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Combined Heterozygous Genetic Variations in Complement C2 and C8B: An Explanation for Multidimensional Immune Imbalance?

Marco Mannes[a] Rebecca Halbgebauer[a] Lisa Wohlgemuth[a]
David Alexander Christian Messerer[a, b] Susa Savukoski[a] Anke Schultze[a]
Bettina Berger[a] Christiane Leonie Knapp[a] Christoph Q. Schmidt[c]
Daniel Fürst[d, e] Morten Hillmer[f] Reiner Siebert[f] Oskar Eriksson[g]
Barbro Persson[g] Bo Nilsson[g] Kristina Nilsson Ekdahl[g, h] Markus Huber-Lang[a]

[a]Institute of Clinical and Experimental Trauma Immunology, University Hospital of Ulm, Ulm, Germany; [b]Department of Transfusion Medicine and Hemostaseology, Friedrich-Alexander University Erlangen-Nürnberg, University Hospital Erlangen, Erlangen, Germany; [c]Institute of Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, Ulm, Germany; [d]Institute of Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Transfusion Service, Baden-Württemberg – Hessen, Ulm, and University Hospital of Ulm, Ulm, Germany; [e]Institute of Transfusion Medicine, Ulm University, Ulm, Germany; [f]Institute of Human Genetics, Ulm University and Ulm University Hospital Center, Ulm, Germany; [g]Department of Immunology, Genetics and Pathology (IGP), Rudbeck Laboratory C5:3, Uppsala University, Uppsala, Sweden; [h]Linnaeus Center of Biomaterials Chemistry, Linnaeus University, Kalmar, Sweden

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Keywords
Genetic complement variants · Complement imbalance · Complement reconstitution

Abstract
The complement system plays a crucial role in host defense, homeostasis, and tissue regeneration and bridges the innate and the adaptive immune systems. Although the genetic variants in complement C2 (c.839_849+17del; p.(Met280Asnfs*5)) and C8B (c.1625C>T; p.(Thr542Ile)) are known individually, here, we report on a patient carrying their combination in a heterozygous form. The patient presented with a reduced general condition and suffers from a wide variety of autoimmune diseases. While no autoimmune disease-specific autoantibodies could be detected, genetic analysis revealed abnormalities in the two complement genes C2 and C8B. Therefore, we performed a comprehensive investigation of the innate immune system on a cellular and humoral level to define the functional consequences. We found slightly impaired functionality of neutrophils and monocytes regarding phagocytosis and reactive oxygen species generation and a diminished expression of the C5aR1. An extensive complement analysis revealed a declined activation potential for the alternative and classical pathway. Reconstitution with purified C2 and C8 into patient serum failed to normalize the dysfunction, whereas the addition of C3 improved the hemolytic activity. In clinical transfer, in vitro supplementation of the patient's plasma with FFP as a complement source could fully restore full complement functionality. This study describes for the first time a combined heterozygous genetic variation in

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Correspondence to:
Markus Huber-Lang, markus.huber-lang@uniklinik-ulm.de

complement C2 and C8B which, however, cannot fully explain the overall dysfunctions and calls for further complement deficiency research and corresponding therapies.

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Introduction

The complement system plays a decisive role in the immune response. It works as a fluid-phase system which rapidly recognizes and clears pathogens. Opsonization of foreign agents, release of anaphylatoxins, and the membrane attack complex-induced lysis of cells are the most prominent effector functions of the complement cascade [1]. Moreover, complement orchestrates several intersections between the innate and adaptive response, underlining its importance for an effective overall immune response. It is therefore not astonishing that disturbances or disbalances of the cascade which are caused, e.g., by acute diseases like traumatic or infectious injuries can have severe negative impacts on the body's defense performance and, thereby, increase the susceptibility for septic complications [2, 3]. Besides such acute scenarios, also, inherited alterations or deficiencies in complement genes can manifest in a variety of complement-mediated allergic, infectious, or autoimmune diseases [4, 5]. While recurrent pyogenic infections associate with alterations in the C3 gene, deficiencies of terminal pathway components are specifically associated with *Neisseria* infections [6]. Genetic defects in the classical pathway cause typical autoimmune conditions like systemic lupus erythematosus (SLE) [7]. Complement-mediated autoimmunity can be explained either by inefficient opsonization and clearance of apoptotic cells, cell debris or immune complex deposits that consequently represent a source of self-antigens, or by a hampered self-tolerance [8]. In support on the latter notion, murine studies revealed that complement contributes to the clearance of self-reactive lymphocytes during immune system maturation [9, 10]. In addition to these direct genetic alterations in complement genes, some genetic defects indirectly disturb functioning of the complement system. Paroxysmal nocturnal hemoglobinuria patients carry an acquired pathogenic variant in the *PIG-A* gene which causes a lack in GPI-anchored proteins which affects two important complement regulators [11]. Clinically, such an alteration eventually leads to a diminished self-protection and an increased susceptibility to complement-mediated attack. In addition, nephritic factors against C3 or C4, which prolong the half-life of the respective convertases, have been associated with different clinical manifestations like membranoproliferative glomerulonephritis or partial lipodystrophy [12]. Prospectively, it is of utmost help to link clinical phenotypes to genetic abnormalities in the complement genes to define their role in these scenarios which eventually could inform on new causative strategies for complement-directed therapies.

In this report, we present a patient suffering from a wide range of autoimmune diseases associated with a so far unknown combined heterozygous genetic variant in the complement genes for C2 and C8B. We evaluated the impact of this dual genetic alteration on the overall clinical phenotype. Besides a comprehensive genetic investigation, we conducted a functional cellular immune profiling and analyzed the humoral fluid-phase response with a focus on the complement system. Most likely, our data indicate that the patient would benefit from a complement substitution therapy, thus opening the door for novel intervention options.

Material and Methods

Blood and Urine Collection

An informed written consent was obtained from the patient for the extended laboratory diagnostics and genetic analysis. Venous blood was drawn into Li-heparin (04.1954, Sarstedt, Germany), serum (01.1601.001, Sarstedt, Germany), citrate (02.1067.001, Sarstedt, Germany), and EDTA (01.1605.001, Sarstedt, Germany) monovettes. Urine was collected in urine monovettes (10.258.020, Sarstedt, Germany). The patient presented twice to our institute (September 30, 2020, and June 28, 2021). Corresponding samples and data from the three sex- and age-matched healthy volunteers were used (obtained after written informed consent; ethic votum #319/20 from the independent Ethics Committee of Ulm University, Ulm, Germany). Detailed processing of the samples is indicated in the following sections.

Molecular Genetic Investigation via Next-Generation Sequencing

Genomic DNA was isolated from peripheral blood using the FlexiGene DNA Kit (QIAGEN N.V., Venlo, Netherlands). For whole genomic detection of copy number variations (CNVs), molecular karyotyping was performed using the CytoScan HD Array (Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's guidelines. The reporting threshold of the CNVs was set at 50 kb with a marker count of ≥20. Data sets were analyzed by the Affymetrix Chromosome Analysis Suite (ChAS) software version 4.0 (Affymetrix). Detected CNVs were aligned with known CNVs that are listed in the publicly available database Database of Genomic Variants (DGV).

Next-generation sequencing was performed using a NextSeq platform (Illumina). For enrichment, the NextSeq High Output Kit v2.5 (150 cycles; Illumina: TruSight One®) was used on ~50 ng DNA, and data were analyzed using the Sequence Pilot SeqNext Module (JSI medical systems Corp., NY, USA) and filtered

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according to Human Phenotype Ontology list "abnormality of complement system" (HP: 0005339, state 11/20) and the HUGO gene list for the complement system (state 11/20). In addition, exome sequencing was performed on ≈50 ng of genomic DNA using the NextSeq High Output Kit v2.5 (300 cycles; TWIST™ Exome Kit as well as Illumina: Nextera™ Exome Kit). Analysis of the exome data included all genes using the varvis software version 1.19.1 (Limbus Medical Technologies GmbH, Rostock).

HLA Typing

HLA typing was performed with a CE-IVD marked kit (H-Seq-LR-NGS, DRK-BSD, Baden-Württemberg Hessen, Ulm) designed for clinical typing purposes using a gene-specific long-range PCR for the genes HLA-A, -B, -C, -DRB, -DQA1, -DQB1, -DPA1, and -DPB1. Library preparation was performed with the QIAseq FX DNA Library Kit (QIAGEN N.V., Venlo, Netherlands). It was sequenced on an Illumina Miseq NGS platform using V2-300 chemistry. Data analysis and allele assignment were performed with the NGSengine software (GenDX, Utrecht, Netherlands).

Clinical Chemistry

EDTA- and Li-heparin-anticoagulated blood as well as urine samples were handed to the clinical routine laboratory of the University Hospital of Ulm for validated diagnostics.

Plasma/Serum Collection

Citrated and EDTA-anticoagulated blood was processed immediately after blood drawing, while for serum collection, blood clotting was allowed for 45 min at RT. Samples were centrifuged at 2,200 *g* for 15 min at 4°C. Plasma/serum was collected, aliquoted, and stored at −80°C until further use.

Analysis of Autoantibodies

Serum samples were tested for the presence of autoantibodies including (1) antinuclear antibodies, (2) anti-neuronal antibodies (autoimmune encephalitis), (3) dermatomyosite antibodies, (4) antiphospholipid antibodies (anti-cardiolipin IgG and IgM, anti-β2-glycoprotein IgG and IgM), and (5) anti-GAD IgG using clinical standard assays at the Department of Clinical Immunology, Uppsala University Hospital, Sweden.

Semi-Quantification of C2 and C8 Serum Concentration by Western Blot

For determination of C2 and C8B serum content, 1 μL serum was mixed with a 2× Laemmli sample buffer (161-0737, BioRad, Germany) and run under nonreducing conditions for 45 min at 200 V on a precast stain-free gel (4–15%, 4568083, BioRad, Germany). Native purified C2 and C8 (CompTech, USA) served as positive controls and were loaded at 10, 25, 50, 75, and 100 ng. Afterward, protein transfer to an Amersham hybond membrane (10600023, GE Healthcare, USA) was performed by a 7-min blotting program for mixed-molecular-weight proteins. The membrane was blocked in 5% dry skim milk (70166, Sigma Aldrich, Germany) in TBST at RT for 1 h before the primary antibody was added for an overnight incubation under rolling conditions at 4°C. In case of C2 detection, the polyclonal rabbit antihuman C2 antibody (PA5-107085, Thermo Fisher Scientific, Germany) was used at a 1:1,250 dilution. For C8B, the polyclonal rabbit antihuman C8B antibody (PA5-31290, Thermo Fisher Scientific, Germany) was diluted 1:2,500.

The next day, three washing steps in TBST for 15 min were followed by incubation in a goat anti-rabbit HRP-linked antibody (A27036, Thermo Fisher Scientific, Germany) for 1 h at a 1:4,000 dilution. For labeling the unstained marker, a 1:15,000 diluted precision protein streptactin-HRP conjugate (L001653A, BioRad, Germany) was added as well. After washing the membrane twice in TBST for 15 min and once in TBS for 10 min, it was developed in a clarity Western ECL solution (170-5061, BioRad, Germany) for 40 s before a chemiluminescence picture was taken with the ChemiDoc XRS+ device (BioRad, Germany). The densitometric analysis was conducted with the image lab software from BioRad, Germany.

CP Hemolysis Assay

Sheep red blood cells (shRBCs, Fiebig Nährstofftechnik, Germany) were sensitized with rabbit anti-sheep hemolysin (Colorado Serum Company, USA) for 20 min at 37°C. Afterward, cells were washed twice with PBS-EDTA (5 mM) and once with PBS+/+. Cells were kept at RT until further use. Concomitantly, serum was mixed with an equal amount of washed and packed shRBCs and incubated on ice for 40 min to allow absorption of all naturally occurring antibodies against shRBC. Then, serum was separated from cells by centrifugation at 2,500 *g* for 3 min at 4°C, collected, and stored on ice until further use.

Pre-absorbed serum was mixed with PBS−/−, 30 μL PBS+/+, and 10 μL shRBCs to a final volume of 100 μL and incubated for 60 min at 37°C. Optionally, C3, C8, and/or C2 (CompTech, USA) were titrated into the mixtures at concentrations noted in the respective figure legends. Afterward, 50 μL of 30 mM PBS-EDTA solution was added to stop complement activity. Hemolytic activity was quantified by measuring the absorbance of the supernatant at 405 nm.

AP Hemolysis Assay

Rabbit red blood cells (rRBCs, Fiebig Nährstofftechnik, Germany) were washed three times with PBS−/− and adjusted to a standardized water lysis. Serum was mixed with PBS−/−, Mg-EGTA (5 mM final concentration), and 10 μL rRBCs to a final volume of 100 μL and incubated for 20 min at 37°C under shaking conditions. Optionally, purified C3 (A113, CompTech, USA) and/or C8 (A125, CompTech, USA) was titrated into the mixtures at concentrations noted in the respective figure legends. Reaction stop and determination of the hemolytic activity were performed in accordance with CP hemolysis assay. When performing the assay in citrate-plasma instead of serum, final Mg-EGTA concentration was increased from 5 mM to 20 mM to overcome the Mg-capturing effect of citrate.

Surface Deposition after AP/CP Activation Measured by Flow Cytometry

The assay was performed in accordance with those described in AP/CP hemolysis assay but with the addition of eculizumab and Coversin (final concentration: 0.5 μM for AP assay, 10 nM for CP assay) during the serum incubation step to prevent cells from complement-mediated lysis. After centrifugation, serum was discarded, cells were resuspended in 100 μL PBS−/−, and washed twice. Then, 50 μL of a 1:100 diluted anti-C3d (AP and CP assay, A702, Quidel, USA) or anti-C4d (only for CP assay, A704, Quidel, USA) antibody was added for 30 min in darkness. After another two washing steps, a 1:100 diluted APC streptavidin conjugate

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(405207, BioLegend, USA) was added in a total volume of 50 µL for another 30 min. Finally, cells were washed twice, resuspended in 500 µL PBS−/−, and measured by flow cytometry. Alternatively, for detection of C3 deposition, a FITC-conjugated antihuman C3/C3b/iC3b antibody (CL7636F, Cedarlane, Canada) was used.

LP Activation Assay

Assessment of LP activation was measured as previously described with some modifications [13]. In brief, 10 µg/well mannan (M7504 Sigma) diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6) were coated in a 96-well plate (MaxiSorb, 446612 Thermo Fisher Nunc) overnight at 4°C. The next day, wells were washed (wash buffer: 10 mM Tris, 140 mM NaCl, 1.5 mM NaN_3, 5 mM $CaCl_2$, 0.05% Tween 20, pH 7.4) and incubated with blocking buffer (10 mM Tris, 140 mM NaCl, 1.5 mM NaN_3, 0.1% BSA, pH 7.4) for 1 h at RT. After washing wells, serum spiked with 10 µM Cp40 was added in different dilutions (dilution buffer: 20 mM Tris, 10 mM $CaCl_2$, 2 M NaCl, 0.05% [v/v] Triton X-100, 0.1% BSA, pH 7.4) at hypertonic conditions for 2 h at 4°C. Then, wells were washed, and purified C4 (A105, CompTech) was added for another 1.5 h at 37°C. Non-bound C4 was washed out, and 50 µL of a 1:2,000 diluted biotinylated anti-C4d antibody (A704, Quidel) was added for 30 min at RT followed by washing and incubation with 50 µL of a 1:2,500 diluted HRP-streptavidin conjugate (554066, BD Biosciences). Finally, after washing, 100 µL of a TMB substrate (555214, BD OptEIA) was added for 15 min in darkness before 50 µL of 2 N H_2SO_4 stopped the reaction. The absorbance was measured at 450 nm.

Quantification and Functional Analysis of Complement Components

The concentrations of C3, C4, factor B, and C1-INH (concentration and function) were analyzed using nephelometry (IMMAGE, Beckman Coulter, Bromma, Sweden; at the Department of Immunology, Uppsala University, Sweden). In-house MagPix assays were used to quantify C1q, properdin, factor I, factor H, and C5. The active binding of MBL to mannan and ficolin-3 to acetylated BSA was quantified by ELISA.

Commercial ELISAs

All ELISAs were performed in accordance with the manufacturer's instructions if not stated otherwise. For measuring the anaphylatoxin concentrations in EDTA-plasma, the C3a ELISA kit (A031, Quidel, USA) and the C5a ELISA kit (EIA-3327, DRG international, USA) were used. Levels of thrombin/antithrombin complexes (ab108907, Abcam, USA) and circulating immune complexes with C3 fragments (A002, Quidel, USA) were determined in EDTA plasma as well.

Determination of IgG Subclasses

IgG subclasses were measured with a Human IgG Subclass Kit from the binding site using a Beckman IMMAGE 800 analyzer.

Rotational Thromboelastometry

Citrated blood samples were analyzed for detailed coagulative parameters by rotational thromboelastometry (ROTEM® delta, Werfen, Germany). After recalcification using star-tem20 (000503-10, Werfen GmbH, Germany), the extrinsic and intrinsic coagulation pathways were stimulated specifically by the addition of ex-tem (000503-05, Werfen GmbH, Germany) or in-tem (000503-02, Werfen GmbH, Germany) reagents, respectively. Recording was stopped 60 min after recalcification.

Cellular Immune Status Measured by Flow Cytometry

10 µL of the respective whole blood sample were stimulated in a total volume of 50 µL with fluorescent-labeled reagents, antibodies, and the following stimuli: platelet-activating factor (PAF) (18779, Cayman Chemical, USA, final conc.: 500 ng/mL), fMLP (F3506, Sigma Aldrich, Germany, final conc.: 100 nM), or C5a (A144, CompTech, USA, final conc.: 100 ng/mL). All used antibodies and their respective isotype controls are listed in the following: anti-CD59 (final conc.: 4 µg/mL, PE-conjugated, 304708, BioLegend, USA; isotype: 400212, BioLegend, USA), anti-CD46 (2 µg/mL, FITC-conjugated, 315304, BioLegend, USA; isotype: 400108, BioLegend, USA), anti-CD62L (62.5 ng/mL, PE-conjugated, 304806, BioLegend, USA; isotype: 400112, BioLegend, USA), anti-CD16 (0.2 µg/mL, PerCP-conjugated, 302030, BioLegend, USA; isotype: 400148, BioLegend, USA), anti-CD11b (7.5 ng/mL, APC-conjugated, 101212, BioLegend, USA; isotype: 400612, BioLegend, USA), anti-CD274 (2 µg/mL, BV785-conjugated, 329735, BioLegend, USA; isotype: 400356, BioLegend, USA), anti-CD35 (8 µg/mL, FITC-conjugated, 332406, BioLegend, USA; isotype: 400108, BioLegend, USA), anti-C5L2 (10.7 µg/mL, PE-conjugated, 342404, BioLegend, USA; isotype: 400212, BioLegend, USA), anti-C3aR (66 ng/mL, PE-Cy7-conjugated, 345808, BioLegend, USA; isotype: 400326, BioLegend, USA), anti-CD88 (134 ng/mL, APC-conjugated, 344310, BioLegend, USA; isotype: 400222, BioLegend, USA). Reactive oxygen species production was measured in heparinized blood by using the CellROX™ Deep Red Reagent (C10422, Thermo Fisher Scientific, Germany) in accordance with the manufacturer's instructions. The ability of phagocytosis was determined in heparinized blood by the addition of fluorescent-labeled phagocytosis beads (18339-10, Polysciences Inc., USA). Heparinized and citrated blood samples were incubated at 37°C in darkness for 30 min or 15 min, respectively. Afterward, the complete content was transferred to 950 mL of 1:10 diluted FACS™ lysing solution (349202, BD Biosciences, USA) and incubated for 30 min at RT in darkness. Samples were centrifuged at 340 g for 5 min, the supernatant was discarded, and cells were resuspended in PBS−/− containing 1% BSA. Finally, samples were stored in the fridge until measurement.

Statistics

For statistical analysis and graphical representation, the software GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) was used. Results are presented as mean and standard deviation if not stated otherwise. p values <0.05 were denoted to be statistically significant.

Results

Medical History

The 48-year-old female patient presented with a long-lasting history of recurrent infections of the upper respiratory tract. At two times during her childhood, her nasal polyps were removed combined with tonsillectomies. Of note, any surgical intervention (an

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Table 1. Medication

Drug	Amount	Taking	Comment	First admission	Second admission
Sandimmun optoral	25 mg	1 – 0 – 1	–	Yes	No
Mycophenolate mofetil	250 mg	1 – 0 – 1	–	Yes	No
Prednisolone	5 mg	1 – 0 – 0	In a shock condition up to 20 mg	Yes	Yes
Acetylsalicylic acid	100 mg	0 – 1 – 0	–	Yes	Yes
Amlodipine	2.5 mg	1 – 0 – 0	–	Yes	No
Colecalciferol	1000 IU	1 – 0 – 0	–	Yes	Yes
Pantoprazole	20 mg	0 – 1 – 0	–	Yes	Yes
L-Thyroxine	25–50 µg	1 – 0 – 0	Individual adjustment	Yes	Yes
Levetiracetam	500 mg	0 – 0 – 1	–	Yes	Yes
Iron tablets	100 mg	0 – 1 – 0	–	Yes	Yes
MCP	PRN 10 mg				
Paracetamol	PRN 500 mg				
Buscopan	PRN 10 mg				
Hylo-gel AT	PRN				

Taking has changed when the patient presented to our institute the second time.

appendectomy and two corrective osteotomies of the knee joint) resulted in severe wound healing problems, all of which required further surgical interventions. An existing hiatal hernia has not been surgically addressed based on this enhanced postoperative infection risk. Recurrent syncopes occurred associated with verified cerebral gliosis foci in the MRI imaging and oligoclonal bands in the cerebrospinal fluid. Furthermore, the patient reported on multiple allergies against mites, grasses, and medical plaster. There are existing vaccinations among others against neisseria, meningococcus, pneumococcus, herpes zoster, and hepatitis. To our knowledge, no comprehensive microbiological workup at her recurrent episodes has been performed. No cardiovascular risk factors were reported, and no alcohol, drug, or substance abuse was existent. The patient's current medication is shown in Table 1. Her medication has changed between the two visits; however, the listed medications had been taken for weeks, respectively. Based on the patient's report, we sketched a pedigree representing severe medical problems over generations (Fig. 1a).

Clinical Status with Current Problems

The status during the patient's examination was characterized by recurrent febrile episodes (occurring between 4 and 8 weeks, fever around 38.5°C to a maximum of 40.4°C) of an overlap connective tissue disease (overlap CTDs, overlap collagenosis) with features of the following diseases: SLE; systemic sclerosis; Sjögren syndrome; and autoimmune diseases of Hashimoto hypothyreosis, Morbus Basedow, and Morbus Raynaud. Externally, the patient presented with an SLE-typical butterfly rash (Fig. 1b) and classical signs of vasculitis on the arms and legs (Fig. 1c). The patient depicted an increasing accumulation of neurological problems with speaking, concentration disorders, ataxia, inclination to fall to the left side, and a left paresthesia. Besides this, elevating breathing problems marked by shortness of breath during walking (reduction in FEV1 by 63%, DLCOVa 35%) were noticed. A more detailed physical examination is listed in Table 2. At admission, we analyzed blood and urine levels (Table 3). Remarkably, we detected higher urine levels of albumin and the presence of red blood cells/hemoglobin/myoglobin in the urine at the second admission, indicative of first signs of a barrier dysfunction in the kidneys.

The Presence of Autoantibodies

To closer define the patient's autoimmune phenotype on a diagnostic level, we screened for the presence of typical antinuclear, anti-neuronal, antiphospholipid, anti-GAD, and dermatomyosite autoantibodies. A detailed list of screened antigens is shown in Table 4. All tested antibodies were below the detection limit of the respective assays. This contrasts with a former diagnostic finding (1 year before the patient's first admission) on positive nucleosome antibodies, positive ANA (1:100 nucleolar), positive anti-Th/To-Ab, positive PM-ScI75, and positive dsDNA and ssDNA.

Double Genetic Effect and HLA Typing

Consequently, we decided to perform a comprehensive investigation on the genetic level. Molecular karyotyping neither did reveal any clinically relevant constitutional

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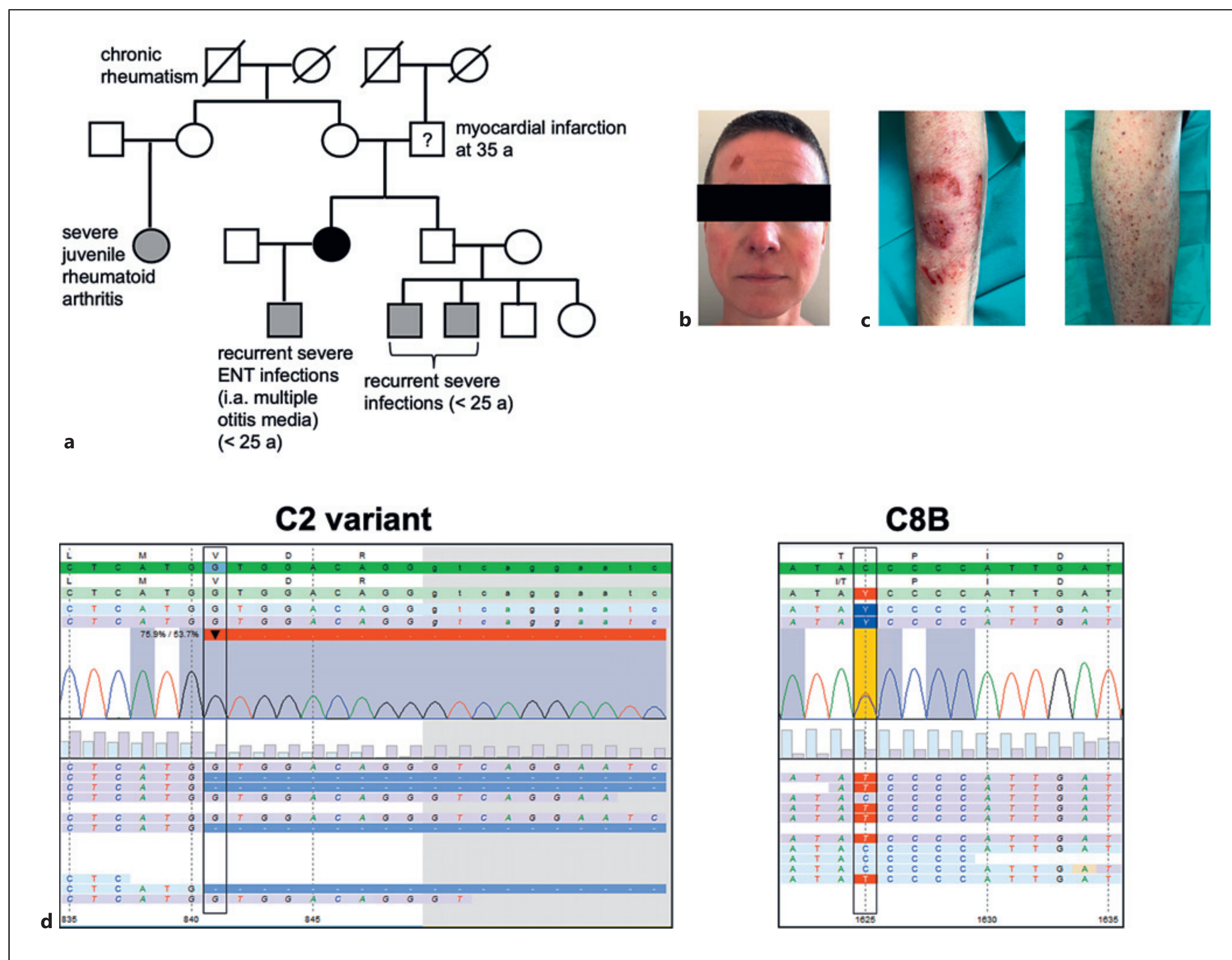

Fig. 1. Clinical phenotype and genetic analysis. **a** Pedigree with severe diseases. This compilation was performed on the basis of the patient's report. **b** Butterfly rash. A picture of the SLE-typical butterfly rash was taken at the patient's first admission. **c** Vasculitis on the arms and legs. **d** Genetic variations in C2 and C8B. Variation sites of C2:c.839_849+17del and C8B:c.1625C>T as shown in SEQUENCE PILOT (JSI medical systems Corp., NY, USA).

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Table 2. Physical examination

Body part	Evaluation
General	Reduced general condition, BMI 17 (loss of weight 8 kg/8 years), reduced appetite, enhanced demand for sleep
Head	Butterfly rash (Fig. 1), good dentation, no swelling, no cyanosis
Chest	Regular breathing with some effort, normal breathing rate, the absence of wheezing, rhonchi, and crackles
Heart	n/d, hemodynamic stable
Abdomen	Soft, tender, nondistended, the absence of pain, alternating diarrhea/obstipation
Extremities	No cyanosis, no edema, puffy fingers, petechiae and purpura in the lower leg and lower arm at both sides (see Fig. 1)

This list relies on the patient's anamnesis.

Table 3. Blood and urine levels

Blood				Urine			
parameter	first admission	second admission	unit	parameter	first admission	second admission	unit
Bilirubin	nd	6	μmol/L	Albumin	11.9	52.8	mg/L
Creatinine	nd	56	μmol/L	Calcium	1.7	3.3	mmol/L
C-reactive protein	nd	0.6	mg/L	Chloride	17.7	21	mmol/L
Erythrocytes	4.4	4.5	Tera/L	Creatinine	2.3	4.6	mmol/L
Hematocrit	0.39	0.41	L/L	RBC/Hb/myogl	nd	Positive	–
Hemoglobin	13.5	14.4	g/dL	Glucose	nd	4	mg/dL
IL-6	nd	3	pg/ml	IgG	nd	4.6	mg/L
Leukocytes	7.9	8.9	Giga/L	Magnesium	2.3	3.3	mmol/L
MCH	31	32.2	pg	Osmolarity	130	204	mosm/kg
MCHC	34.7	35.6	g/dL	Phosphate	3.3	9.1	mmol/L
MCV	89.2	90.6	fl	Potassium	11.8	23.2	mmol/L
Procalcitonin	nd	0.024	μg/L	Protein	36	95	mg/L
Thrombocytes	145	244	Giga/L	Sodium	13.5	14	mmol/L
				Urea	65.1	94.7	mmol/L
				Uric acid	nd	1.1	mmol/L

Table 4. List of antigens included in the panels for screening of autoantibodies

Category	Antigen
Anti-neuronal	Amphiphysin, CV2, PNMA2/Ta, Ri, Yo, Hu, Recoverin, SOX1, Titin, Zic4, Tr(DNER)
Antiphospholipid	Cardiolipin (IgG + IgM), β2-glycoprotein (IgG + IgM)
Anti-GAD	GAD65
Dermatomyosite	Mi-2alpha, Mi-2beta, TIF-1gamma, MDA5, NXP2, SAE1, Ku, PM-Scl100, PM-Scl75, Jo-1, SRP, PL-7, PL-12, EJ, OJ, Ro52

imbalances nor long copy number neutral stretches of homozygosity. By using clinical and whole-exome sequencing in a total of three different approaches, we could identify a variant in C2 (c.839_849+17del; p.(Met280-Asnfs*5), alternatively known as C2:c.841_849del; p.(Val281_Arg283del)) in heterozygous constellation (Fig. 1d). It comprises of a 28-bp deletion which leads to a loss of exon 6 due to a failed splicing process [14]. In the clinically relevant databases HGMD professional and ClinVar, this variation is scored to be likely pathogenic. Since C2 is encoded on the short arm of chromosome 6 and surrounded by genes of the major histocompatibility complex, a strong association has been reported between C2 deficiencies and a certain HLA haplotype [14–16]. Our investigation of the HLA gene loci revealed that the patient also carries the common haplotype HLA-A25, B18, DRw2 that is linked to the 28-base pair deletion in C2 (Table 5). In addition to this, a single missense variant was detected in the C8B gene (c.1625C>T; p.Thr542Ile) in the heterozygous constellation which was classified as likely benign (Fig. 1d). No small or copy number variant affecting the second allele of the C2 or C8B genes, respectively, was detected. Moreover, evaluation of the whole-exome sequencing data revealed no other pathogenic or likely pathogenic variants which could be linked to the phenotype. In particular, we could not identify a pathogenic or likely pathogenic variant in any of 406 genes associated with inborn errors of the immunity [17].

Impaired Immune Function of Innate Leukocytes

In order to characterize the consequences of such a combined heterozygosity for the described C2 and C8B gene variants on the overall immune function on a cellular level, we performed a static and functional immune profiling of innate polymorphonuclear (PMN) cells and monocytes. A special focus was set on complement receptors and corresponding regulators. In an in vitro setting, blood samples of the patient were exposed either to fMLP (100 nM; a well-established strong activator of these cells [18]), C5a (100 ng/mL; being the most prominent pro-inflammatory anaphylatoxin of the

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Table 5. HLA typing

	Allele 1	Allele 2
HLA-A	03:01:01	25:01:01
HLA-B	18:01:01	35:01:01
HLA-C	04:01:01	12:03:01
DRB1	01:01:01G	15:01:01
DRB5	01:01:01	
DQB1	05:01:01	06:02:01
DQA1	01:01:01G	01:02:01G
DPB1	03:01:01G	04:02:01G
DPA1	01:03:01	01:03:01

complement cascade), or PAF (500 ng/mL, thereby also addressing the thromboinflammatory axis [19, 20]). Incubation of the blood exclusively with PBS−/− served as a negative control. Investigating the general innate leukocyte activation statues, we observed no differences of CD11b expression levels on PMNs and monocytes between the patient and controls. However, provoked shedding of CD62L, which serves as another well-established activation marker, was more pronounced in healthy controls (Fig. 2a). Furthermore, no significant alterations in the expression level of CD16 and CD274 were detectable between healthy volunteers and the patient (online suppl. Fig. 1a; for all online suppl. material, see www.karger.com/doi/10.1159/000528607). For evaluation of PMN and monocyte functionality, we focused on the vital functions of phagocytosis and reactive oxygen species generation. While the phagocytotic activity of monocytes was mainly on the same level, PMNs from healthy donors were significantly more active in comparison to those of the patient. Reactive oxygen species generation was clearly impaired in PMNs and monocytes obtained from the patient irrespective of their resting or activation state (Fig. 2a).

When focusing on complement-related surface proteins, no alterations in the expression levels of the regulators CD46 and CD59 as well as the receptors CR1 and C5aR2 were observable (Fig. 2b; online suppl. Fig. 1b). Moreover, no abnormalities in the expression level of the C3aR could be detected on neutrophils, while it seemed to be declined on monocytes. Remarkably, attenuated expression of C5aR1 was evident on both monocytes and PMNs (Fig. 2b).

Alterations in the Adaptive Profile

Although focusing predominantly on the innate immune system, we also evaluated features on adaptive leukocytes out of the experiment designed for innate leukocytes. By defining the lymphocyte population by size and granularity, we recognized slightly declined expression of both C5a receptors, C5aR1 and C5aR2, in comparison to respective controls (online suppl. Fig. 2a). Remarkably, the expression of CD62L is greatly enhanced on the patient's cells. We further assessed the humoral status of the adaptive immune system by measuring IL-38, IL-10, and IL-2R plasma levels which were clearly elevated in comparison to controls (online suppl. Fig. 2b). Interestingly, all markers were higher at the first admission. A former diagnostic finding reported on normal immunoglobulin levels with the following values: IgG 913 mg/dL (reference range: 700–1,600), IgA 92 mg/dL (reference range: 70–500), IgM 92 mg/dL (reference range 40–280). We now specifically addressed IgG subclass concentrations and determined normal IgG1, 2, and 4 levels with slightly declined IgG3 levels (online suppl. Fig. 2c).

Effects on the Hemostatic System

Under the shield of the intravascular innate immune system, complement is known to tightly interact and cooperate with the hemostatic system [21, 22]. Therefore, we investigated whether the present genetic alterations in the complement genes elicit alterations in the hemostatic process. Interestingly, thrombin-antithrombin complexes which are formed upon thrombin generation were clearly elevated in the patient's plasma (online suppl. Fig. 3a). On the other hand, rotational thromboelastometry analysis with specific activation of either the extrinsic or intrinsic pathway revealed no considerable differences between the patient and the healthy controls (online suppl. Fig. 3b). Moreover, the patient did not report on any clinical manifestation of a coagulation disorder.

Alterations in Complement Function

In order to determine the functionality of the complement system, the impact of the genetic alterations was assayed in a wide range of experiments. While no significant semiquantitative differences in the plasma C2 and C8B levels could be observed in Western blot experiments (Fig. 3a, b), a functional impairment for the C2 protein was identified by an external validated complement diagnostics laboratory (Table 6). In comparison, C8 function was within the reference range which substantiates that the sequence change in C8 likely is a benign variation. Besides this, normal levels of C4 and low levels of C3 have been measured, both agreeing with previous laboratory diagnostics (data not shown). Normal levels of factor I, factor H, and C5 were determined in-house as well. Furthermore, the analysis showed no hints of increased complement activation, evidenced by normal

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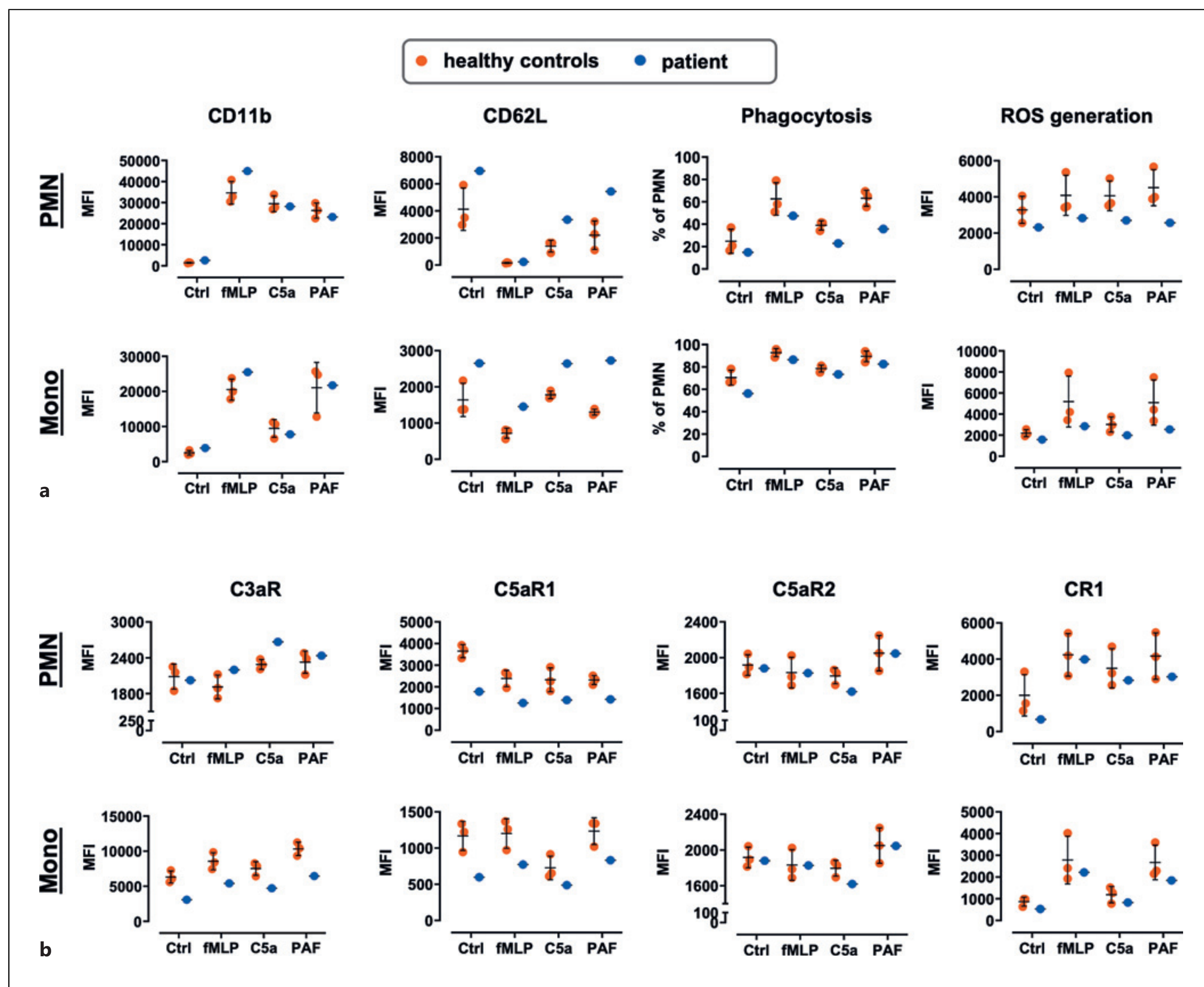

Fig. 2. Immune profile of PMN cells and monocytes. Citrated and heparinized blood were stimulated with either PBS–/– (neg. Ctrl), PAF (500 ng/mL), fMLP (100 nM), or C5a (100 ng/mL) and analyzed for general functional parameters (**a**) and complement receptor expression (**b**). Blood from the patient (blue) was compared with three age- and sex-matched healthy controls (orange). All samples were stimulated and measured in triplicates, and the mean value with standard deviation is shown.

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plasma levels of complement activation products (C3a, C5a, C3d, Bb, sC5b-9; Table 6; online suppl. Fig. 4a). In contrast, considerably higher levels of circulating immune complexes with attached C3 activation fragments were measured in comparison to controls (online suppl. Fig. 4b).

The functional complement tests revealed diminished complement activity in the alternative (57%, normal range: 60–140%, at first admission) and classical (71%, normal range: 74–151%, at first admission) pathways (Table 6). This is in line with our in-house hemolytic assays mirroring these results (Fig. 4a, b). rRBCs (for AP activation) or sensitized shRBCs (for CP activation) were exposed to different amounts of serum, and cell lysis was determined by measuring the released hemoglobin. The patient's serum showed a clear right shift, indicating a declined hemolytic activity in both pathways. However, when performing these assays with serum from the patient at the second visit, the differences to healthy controls were less pronounced (online suppl. Fig. 5a, b).

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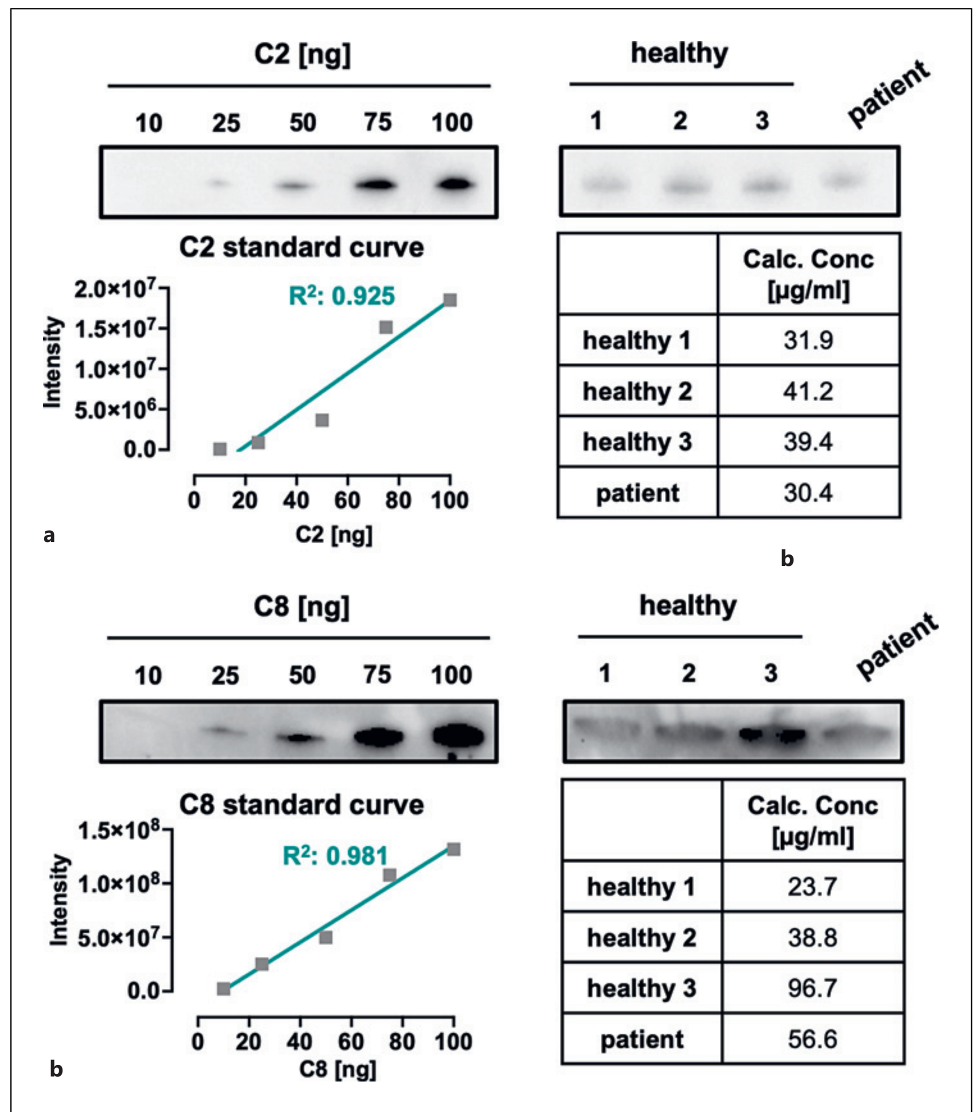

Fig. 3. Semi-quantification of C2 and C8 serum levels. **a** C2 Western blot: serum from the patient and three healthy donors was separated under nonreducing conditions on a 4–15% SDS-PAGE gel and transferred onto a PVDF membrane. For the positive control, different amounts (10 ng, 25 ng, 50 ng, 75 ng, 100 ng) of purified native C2 (CompTech) were loaded as well. C2 proteins were detected by a polyclonal anti-C2 antibody. Band intensity of purified proteins was used to establish a standard curve which eventually allowed a rough calculation of C2 serum concentration. **b** C8 Western blot as in (**a**) but with purified C8 (CompTech) and a polyclonal antibody against C8B.

Accordingly, validated tests revealed further low functionality for the AP but within the reference range (68%, reference range: 50–150%, Table 6). Furthermore, on the basis of a previous publication [13], we established an in-house assay which allows us to evaluate the initial LP activation step on a mannan-coated surface. Interestingly, the patient seems to activate more strongly as determined by elevated C4 deposition (Fig. 4c). In agreement with this observation, the MBL activity (capacity to bind mannan) was enhanced in comparison to the controls (Table 6). However, the activity of ficolin-3 (binding to acetylated bovine serum albumin), another strong activator of the LP, was reduced by approximately 70%. Next, we tested whether the full hemolytic activity could be reconstituted by adding the purified components C8 (for AP and CP) and/or C2 (only for CP) (Fig. 4d, f). None of these single or combined titrations had a beneficial effect, while the addition of C3 could at least partially increase the

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Table 6. Validated complement diagnostics

Analysis	First admission	Second admission	Reference range	Unit
Classical pathway (function)	71	78	74–151; 80–120[a]	%
Alternative pathway (function)	57	68	60–140; 50–150[a]	%
C1-INH (concentration)	nd	0.246	0.13–0.29	g/L
C1-INH (function)	nd	119	72–129	%
C1q (concentration)	nd	86.6	102.2[b]	mg/L
C3 (concentration)	0.93	0.71	0.92–1.56; 0.67–1.29[a]	g/L
C4 (concentration)	0.21	0.17	0.13–0.35; 0.13–0.32[a]	g/L
C5 (concentration)	nd	67.1	58.3[b]	mg/L
MBL (activity)	nd	183.2	69.3[c]	%
Ficolin 3 (activity)	nd	60.8	88.3[c]	%
Factor B (concentration)	nd	0.21	0.19–0.5	g/L
Properdin (concentration)	nd	11.1	19.1[b]	mg/L
Factor I (concentration)	nd	20.3	21.2[b]	mg/L
Factor H (concentration)	nd	0.432	0.615[b]	g/L
C3d (concentration)	14	nd	<40	mU/L
sC5b-9 (concentration)	158	nd	58–239	ng/mL
C2 (function)	55	nd	65–135	%
C8 (function)	80	nd	50–150	%
C3 nephritis factor (presence)	nd	Slightly positive	Negative	–
Anti-C1q IgG auto antibody	nd	3	<10	U/mL

Central complement components were assayed for their concentration and functional tests. [a]Deviating reference ranges between the first and second admission due to change in the analytical laboratory. [b]Mean of three age- and sex-matched controls, magnetic bead assay. [c]Mean of three age- and sex-matched controls, active binding ELISA.

hemolytic activity in both AP and CP. Since the activation of the terminal pathway and the subsequent formation of the membrane attack complex are strongly associated with the surface opsonization [23, 24], we addressed the C3 deposition on rabbit erythrocytes and sensitized sheep erythrocytes after AP (Fig. 4e) and CP (Fig. 4g) activation, respectively. In both cases, C3 opsonization was clearly diminished in case of the patient's serum, while upstream C4 attachment to the surface of sensitized shRBCs was comparable in case of CP activation (Fig. 4h). Since our analysis revealed no genetic alteration in C3, we searched for putative reasons explaining the decreased ability of C3 activation. We identified low plasma concentrations of factor B and properdin in comparison to the healthy controls (Table 6). However, re-substitution of properdin into the patient's plasma showed no beneficial effect (data not shown). Besides, we detected a slight positive signal for C3 nephritis factor which can further modulate C3 function in terms of convertase stabilization, thereby causing complement dysregulation (Table 6). With respect to sings of arising kidney damage, we screened for anti-C1q autoantibodies which are typical for lupus nephritis, a concomitant phenomenon of SLE (Table 6). However, we could not detect those in plasma from the patient's second admission.

Complement Reconstitution with Fresh Frozen Plasma

Up to date, no standardized complement add-on therapy is available to balance hypocomplementemia. Since our data clearly revealed an improvement in complement activity by the addition of C3, we searched in vitro for a therapeutical axis to restore complement functionality. Few reports indicated favorable outcomes for patients deficient or defective in C2 or C3 after fresh frozen plasma (FFP) infusion [25–27]. We therefore tested in vitro the effect of blood group-matched FFP addition as a source of complement proteins. For this purpose, we established a protocol for measuring the hemolytic activity in citrated plasma instead of serum (online suppl. Fig. 5c, d). The patient's citrated plasma was then substituted with increasing amounts of three different (blood group-matched) FFP samples, and the hemolytic activity and C3 deposition were assessed (Fig. 5a, b). Of note, albeit the C3 opsonization level of healthy volunteers was not reached, 40% substitution with FFP completely restored complement functionality of the patient (Fig. 5a, b).

Discussion

The present report presents a patient with a wide spectrum array of overlapping autoimmune diseases,

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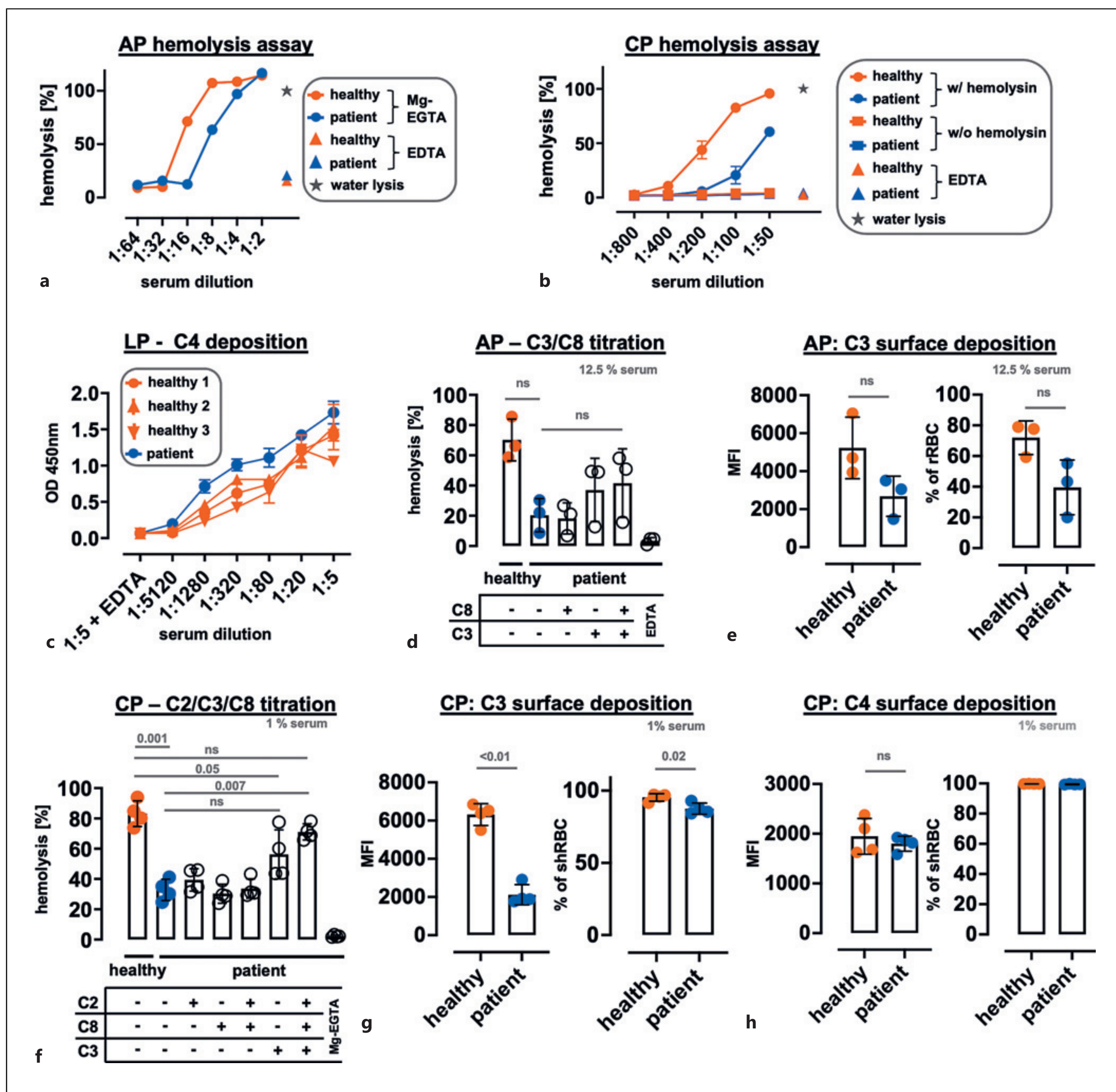

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resulting in a high individual burden. Although clinically not exhibiting classical acute infection patterns, we cannot completely rule out (opportunistic) microbe-driven/modulating effects during the recurrent fever episodes since an advanced microbiological workup is missing. However, the genetic workup exhibited only two heterozygous variants in the complement genes C2 and C8B, classified as likely pathogenic and likely benign, respectively. Complement deficiencies in the classical pathway are generally associated with SLE or SLE-like conditions, but comprehensive diagnostic screening including autoimmune-specific antibodies rules out these conditions.

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At a first glance, such a heterozygous variant may not suffice to explain the severe clinical manifestation. However, as nicely pointed out by Vignesh et al., complement can act as a kind of double-edged sword in the context of autoimmune diseases. As an effector arm of the innate immune system, disbalanced complement is significantly involved in tissue damage, while on the opposite, a genetic variant or resulting deficiency can also cause autoimmunity [9]. The existing variant in C8B occurs in 0.86% of the European population and is classified to be "likely benign" by the database ClinVar. This is in line with our data since neither a quantitative nor a functional impairment could be detected which would be explained by a functional impairment of C8. In general, homozygous C8β deficiencies are susceptible to meningococcal disease, while a heterozygous constellation does not result in increased disease risk in a Russian population [28]. Heterozygous C8β deficiency did not alter functional activity of classical and alternative pathways and did not correlate with neisserial infections in an Italian population [29]. With respect to the special composition of C8 consisting of an alpha-gamma subunit that is non-covalently linked to the β-subunit, it was shown that the soluble terminal complement complex can be formed in sera deficient for C8β [30]. These data reveal that further investigation is necessary to clarify precise functionalities of the respective subunits to evaluate their roles in diseases. Although no quantitative reduction in the plasma C2 level could be determined as well, functional tests revealed a slight impairment. Interestingly, the detected C2 variant is one of the most common genetic alterations in complement C2 with an appearance of 0.74% in the non-Finnish European population. With respect to the ACMG guideline, the variant is scored as "likely pathogenic" [31]. No further pathogenic variants with unknown significance were found in C2 or in other associated genes which could lead to a combined heterozygous deficiency of proximal classical pathway components as described by Hartmann et al. [32]. Nonetheless, there are also reports that implicate a partial complement defect only due to heterozygous mutations in C2 [33]. In general, two major C2 deficiencies are known: the present type I deficiency with a 28-base pair deletion resulting in the complete loss of exon 6 as well as a type II deficiency with a selective block in C2 secretion [14]. C2 deficiencies often manifest with mild phenotypes, and only a minority of C2-deficient patients develop autoimmune diseases [7, 9], suggesting that the specifically activated complement pathways are not purely reliant on the functionality of C2. Indeed, one recent report suggested a C2 bypass activation in which C2 is replaced by the alternative pathway homologue Bb, thus forming a hybrid C3 convertase C4bBb [34]. Another study revealed the MBL-driven in vitro activation of the alternative pathway in sera from C2- and C4-deficient patients [35]. Interestingly, the addition of purified C2 into our assays did not improve complement functionality which could support such concepts of bypassing C2. However, addition of C3, which was inconspicuous from the genetic point of view in this case, enhanced the hemolytic activity in both classical and alternative pathways. Besides the C3-bearing immune complexes, we could not determine any significant complement consumption as measured by complement activation products which could explain the relatively low plasma levels of C3. Interestingly, we also measured relatively low levels of FB and properdin. Both components fulfill an essential role in the alternative pathway amplification loop. It is tempting to speculate that the combination of low C3, FB, and properdin levels potentiates and eventually results in

Fig. 4. Complement functionality. **a** AP hemolysis assay: alternative pathway-mediated lysis of rRBCs was measured at different serum dilutions by the release of hemoglobin. The patient's serum (blue) was compared to an age- and gender-matched control (orange). Incubation of cells in water (gray star) served as the positive control and the addition of PBS-EDTA (10 mM, triangle) to 1:2 diluted serum as the negative control. Data points are single values from one experiment. **b** CP hemolysis assay: sensitized (round) or non-sensitized (square) shRBCs were treated in preabsorbed serum from healthy donors (orange) and patient (blue), and lysis was normalized to the water sample (gray star). Data points are the mean average with standard deviation from three independently performed assays. **c** LP activation assay: LP activation was measured on a mannan-coated 96-well plate in the form of C4 deposition. The graph represents one of three independently performed assays in which all conditions were measured in duplicates. **d** AP – C3/C8 titration: purified C3 (CompTech) and C8 (CompTech) was added at 400 nM and 45 nM, respectively. The graph shows average and standard deviation of three assay repetitions. **e** C3 deposition after AP activation as in (**a**) but with eculizumab and Coversin to prevent cell lysis. Surface-deposited C3 was determined with an anti-C3/C3b/iC3b antibody (Cedarlane) by flow cytometry. Mean fluorescence intensities and the percentage of positive cells from 3 independent experiments are shown. **f** CP – C2/C3/C8 titration: sensititzed shRBCs were incubated in 1% serum optionally supplemented with C2 (2.1 nM), C3 (320 nM), and/or C8 (3.6 nM). Average and standard deviation of 4 independent assays are shown. **g** C3 deposition after CP activation as in (**e**) but after CP activation on shRBCs in 1% serum. C3 deposition was measured with a biotinylated anti-C3d antibody (Quidel). Mean with standard deviation of 4 independent experiments is shown. **h** C4 deposition after CP activation as in (**g**) but for C4. Deposition was measured using a biotinylated anti-C4d antibody (Quidel).

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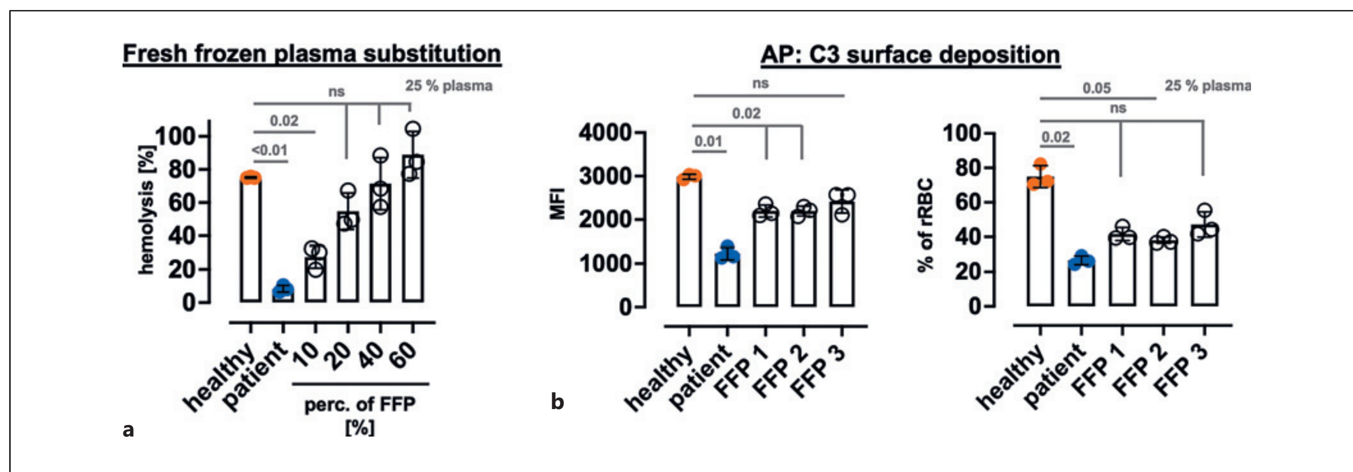

Fig. 5. Reconsititution of the normal hemolytic level by FFP. **a** FFP substitution: lysis of rRBCs was assayed in 25% citrate plasma. The patient's plasma was substituted with 10, 20, 40, or 60% of FFP. The graph shows mean and standard deviation of three independent assays with FFP derived from three different donors. **b** C3 surface deposition after FFP substitution as in (**a**) with 40% substitution but with eculizumab and Coversin for lysis inhibition. C3 deposits on rRBCs were measured with an anti-C3/C3b/iC3b antibody (Cedarlane) by flow cytometry.

the present attenuated complement activity. Significant proteinuria as evidenced by a high protein/creatinine ratio in the urine, occasional diarrhea, and rapid weight loss within a short period provide plausible explanations for low protein levels. Accordingly, studies in anorexia nervosa and obesity patients revealed a connection between concentrations of AP complement components and weight [36, 37]. Furthermore, we identified a slight positive signal for the presence of C3 nephritis factor which can further influence to C3-modulating effects. Those autoantibodies are counted among immunoconglutinins which represent a class of autoantibodies against fixed complement factors. Raised levels can be found in response to acute or chronic infections in a wide array of rheumatic diseases [38, 39]. In general, C3 nephritis factors stabilize the AP convertase and prevent its decay, thus causing C3 consumption [40]. As the central hub of the cascade, C3 plays a significant role in fulfilling essential effector functions [41]. Activated fragments on foreign surfaces induce the complement receptor-mediated clearance of those particles by immune cells [42], and densely deposited surfaces have been recently shown to be essential for effective terminal pathway activation [23, 24]. Therefore, we aimed to counteract this impairment by an in vitro plasma substitution with FFP. Indeed, we could successfully restore hemolytic activity by increasing surface C3 deposition. Former in vivo studies have indicated such FFP substitutions as a reliable therapeutic intervention therapy. Steinsson et al. [26] treated C2-deficient SLE patients who were nonresponsive to conventional therapy with repetitive plasma infusions over almost 1 year. They observed a complete remission of the disease phenotype and a temporary recovery of the hemolytic activity. Most importantly, the therapy appeared to be safe with no observable adverse effects. Moreover, an SLE-like patient with dysfunctional C3 received plasma transfusion which resulted in the disappearance of the Raynaud's phenomenon and a significant reduction of immune complex load [27]. In conjunction with that, the patient's fatigue gradually disappeared and life quality improved. Nonetheless, the authors recognized a potential risk of (among others) fever development, diarrhea, and hematuria which could aggravate tissue damage. Also, in other diseases in which plasma infusion or replacement therapy is performed, adverse effects are reported. Rosenkvist et al. [43] described attacks of urticaria in half of myasthenia gravis patients who underwent an infusion. In general, there is always the risk of sensitization or transfusion-related acute kidney injury during a prolonged infusion of plasma [44].

Taken together, plasma substitution with FFP so far remains the only putative option to restore complement function in vivo. In the present case, an emergency treatment strategy has been developed for the patient based on our present findings which includes FFP substitution in case of severe tissue trauma or necessary operations to provide enough complement functionality for sufficient (also temporary) fluid-phase defense purposes. However, more

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clinical evaluation and characterization of such a therapeutic approach must be gained to define an optimal long-term therapy scheme with minimal adverse effects. This case report emphasizes that besides focusing on complement inhibition, there is an unmet need to further study the clinical implications of complement reconstitution.

Statement of Ethics

The study protocol was reviewed and approved by the independent Ethics Committee of the University of Ulm, Ulm, Germany, approval number 319/20. An informed written consent was obtained from the patient for the extended laboratory diagnostics and genetic analysis. Corresponding samples and data from the three sex- and age-matched healthy volunteers were used after having obtained written informed consent as well.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Marco Mannes, Anke Schultze, Bettina Berger, Barbro Persson, Oskar Eriksson, Rebecca Halbgebauer, Lisa Wohlgemuth, Christiane Leonie Knapp, and Susa Savukoski performed the experiments. Reiner Siebert, Morten Hillmer, and Daniel Fürst conducted the genetic analysis. David Alexander Christian Messerer, Christoph Q. Schmidt, Kristina N. Ekdahl, and Bo Nilsson helped designing the experimental set-up and provided reagents. Marco Mannes and Markus Huber-Lang wrote the manuscript and prepared the graphs. Marco Mannes, Rebecca Halbgebauer, Lisa Wohlgemuth, David Alexander Christian Messerer, Susa Savukoski, Anke Schultze, Bettina Berger, Christiane Leonie Knapp, Christoph Q. Schmidt, Daniel Fürst, Morten Hillmer, Reiner Siebert, Oskar Eriksson, Barbro Persson, Bo Nilsson, Kristina N. Ekdahl, and Markus Huber-Lang critically reviewed the manuscript.

Data Availability Statement

Research data sets are not publicly available on ethical grounds. All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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