregulated. Despite the blockade of adaptive cellular immunity, the lymphocytes in the analyzed group reveal sustained proinflammatory status with decreased ability to regulate translation. This potentially affects pregnancy and immunity.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2022 The Authors. *Clinical and Translational Science* published by Wiley Periodicals LLC on behalf of American Society for Clinical Pharmacology and Therapeutics.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Research on the impact of pharmacological immunosuppression on pregnancy are limited due to ethical issues.

WHAT QUESTION DID THIS STUDY ADDRESS?

The study addresses the question how the most common immunosuppressive regimen introduced after kidney transplantation shapes the complex biology of lymphocytes. Omics- technology has been involved.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study delivers novel data on up- and downregulated proteins in lymphocytes during pregnancy and under immunosuppressive treatment. Some of these have been successfully assigned to particular molecular pathways. The others have been interpreted in the context of early findings, up-to-date immunologic knowledge, and clinical practice.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

The findings of this study cast a new light on specific routine procedures in pregnancy and chronic kidney disease (e.g., iron supplementation), but also on the pathomechanisms of frequent adverse events related to post-kidney transplantation pregnancy.

INTRODUCTION

End-stage solid organ dysfunctions remain as organ transplantation as the optimal therapeutic option, which includes kidney transplantation for end-stage kidney disease. Various immunosuppressive agents have been introduced in clinical transplantation history, starting with azathioprine in the early 1960s.[1] Regarding an increasing experience in this area, calcineurin inhibitors (CNIs) appeared critical in combating acute cellular rejection—the major challenge in the early post-transplant period—due to their unique ability to interfere with interleukin-2 production via calcineurin and the nuclear factor of activated T cells (NFAT)-dependent pathway.[2] The current guidelines suggest tacrolimus (TAC; CNI representative), mofetil mycophenolate (MMF; next-generation antimitotic), and glucocorticosteroids (GCS) as the first choice immunosuppressive treatment following kidney transplantation.[3]

As mentioned above, CNI activity is based on the inhibition of the calcineurin-dependent pathway of lymphocyte activation. Normally, calcineurin mediates intracellular signal transduction following T cell receptor ligation. Further, it gets activated with an increased intracellular calcium level and induces a downstream pathway of protein phosphorylation.[3-10] Subsequently, CNI activity results in the downregulation of NFAT-dependent genes. Simultaneously, MMF inhibits de novo purine synthesis by suppressing monophosphate dehydrogenase.[11]

Nevertheless, the method resolution limited the understanding of how immunosuppressive regiments shape lymphocytes' status. Currently, the systemic approach and bioinformatic tools that have evolved, enabled a pipeline model for in-depth cellular biology investigation. This multi-omic pipeline starts on a genomic level and comes across transcriptomics and proteomics to eventually finish with metabolomics.[12]

Whereas genomics and transcriptomics are based on next-generation sequencing (NGS), proteomics and metabolomics rely on mass spectrometry (MS), known much longer than NGS. However, more sophisticated mass detection systems and increasing computational power of bioinformatics tools have transformed MS into truly high throughput technology capable of performing untargeted analyses. Compared to NGS-based genomics and transcriptomics, proteomics and metabolomics provide data closer to the phenotypic status of the biological systems (cells, tissues, body fluids, etc.).[12] Consequently, systems biology provides a complex insight into the downstream mechanisms between the genome and the phenotype, and it has become outstandingly significant for basic and applied research. Subsequently, the systemic approach has significantly impacted immunology and molecular medicine.[13] According to modern concepts, molecular medicine is less predestined to revolutionize the understanding of pathology but rather to make it more precise. Moreover, this is thanks to the complex insight into the network of molecule interactions.[14] Still, despite increasing clinical experience, this is not extensively utilized in transplantation medicine.

It is also true for pregnancy after kidney transplantation. Due to improved fertility and sexuality compared

to the pre-transplant status, pregnancy post-transplant is recognized more frequently.[15] However, this is still less common than in the general population and is considered a higher risk. There have been many unresolved questions regarding successful pregnancy post-transplant (e.g., the optimal conception moment, hematopoiesis stimulation therapy, and, eventually, the optimal immunosuppression).[15,16] At the same time, high throughput technologies, such as proteomics, have not been implemented in this area.

Our center previously performed experiments targeting the cytokine profiles of lymphocytes obtained from pregnant female Wistar rats and their offspring treated with different immunosuppressive regimens and further stimulated with concanavalin A (ConA).[17] We have indicated several differences regarding the cytokines' secretion. These were mostly related to interleukin-17 (IL-17). We have also observed a robust teratogenic effect of this treatment, mostly associated with MMF and everolimus activity. Furthermore, MMF appeared more toxic when combined with calcineurin inhibitors than itself. Despite the recommendation to modify the immunosuppressive therapy at least 6 weeks before conception (e.g., switching MMF or mTOR inhibitors to azathioprine), unscheduled pregnancies remain relatively common among kidney recipients. As mentioned, TAC and MMF-based treatment represent the first choice in kidney recipients.

Consequently, the pregnancy itself is often diagnosed in the period of early organogenesis, which is, in fact, too late for treatment modification. Hence, it is still reasonable to assume that this potentially teratogenic treatment acts at an early and critical stage of pregnancy. Considering the circumstances and the abovementioned questions about post-transplant pregnancy, we have chosen this protocol for further proteomic study.[17] It needs to emphasize that prospective molecular assessment of a drug's cellular toxicity is possible only in an animal model. Such an experiment appears highly ethically questionable in humans. Consequently, this study aimed to investigate the proteomic profiles of lymphocytes in pregnant female Wistar rats treated with the most common but potentially teratogenic and toxic immunosuppressive regimen in kidney transplant recipients: TAC, MMF, and GCS.

MATERIALS AND METHODS

Animals' treatment and lymphocytes isolation

The methodology related to animals' treatment and lymphocytes isolation has been described elsewhere. Briefly, female Wistar rats were used in the experiment, five received immunosuppressive therapy (TAC, MMF, and GCS), and five were treated as a control. Males were used for mating only (purchased from a licensed breeder — Center of Experimental Medicine, Medical University in Bialystok, Poland). This study was approved by the Local Ethical Committee for Experiments on Animals in Szczecin (No. 12/2013, dated October 24, 2013). At the start of the experiments, the age of the rats was 12 weeks. The experiments were performed using the pharmaceutical form of each drug. The animals received drugs by oral gavage at a dose volume of 5 ml/kg daily. As mentioned, five rats did not receive treatment. They formed a control group and were given the vehicle and olive oil under identical conditions as others used in experimental rats. The drug doses were based on data available in the literature, as follows: TAC (Prograf; Astellas, Tokyo, Japan): 4 mg/kg/day; MMF (CellCept; Roche, Basel, Switzerland): 20 mg/kg/day; prednisone (Encorton; Polfa, Warsaw, Poland): 4 mg/kg/day. The animals received medication every 24 h for approximately 5 weeks (2 weeks after acclimatization before mating – when placed with males 1:1 in separate cages – and later after mating during 3 weeks of pregnancy). After delivery, the treatment was stopped. The dams were euthanized at weaning (day 21 after delivery) – we decided not to euthanize them earlier than their offspring as they had to stay alive for further studies. The female rats were euthanized by pentobarbital sodium (Polpharma) injection administered intraperitoneally at 40 mg/kg body weight. The spleen was cut into slices and pressed through nylon to RPMI-1640 medium with phenol red and L-glutamine. The liquid was transferred into a centrifuge tube containing the same volume of lymphocytes separation medium and centrifuged at 2000 ***g*** for 20 min at room temperature. The middle cloud layer was placed in a lymphocytes isolation medium and centrifuged at 1000 ***g*** for 10 min. The pellets were resuspended in an RPMI-1640 medium.

Incubation of lymphocytes

Lymphocytes were incubated on wells plates for 72 h at 37°C under an atmosphere containing 5% CO_2. Cell suspensions at a density of 2×10^6 were placed in wells – 0.5 ml of cell suspension in 1 ml of the incubation medium. The incubation medium consisted of RPMI, 10% bovine serum, 50 μg of gentamicin, and increasing amounts of ConA (0; 2; 5 μg/ml). After the incubation, cell suspensions were centrifuged at 1000 ***g*** for 10 min. The cell pellets were harvested and frozen at −70°C until further analyses. The pellets from suspensions stimulated with 5 μg/ml ConA were chosen for further proteomic analyses.

Proteomic analysis

Samples were prepared for MS analysis in a Filter Aided Sample Preparation (FASP) procedure with proteolytic digestion by LysC/Trypsin mix. Each sample was mixed with the lysis solution containing 1% SDS, 100 mM Tris–HCl pH 8.0, 50 mM dithiothreitol, and incubated at 95°C for 10 min. Protein concentration was measured using a spectrophotometer at 280 nm, and about 100 μg of each sample was transferred to separate 10 kDa Microcon filters (Merck-Millipore). Samples were later processed according to standard FASP procedure. Briefly, filters were washed multiple times with the solution containing 8 M urea in 100 mM Tris–HCl, pH 8.5, by centrifugation at 10000 ***g***. Next, proteins were alkylated by 20 min incubation in darkness at room temperature with the 55 mM iodoacetamide solution in the urea buffer. Filters were washed with the urea buffer, followed by 50 mM Tris–HCl to prepare proteins for digestion. LysC/Trypsin mix was added to samples in a 1:50 weight ratio, and samples were incubated at 37°C overnight. After peptide elution with 50 mM Tris–HCl, 10 μg of peptides from each sample were desalted on STAGE Tips containing Empore C18 resin (3 M, Maplewood, MN). In the final step, peptides were eluted from STAGE Tips with 60% acetonitrile and 1% acetic acid in the water and concentrated to 30 μl in a SpeedVac. Liquid-chromatography tandem mass spectrometry measurements were conducted on a Triple time-of-flight (TOF) 5600+ MS (SCIEX) operating in a positive ion mode coupled with an Ekspert MicroLC 200 Plus System (Eksigent). Liquid chromatography separation was performed on the ChromXP C18CL column (3 μm, 120 Å, 150 × 0.3 mm) in a gradient of 7-35%B in 60 min (buffer A: 0.1% formic acid in the water, buffer B: 0.1% formic acid in acetonitrile). The instrument was operated by Analyst TF 1.7.1 software (SCIEX, Framingham). Precursor ions were fragmented by collision-induced dissociation. Data-dependent acquisition (DDA) runs consisted of a TOF scan in the m/z range of 400–1200 Da in 100 ms and a subsequent Product Ion scan in the m/z range of 100–1800 Da in 50 ms, resulting in the cycle time of 1.15 s. Data independent acquisition (DIA) measurements were conducted with the Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS) method in the high sensitivity mode in 25 overlapping m/z windows of variable width covering the range of 400–1200 m/z, constructed with swathTUNER with the focus on equalized ion frequency. Each SWATH MS run consisted of a TOF survey scan in the m/z range of 400–1200 Da in 100 ms and Product Ion scans in the m/z range of 100–1800 Da in 50 ms, resulting in a cycle time of 1.4 s. All samples were measured in the DDA mode for the spectral library construction and in triplicate by the described SWATH-MS method for relative quantification. Database search was carried out in ProteinPilot 4.5 software (SCIEX) using the Paragon algorithm against the UniprotKB Rattus norvegicus database (version from 23.10.2019) with an automated false discovery rate (FDR) analysis with the focus on biological modifications. Only proteins identified at 1% FDR were considered valid identifications. A joint database search result group file of all samples analyzed in the study was subsequently used for the spectral library construction in the PeakView 2.2 software (SCIEX) with the exclusion of shared peptides. Files from SWATH-MS runs were processed with the library as previously described. The result was used to calculate the concentration of proteins by total protein approach (TPA) with the equation described before in a DIA-TPA experiment.

Computational analysis

Concentrations were imported into the Perseus 2.0.3.1 software, where the three measurement (technical) replicates of each biological replicate were median-averaged, and the resulting values were log2-transformed and normalized by z-score. The normality of the data was confirmed in histograms. A priori power calculations were not performed due to the lack of comparable data. Two-tailed Student's t-test between the test ($n = 5$) and control ($n = 5$) groups and a threshold p value of 0.05 was used to truncate the results. Functional enrichment analysis and interaction network construction were carried out in Cytoscape 3.8.2 using the STRING 11 database. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035571 (reviewer account username: reviewer_pxd035571@ebi.ac.uk, password: 2eVBFczU).

All methodological references were listed in the Supplementary Material S1.

RESULTS

Analysis of the rat lymphocyte proteome

All MS experiments identified 2082 proteins (see Table S1). The searches of all measurements performed in the test and control groups yielded 1306 (877.4 ± 201.43 average per sample) and 1515 (1006.7 ± 301.24 average per sample) identifications, respectively. The spectral library for the SWATH-MS quantification built from all DDA measurements conducted in this study included 1646 proteins (see Table S2), 805 of which could be analyzed quantitatively (see Table S3). The three most abundant proteins,

histones H4, H2B, and H1.1, constituted almost 27% of quantified proteins' mass.

Differences in lymphocyte proteomes of treated and untreated rats

Four hundred forty-nine proteins were identified only in the control group of samples, and 240 were unique to the test group samples of rats receiving treatment (see Table S1). We conducted a *t*-test on the normalized concentration values to discern quantitative differences between the groups and found 95 proteins with a p value less than 0.05 (see Table S4). The interaction network constructed in Cytoscape is presented in Figure 1. Seventy-six proteins were present at decreased concentrations in the treatment-receiving group, constituting the core of two clusters visible in the interaction network. The dominant cluster mainly consists of proteins from the ribonucleoprotein complex, such as GNB2L1, RPS27A, RPL7A, RPLP1, RPS2, RPS12, NOP58, RPS3, RPS15A, RPL18A, RPS16, RPS25, RPL11, and RPL3. The other cluster contains proteins of the minichromosome maintenance complex (MCM7, MCM4, MCM6, MCM3, and MCM2). These representing ribonucleic complex were annotated to nonsense-mediated pathway enhanced by the exon junction complex (NMD), L13a-mediated translational silencing of ceruloplasmin expression, GTP hydrolysis, and joining of the 60S ribosomal subunit. Nineteen proteins were present at increased concentrations in the treatment-receiving group, and displayed significantly fewer interactions in the presented network. Of those, GLO1, HMOX1, A2M, FTH1 and C3 were involved in immunity, and ACSF2, LIPA, were associated with cellular lipid metabolic processes. The changes in concentration were more than two-fold for 18 proteins present at statistically significant differences in the test group (see Table 1). There was also a group of unique proteins in the

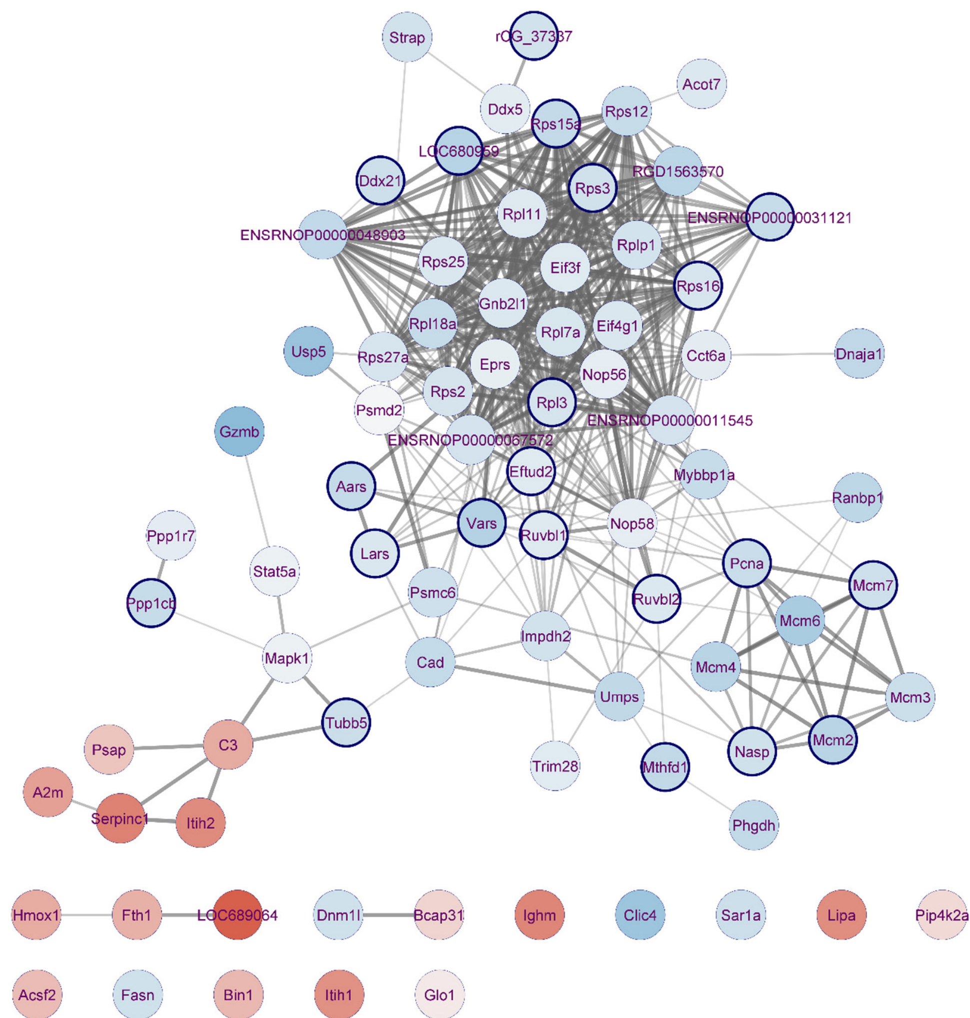

FIGURE 1 The interaction network of the proteins present at statistically significant differences between studied groups (p value < 0.05). The fill color of the nodes corresponds to fold change (FC) value (red – FC > 1, blue – FC < 1), and the thicker blue borders indicate p value < 0.01.

TABLE 1 Proteins present at greater than two-fold concentration difference at p value < 0.05 between the studied groups

Uniprot ID	Protein name	Median of control group [pmol/mg]	Median of test group [pmol/mg]	Median Fold Change	Student's *t*-test *p* value
P06238	Alpha-2-macroglobulin	2.37	6.43	2.71	4.85E-02
Q9Z0W7	Chloride intracellular channel protein 4	10.86	4.58	0.42	3.38E-02
M0RBJ7	Complement C3	1.65	3.95	2.40	1.06E-02
A9CMB8	DNA helicase	1.60	0.77	0.48	1.73E-02
A0A0G2JXT3	Farnesyl pyrophosphate synthase	7.16	3.32	0.46	4.60E-04
Q66HI5	Ferritin	3.67	8.17	2.23	3.33E-02
P18291	Granzyme B	10.03	3.59	0.36	1.20E-02
P06762	Heme oxygenase 1	3.63	8.55	2.36	2.03E-02
P11517	Hemoglobin subunit beta-2	21.91	123.23	5.62	4.46E-02
F1LPR6	Immunoglobulin heavy constant mu	1.53	5.56	3.63	1.65E-02
B2RYM3	Inter-alpha trypsin inhibitor, heavy chain 1	0.55	1.76	3.20	1.97E-02
D3ZFH5	Inter-alpha-trypsin inhibitor heavy chain 2	2.68	9.18	3.43	2.20E-02
Q6IMY6	Lipase	11.25	37.54	3.34	1.37E-02
F1LMX1	Myc box-dependent-interacting protein 1	1.61	3.32	2.07	3.16E-02
A0A0G2K9E0	Ribonuclease inhibitor	3.45	1.55	0.45	3.66E-02
Q5M7T5	Serine (or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1	2.73	10.47	3.83	1.53E-02
A0A0G2JSH5	Serum albumin	155.57	613.20	3.94	3.37E-02
D3ZVQ0	Ubiquitin carboxyl-terminal hydrolase	2.59	1.06	0.41	2.16E-02

test group, which were annotated to metabolic processes, membrane-related structural processes, antigen presentation, cornification, and a few unique NMD components absent in the control group (see Table S5).

DISCUSSION

The study was inspired by remaining questions about the risk factors for post-kidney transplant pregnancy. In addition, our observation that the immunosuppressive treatment MMF-inclusive revealed the most potent teratogenic activity. According to the clinical data, whereas most pregnancies in kidney recipients appear successful, complications such as eclampsia preterm birth are more common and achieve a prevalence value of 40%.[18] The lymphocyte-directed effect of the analyzed drugs becomes more interesting when an embryo represents an allogenic graft that typically survives under natural local immunosuppression. Despite the clear evidence on shaping IL2-dependent immune reaction by CNI-based therapy, it was hypothesized that non-targeted analysis would provide more profound insight into lymphocyte biology under such treatment. Hence, the current study was focused on proteomic profiling of lymphocytes obtained from pregnant Wistar rats treated with the most common TAC-based immunosuppressive therapy and stimulated with ConA. The tested group had downregulated two significant translation pathway's control, associated with ribonucleic complex: exon junction enhanced NMD and L13a mediated translational silencing of ceruloplasmin expression.

In contrast, several proteins were found to be upregulated. The upregulated proteins do not reveal annotation to a particular well-defined intracellular pathway. However, functionally, these represent innate immune response factors and lipids metabolism regulators. As we used ConA, a T-cell-specific stimulant, we assume the abovementioned changes refer to the T cells population. Below, we will propose the possible impact of our findings on understanding lymphocyte status and post-transplant pregnancy. The upregulated proteins can be biologically indicated as immunologically active, but most of these represent clinically relevant groups of acute-phase proteins. It refers primarily to the C3 complement component, alpha 2 macroglobulin, ferritin, antithrombin III, and inter-α-antitrypsin inhibitor heavy chains (ITIH). There is a different amount of data available on each of these in the context of lymphocyte function, which will be discussed below.

The role of ferritin in chronic inflammation seems clear; however, little is known about its function in lymphocytes. Apart from relatively early findings that lymphocytes produce ferritin,[19] very little has been investigated in this area to the authors' knowledge. Still, iron metabolism remains within the scope of interest in the context of feto-maternal unit wellbeing. In the recent experimental study by Fisher et al., excess iron enhances embryo injury during both acute LPS-induced and chronic obesity-induced inflammation. Such injury is considered to be TNFα-dependent. Moreover, this can be potentially reversed by using antioxidants such as α-tocopherol.[20] The clinical study has also recently indicated that high ferritin levels in the third trimester are correlated with preterm birth and low birth weight.[21] Iron administration is recommended during pregnancy by the World Health Organization (WHO) guidelines,[22] as it is during chronic kidney disease and post-kidney transplant as a component of erythropoiesis-stimulating therapy. Fisher et al. postulate revision of the routine practice of iron administration in pregnancy, regardless of the mother's iron status.[20] Considering our findings, this postulate seems even more valid for kidney-allograft recipients.

In contrast, alpha 2 macroglobulin has gained more attention. Currently, this is recognized as a multifunctional protease inhibitor that pivotally contributes to regulating inflammatory response. It was confirmed in the 1970s that lymphocytes could produce alpha 2 macroglobulins, and, further, studies indicated its regulatory potential on lymphocytes' function.[22,23] It has also been indicated that the primary source was the metrial gland in pregnant female rats and, lymphocytes in males.[24] Furthermore, alpha 2 macroglobulin has been found to promote implantation, placental vessel growth, and subsequent local immune tolerance in pregnancy.[25] Hence, our findings suggest that immunosuppressive treatment aggravates the immunomodulatory function of pregnancy-related immune status, at least regarding alpha 2 macroglobulin activity. However, unlike the previous studies that suggest the metrial gland as the significant source of alpha 2 macroglobulin in pregnancy, our findings suggest an increased role of lymphocytes in alpha 2 macroglobulin production during immunosuppressive treatment.

Similarly, antithrombin III is recognized as a highly pregnancy-related protein. Apart from its function in the clotting system, it also represents strong modulatory properties in inflammation.[26] In the early experimental animal models, it has been indicated that cortisol rather than progesterone impacted antithrombin III production. It potentially corresponds with the steroid component of the treatment implemented in our study.[27] Nevertheless, little is known about its lymphocytic origin. Both alpha 2 macroglobulin and antithrombin III represent a phylogenetically old group of serine proteases inhibitors called serpins involved in innate immunity regulation.[28]

ITIH 1 and 2 found upregulated in our study also represent acute phase proteins activity. Under inflammatory conditions, these modulate hyaluronic acid molecules, and these further create serum-derived hyaluronan-associated proteins complex and build tunnels for the leukocytes migrating within the extracellular matrix.[29] Similar to α-2-macroglobulin and antithrombin III, ITIH is assigned to proteases inhibitors. However, apart from their inflammatory contribution, ITIH 1 and 2 are also recognized for regulating implantation and placentation.[30] Liver overproduction of ITIH 1 has been associated with pregnancy diabetes mellitus.[31] Furthermore, ITIH 1 itself has been considered a predictive biomarker for rheumatoid arthritis, knee osteoarthritis, and inflammatory bowel disease (IBD).[32,33]

C3 complement component has been found upregulated in our study. The role of the C3 component in adaptive (apart from innate) immunity has also been well-recognized. C3 and its receptors are expressed in lymphocytes and contribute to T cells differentiation and the crosstalk between T cells and dendritic cells. Lacking the C3 component diminishes the ability of dendritic cells to produce both IL-12 and IL-23. Consequently, it is assumed that C3 can promote Th1/Th17 rather than Th2 subsets. Intracellular activity of cathepsin L downregulates C3 expression, and cathepsin L inhibition further inhibits Th17 differentiation.[34–37] On the other hand, in the clinical setting of kidney biopsies, the C3 component has been recognized to promote fibrosis via Th17 cells activation.[38] However, this finding was not done in the population exposed to immunosuppressive treatment. In our previous study, IL-17 and the other cytokines production were blocked in the females treated with a TAC-based therapy.[17] Hence, the findings on the possible C3 role within lymphocytic subsets' differentiation under immunosuppression appear contradictory.

Nevertheless, complement remains significant for a successful pregnancy. Within the local immunotolerant environment, the complement system remains one of the primary defense mechanisms, controlled by locally active complement inhibitors.[39] However, excessive C3 complement activity has been associated with adverse pregnancy events, such as recurrent spontaneous abortions, preterm birth, antiphospholipid syndrome, and pre-eclampsia. Moreover, complement activity is postulated as a useful clinical tool in the differential diagnosis between pre-eclampsia and other pregnancy-related complications (e.g., systemic lupus erythematosus relapse).[40,41]

In the context of available literature data, C3 expression also appears to be nonsense-mediated decay pathway related. Evidence has been found on abnormal NMD pathway function in inherited C3 deficiencies.[42-44] NMD

represents one of the pathways controlling the quality of translation. It is involved in eliminating abnormal, truncated transcripts that, if uncontrolled, could eventually deregulate the cell life cycle in a gain-of-function manner.[45] Hence, it focuses much interest in the context of tumor transformation.[46] As the research is limited in this area, the authors propose considering NMD pathway downregulation as possibly involved in the teratogenic effect of the drugs during pregnancy. Nevertheless, the NMD pathway is also postulated to surveil the process of noncoding RNA survival in a transcript-specific manner. It represents a regulatory function in T cell differentiation, mainly controlling long non-coding RNA (lncRNA) activity. LncRNA is currently considered to contribute to Th17 development.[47] Our finding on the NMD pathway appears attractive regarding these postulates. Therefore, it also regards our previous findings, in which IL-17 production was blocked under TAC-based treatment but sustained under cyclosporine A, MMF and GCS treatment.[17,18]

As mentioned, the problem of lymphocytes proteomics in a post-transplant pregnancy model has not been addressed. Interestingly, both the C3 component and antithrombin III have been upregulated in a rat model of spontaneous tolerance toward liver transplant.[48] However, in this study, the primary role has been assigned to haptoglobin, which in our analysis has not been indicated.

L13a mediated translational silencing of ceruloplasmin expression is another of the downregulated pathways recognized in our study. Similar to NMD, this is a mechanism of translation control, and it contributes to a complex system of IFNγ activated inhibitor of translation (GAIT) recognized to gate inflammatory gene expression. Therefore, it is considered to manage the resolution of the inflammatory process.[49] However, the data on L13a-mediated translational silencing comes from in vitro experiments on monocytic cells, and the data on this pathway in lymphocytes are, in fact, absent.[50] Hence, this is difficult to judge if this downregulation results directly from immunosuppressive drugs activity or indirectly via impaired IFNγ production (as indicated in our previous study). Still, downregulation of potentially inflammatory genes controlling the pathway corresponds with the overall pro-inflammatory protein profile of the treated lymphocytes indicated in this study.

Apart from acute-phase proteins, lipid metabolism-associated protein recognized as lipase has been indicated as upregulated. Lipase, identified as *lipa* gene product, upregulation is challenging to discuss as the literature on this particular protein in lymphocytes is minimal. It has been confirmed that lymphocytes express lipid metabolism, which has been considered to fuel their differentiation, especially within the T CD8 subset.[51] Nevertheless, gene ontology libraries annotate lipase to inflammatory reactions; however, this entry has not been reviewed yet.[52] Still, lipid metabolism ability seems sustained in the tested lymphocytes under immunosuppression.

Interestingly, the proteins unique for the test group were not annotated into immune phenomena except for antigen processing and presenting. These are recognized as professional antigen-presenting cells' rather than lymphocytes' domain. It needs to be emphasized that concerning spleen as the material used and isolation procedure used in this study, the authors cannot exclude the presence of a small subset of APCs within the cells' suspensions. However, this issue is challenging due to the study's limitations discussed below.

Despite the potential usefulness in further translational approaches, this study represents some limitations. One of these is using the whole pool of lymphocytes without the distinction between particular subpopulations, especially between CD4+ and CD8+. It is still worth noting that the overall profile of the upregulated proteins found in our analysis resembles the data retrieved from research on biomarkers useful in chronic inflammatory diseases. In these, C3 and alpha 2 macroglobulin were found to be the most promising by Okada et al.[53] It becomes even more apparent when considering our results both as absolute concentrations and relative SWATH-MS intensities (data not shown) in comparison to Okada's list of IBD-related candidate biomarkers retrieved from SDS-PAGE and subsequent MALDI TOF.

In addition, orthogonal methods of validation, such as multiple testing correction, were not used in this study. However, these are very rigorous and can result in losing some true positive results. Regarding the preliminary character of the study, the authors aimed to create a general report at this stage in order to hint at potential molecular processes, which could be further studied. Furthermore, the computational methodology implemented in the study is compliant with the practice dedicated to similar ones.[54–57]

In summary, our study indicates that, despite the evident blockade of the IL2-dependent adaptive immunity pathway, the proteomic profile of the up-regulated lymphocytes in pregnant female Wistar rats reveals sustained inflammatory character. It is constituted by phylogenetically ancient acute phase proteins and lipid metabolism enzymes. Most of these contribute to feto-maternal unit well-being and inflammation, acting either as its promoters or regulators. Like the complement component C3, some of these have been straightforwardly connoted to pregnancy-related adverse events, corresponding to clinical observations on post-transplant pregnancies. Regarding available basic and at least preliminary clinical studies, the profile of the upregulated proteins resemble the status found during autoimmune disorders, such as IBD and inflammatory arthritis (knee osteoarthritis and rheumatoid arthritis). We proposed some mechanistic explanations of the links between downregulated pathways and the upregulated proteins. However,

more profound insight is required in this area. Regarding our previous and current findings, the phenotype of the lymphocytes resembles Th1/Th17-dependent lineages somewhat. Nevertheless, an exact distinction between these two is not possible due to the limitations of this study. Regarding the link among the NMD pathway, lncRNA, and its postulated role in Th 17 lineage development, we suggest further transcriptomic investigations. These may be lncRNA and defined cells population-directed to uncover molecular mechanisms underlying lymphocyte differentiation in immunosuppressed conditions. The role of lncRNA in chronic inflammation has been preliminarily done in IBD clinical and experimental settings.[58] Eventually, the impact on the NMD pathway downregulation and acute-phase proteins disbalance shall also be considered when investigating the teratogenic activity of the analyzed protocol.

AUTHOR CONTRIBUTIONS

B.W. and J.K.K. designed the research. A.B. and P.C. contributed new analytical tools. P.O. and B.W. analyzed the data. I.W.-K. performed the research. B.W., J.K.-K., K.C., and P.O. wrote the manuscript.

FUNDING INFORMATION

No external funding was received for this work.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

REFERENCES

1. Murray JE, Merrill JP, Harrison JH, Wilson RE, Dammin GJ. Prolonged survival of human-kidney homografts by immunosuppressive drug therapy. *N Engl J Med*. 1963;268:1315-1323.
2. Kel A, Kel-Margoulis O, Babenko V, Wingender E. Recognition of NFATp/AP-1 composite elements within genes induced upon the activation of immune cells. *J Mol Biol*. 1999;288:353-376.
3. Azzi J, Sayegh MH, Calcineurin MSG. Inhibitors: 40 years later, can't Live without .... *J Immunol*. 2013;191(12):5785-5791.
4. Borel JF, Feurer C, Gubler HU, Sta¨helin H. Biological effects of cyclosporin a: a new antilymphocytic agent. *Agents Actions*. 1976;6:468-475.
5. Bram RJ, Hung DT, Martin PK, Schreiber SL, Crabtree GR. Identification of the immunophilins capable of mediating inhibition of signaltransduction by cyclosporin a and FK506: roles of calcineurin binding and cellularlocation. *Mol Cell Biol*. 1993;13:4760-4769.
6. Hurwitz MY, Putkey JA, Klee CB, Means AR. Domain II of calmodulin is involved in activation of calcineurin. *FEBS Lett*. 1988;238:82-86.
7. Klee CB, Draetta GF, Hubbard MJ. Calcineurin. *Adv Enzymol Relat Areas Mol Biol*. 1988;61:149-200. Shenolikar, S. 1994. Protein serine/threonine phosphatases: new avenues for cell regulation. Annu. Rev. Cell Biol. 10: 55–86.
8. Zhang BW, Zimmer G, Chen J, et al. T cell responses in calcineurin Aalpha-deficient mice. *J Exp Ther Med*. 1996;183:413-420.
9. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family:regulation and function. *Annu Rev Immunol*. 1997;15:707-747.
10. Shibasaki F, Price ER, Milan D, McKeon F. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature*. 1996;382:370-373.
11. Ransom JT. Mechanism of action of mycophenolate mofetil. *Therap Drug Monitor*. 1995;17(6):681-684.
12. Horgan RP, Kenny LC. 'Omic' technologies: genomics, transcriptomics, proteomics and metabolomics. *Obst Gynaecol*. 2011;13:189-195.
13. Brodin P, Valentini D, Uhlin M, Mattsson J, Zumla A, Maeurer MJ. Systems level immune response analysis and personalized medicine. *Expert Rev Clin Immunol*. 2013;9(4):307-317.
14. Yadav BS, Tripathi V. Recent advances in the system biology-based target identification and drug discovery. *Curr Top Med Chem*. 2018;18(20):1737-1744.
15. Kleinclauss F, Timsit MO, Thuret R. Sexuality, fertility and pregnancy after kidney transplantation. *Prog Urol*. 2016;26(15):1122-1131.
16. Josephson MA, McKay DB. Pregnancy and kidney transplantation. *Semin Nephrol*. 2011;31(1):100-110.
17. Kabat-Koperska J, Kolasa-Wołosiuk A, Wojciuk B, et al. Ciechanowski K changes in the immune system of female Wistar rats after exposure to immunosuppressive treatment during pregnancy. *Scand J Immunol*. 2016;83(6):418-426.
18. Shah S, Venkatesan RL, Gupta A, et al. Verma P Pregnancy outcomes in women with kidney transplant: Metaanalysis and systematic review. *BMC Nephrol*. 2019;20(1):24.
19. Dorner MH, Silverstone A, Nishiya K, de Sostoa A, Munn G, de Sousa M. Ferritin synthesis by human T lymphocytes. *Science*. 1980;209:1019-1021.
20. Fisher AL, Sangkhae V, Balušíková K, Palaskas NJ, Ganz T, Nemeth E. Iron-dependent apoptosis causes embryotoxicity in inflamed and obese pregnancy. *Nat Commun*. 2021;12:4026.
21. Ahn TG, Li L, Lee SJ, Hu YH, Kim C, Hwang JY. Serum ferritin concentration in the early third trimester of pregnancy and risk of preterm birth and low birth weight based on gestational age. *J Korean Soc Matern Child Health*. 2021;25(1):55-62.
22. WHO. *WHO recommendations on antenatal Care for a Positive Pregnancy Experience*. World Health Organization; 2016.
23. Tunstall AM, James K. Preliminary studies on the synthesis of alpha$_2$-macroglobulin by human lymphocytes *in vitro*. *Clin Exp Immunol*. 1974;17(4):697-701.
24. Miyanaga O, Okubo H, Kudo J, Ikuta T, Hirata Y. Effect of α_2-macroglobulin on the lymphocyte response. *Immunology*. 1982;47(2):351-356.
25. Panrucker DE, Lorscheider FL. Synthesis of acute-phase alpha 2-macroglobulin during inflammation and pregnancy. *Ann N Y Acad Sci*. 1983;417:117-124.
26. Tayade C, Esadeg S, Fang Y, Croy BA. Functions of alpha 2 macroglobulins in pregnancy. *Mol Cell Endocrinol*. 2005;245:60-66.
27. Levy JH, Sniecinski RM, Welsby IJ, Levi M. Antithrombin: anti-inflammatory properties and clinical applications. *Thromb Haemost*. 2016;115(4):712-728.
28. Kobayashi N, Takeda Y. Studies of the effects of estradiol, progesterone, cortisol, thrombophlebitis, and typhoid vaccine on synthesis and catabolism of antithrombin III in the dog. *Thromb Haemost*. 1977;37(01):111-122.

29. Yaron JR, Zhang L, Qiuyun Guo Q, Haydel SE, Lucas AR AR. Fibrinolytic serine proteases, therapeutic serpins and inflammation: fire dancers and firestorms Front *Cardiovasc Med*. 2021;25(8):648947.
30. Petrey AC, de la Motte CA. Thrombin cleavage of inter-α-inhibitor heavy chain 1 regulates leukocyte binding to an inflammatory hyaluronan matrix. *J Biol Chem*. 2016;291(47):24324-24334.
31. Geisert RD, Ashworth MD, Malayer JR. Expression of inter-alpha-trypsin inhibitor heavy chains in endometrium of cyclic and pregnant gilts. *Reproduction*. 2003;126:621-627.
32. Kim TH, Koo JH, Heo HJ, et al. Overproduction of inter-α-trypsin inhibitor heavy chain 1 after loss of $G\alpha_{13}$ in liver exacerbates systemic insulin resistance in mice. *Sci Transl Med*. 2019;11(513):eaan4735.
33. Lourido L, Balboa-Barreiro V, Ruiz-Romero C, et al. A clinical model including protein biomarkers predicts radiographic knee osteoarthritis: a prospective study using data from the osteoarthritis initiative. *Osteoarthr Cartil*. 2021;29(8):1147-1154.
34. Yamaguchi Y, Noda H, Okaniwa N, et al. Serum-derived hyaluronan-associated protein is a novel biomarker for inflammatory bowel diseases. *Digestion*. 2017;95:146-155.
35. Killick J, Moriss G, Sieger D, Astier AL. Complement as a regulator of adaptive immunity. *Semin Immunopathol*. 2018;40:37-48.
36. Strainic MG, Liu J, Huang D, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive $CD4^+$ T. *Cell*. 2008;28(3):425-435.
37. Jiménez-Reinoso A, Marin AV, Regueiro JR. Complement in basic processes of the cell. *Mol Immunol*. 2017;84:10-16.
38. Ma Q, Li D, Carreño R, et al. Complement component C3 mediates Th1/Th17 polarization in human T-cell activation and cutaneous GVHD. *Bone Marrow Transplant*. 2014;49:972-976.
39. Liu Y, Wang K, Liang X, et al. Complement C3 produced by macrophages promotes renal fibrosis via IL-17A. *Secretion Front Immunol*. 2018;22(9):2385.
40. Girardi G, Bulla R, Salmon JE, Tedesco F. The complement system in the pathophysiology of pregnancy. *Mol Immunol*. 2006;43(1–2):68-77.
41. Denny KJ, Woodruff TM, Taylor SM, Leonie K. Complement in pregnancy: a delicate balance. *Am J Reprod Immunol*. 2013;69:3-11.
42. Derzsy Z, Prohászka Z, Jr R, Füst G, Molvarec A. Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol*. 2010;47(7–8):1500-1506.
43. Da Silva RE, Baracho GV, Sousa Lima AS, Farah CS. Lourdes I homozygous hereditary C3 deficiency due to a premature stop codon. *J Clin Immunol*. 2002;22(6):321-330.
44. Reis ES, Nudelman V, Lourdes I. Nonsense-codon-mediated decay in human hereditary complement C3 deficiency. *Immunogenetics*. 2004;55:667-673.
45. Nele Hug N, Dasa Longman D, Javier F, Cáceres JF. Mechanism and regulation of the nonsense-mediated decay pathway. *Nucleic Acids Res*. 2016;44(4):1483-1495.
46. Popp MW, Maquat LE. Nonsense-mediated mRNA decay and cancer. *Curr Opin Genet Dev*. 2018;48:44-50.
47. Zhang Y, Wei Z, Dong H, et al. Regulation of mRNA stability by RBPs and noncoding RNAs contributing to the pathogenicity of Th17 cells. *RNA Biol*. 2021;18(5):647-656.
48. Pan TL, Wang PW, Huang CC, Goto S, Chen CL. Expression, by functional proteomics, of spontaneous tolerance in rat orthotopic liver transplantation. *Immunology*. 2004;113:57-64.
49. Mukhopadhyay R, Abul JJ, Partho A, Ray S, Fox PL. The GAIT system: a gatekeeper of inflammatory gene expression. *Trends Biochem Sci*. 2009;34(7):324-331.
50. Mazumder B, Sampath P, Seshadri V, Maitra RK, DiCorleto PE. Fox PL regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. *Cell*. 2003;115(2):187-198.
51. https://www.uniprot.org/uniprot/Q6IMY6. Accessed October 04, 2021.
52. Howie D, Ten Bokum AT, Necula AS, Cobbold SP, Waldmann H. The role of lipid metabolism in T lymphocyte differentiation and survival. *Front Immunol*. 2018;12(8):1949.
53. Okada K, Hiroshi I, Ikemoto M. Serum complement C3 and α_2-macroglobulin are potentially useful biomarkers for inflammatory bowel disease patients. *Heliyon*. 2021;7(3):e06554.
54. Levin Y. The role of statistical power analysis in quantitative proteomics. *Proteomics*. 2011;11:2565-2567.
55. [2] Tang WH, Shilov IV, Seymour SL. Nonlinear fitting method for determining local false discovery rates from decoy database searches. *J Proteome Res*. 2008;7(9):3661-3667.
56. [3] Graham RLJ, McMullen AA, Moore G, Dempsey-Hibbert NC, Myers B, Graham C. SWATH-MS identification of CXCL7, LBP, TGFβ1 and PDGFRβ as novel biomarkers in human systemic mastocytosis. *Sci Rep*. 2022;12(1):5087.
57. [4] Zhi S, Congcong Z, Zhiling G, et al. Quantitative proteomics of HFD-induced fatty liver uncovers novel transcription factors of lipid metabolism. *Int J Biol Sci*. 2022;18(8):3298-3312.
58. Rankin CR, Theodorou E, Man Law IK, et al. Identification of novel mRNAs and lncRNAs associated with mouseexperimental colitis and human inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol*. 2018;315:G722-G733.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Wojciuk B, Bogucka A, Czaplewska P, et al. Proteomic study on the lymphocytes from pregnant Wistar rat females treated with immunosuppressive regimen. *Clin Transl Sci*. 2023;16:118-127. doi:10.1111/cts.13432