Aus dem Institut für Virologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Autoantibodies Against Type I Interferons in SARS-CoV-2 Infection

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Autoantikörper gegen Typ-I-Interferone bei SARS-CoV-2 Infektion

zur Erlangung des akademischen Grades
Doctor of Philosophy (PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Bengisu Akbil

Datum der Promotion: 30. Juni 2024

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List of abbreviations

ACE2	Angiotensin-converting enzyme 2
AIRE	Autoimmune regulator
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS-1	Autoimmune polyendocrine syndrome type I
BMI	Body mass index
CD8	Cluster of differentiation 8
COVID-19	Coronavirus disease 2019
CRP	C-reactive protein
CSC	Cross-sectional cohort
DC	Dendritic cells
DMV	Double membrane vesicle
E	Envelope
ECMO	Extracorporeal membrane oxygenation
ELISA	Enzyme-linked immunosorbent assay
ERGIC	Endoplasmic reticulum–Golgi intermediate compartment
GAS	Gamma interferon activation site
IFN	Interferon
IFN-AAB	Interferon autoantibody
IFNAR	Interferon alpha/beta receptor
IMV	Invasive mechanical ventilation
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-sensitive response element
JAK	Janus kinase

LDH	Lactate dehydrogenase
LSC	Light signal count
M	Membrane
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma differentiation-associated protein 5
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
N	Nucleocapsid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
Nsp	Non-structural protein
ORF	Open reading frame
pDC	Plasmacytoid dendritic cells
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RTC	Replication-transcription complex
S	Spike
SARS-CoV-2	Severe-acute-respiratory syndrome coronavirus-2
sg	Subgenomic
SLE	Systemic lupus erythematosus
Ss	Single-stranded
STAT	Signal transducer and activator of transcription
TBK1	TANK Binding Kinase 1
TLR	Toll-like receptor
TPE	Therapeutic plasma exchange
TRIF	TIR-domain-containing adapter-inducing interferon-β

TYK	Tyrosine kinase
WHO	World Health Organization

Abstract

Severe-acute-respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), rapidly became a pandemic and has caused 6.6 million deaths since the outbreak started. Although the development and administration of vaccines provided a substantial improvement by decreasing both the number of transmission events and disease severity of infected individuals, emerging viral variants still pose a threat due to their potentially increased transmissibility, immune evasion and virulence. Therefore, there is an ongoing need to better characterize SARS-CoV-2 pathogenicity and the underlying mechanisms to develop alternative strategies for prevention and treatment of COVID-19.

This dissertation is based on two publications investigating interferon (IFN) autoantibodies in patients with autoimmune polyendocrine type I (APS-1) and hospitalized COVID-19 patients, respectively. Despite the known production of neutralizing IFN autoantibodies (IFN-AABs) by patients with autoimmune disorders –like APS-1- and well-characterized, key role of IFNs in immune responses during viral infections, the contribution of IFN-AABs to disease in viral infections have been rarely addressed. With the emergence of the COVID-19 pandemic, several studies revealed presence of IFN-AABs in a proportion of patients with severe COVID-19 and pointed towards a link between IFN-AABs and disease severity.

Firstly, we conducted a prospective study (1) on a small cohort of patients with APS-1. We hypothesized that these patients have a disposition to develop severe COVID-19 in case of a SARS-CoV-2 infection due to pre-existing IFN-AABs. Contrary to our hypothesis, patients within our cohort that reported an infection with SARS-CoV-2, reported mild disease, showing an incomplete clinical penetrance of IFN-AABs to severe COVID-19. Secondly, we performed a large scale, cross-sectional, multi-cohort study (2) on hospitalized patients with COVID-19. Here, we aimed to identify clinical parameters that co-present with neutralizing IFN-AABs. We propose a novel clinical algorithm for rapid identification of neutralizing IFN-AAB-positive patients that can benefit from specific alternative therapies.

Zusammenfassung

Das schwere akute respiratorische Syndrom Coronavirus 2 (SARS-CoV-2), der Erreger der Coronavirus-Krankheit 2019 (COVID-19), wurde schnell zu einer Pandemie und hat seit Beginn des Ausbruchs 6,6 Millionen Todesfälle verursacht. Obwohl die Entwicklung und Verabreichung von Impfstoffen eine wesentliche Verbesserung gebracht hat, indem sowohl die Zahl der Übertragungsereignisse als auch die Schwere der Erkrankung bei infizierten Personen verringert wurden, stellen neu auftretende Virusvarianten aufgrund ihrer potenziell erhöhten Übertragbarkeit, Immunflucht und Virulenz immer noch eine Bedrohung dar. Daher besteht ein anhaltender Bedarf, die Pathogenität von SARS-CoV-2 und die zugrunde liegenden Mechanismen besser zu charakterisieren, um alternative Strategien und zur Prävention und Behandlung von COVID-19 zu entwickeln.

Diese Dissertation basiert auf zwei Publikationen, die Interferon (IFN)-Autoantikörper bei Patienten mit autoimmunem polyendokrinem Typ I (APS-1) bzw. bei hospitalisierten COVID-19-Patienten untersuchen. Trotz der bekannten Produktion von neutralisierenden IFN-Autoantikörpern (IFN-AABs) in Patienten mit Autoimmunerkrankungen – wie APS-1 – und einer gut charakterisierten Schlüsselrolle von IFNs bei Immunantworten während Virusinfektionen, wurde der Beitrag von IFN-AABs zur Erkrankung im Kontext von Virusinfektionen selten untersucht. Mit dem Aufkommen der COVID-19-Pandemie zeigten mehrere Studien die Präsenz von IFN-AABs bei einigen Patienten mit schwerem COVID-19 und wiesen auf einen Zusammenhang zwischen IFN-AABs und der Schwere der Erkrankung hin.

Zunächst führten wir eine prospektive Studie (1) an einer kleinen Kohorte von Patienten mit APS-1 durch. Wir stellten die Hypothese auf, dass diese Patienten eine Neigung haben, im Falle einer Infektion mit SARS-CoV-2 aufgrund prä-existierender IFN-AABs eine schwere COVID-19 Erkrankung zu entwickeln. Entgegen unserer Hypothese berichteten Patienten in unserer Kohorte, die eine Infektion mit SARS-CoV-2 berichteten, von einer leichten Erkrankung, die eine unvollständige klinische Penetranz von IFN-AABs in Bezug auf schwerem COVID-19 zeigte. Zweitens führten wir eine groß angelegte Querschnittsstudie mit mehreren Kohorten (2) von hospitalisierten Patienten mit COVID-19 durch. Hier zielten wir darauf ab, klinische Parameter zu identifizieren, die mit neutralisierenden IFN-AABs assoziieren. Wir schlagen einen neuartigen klinischen Algorithmus zur schnellen

Identifizierung neutralisierender IFN-AAB-positiver Patienten vor, die von spezifischen alternativen Therapien profitieren können.

1 Introduction

1.1 SARS-CoV-2 and COVID-19

1.1.1 Emergence and Spread of SARS-CoV-2

Severe-acute-respiratory syndrome coronavirus 2 (SARS-CoV-2), first identified in an outbreak in Wuhan, China in December 2019 (3), is the causative agent of coronavirus disease 2019 (COVID-19) and has spread worldwide. As of December 2022, more than 640 million people have been infected with SARS-CoV-2, of which 6.6 million died due to COVID-19-related diseases since the beginning of the pandemic (4).
Respiratory droplet exposure and droplet transmission from presymptomatic or symptomatic persons who are carrying the virus is the primary mode of SARS-CoV-2 transmission (5). After a transmission event, the initial viral replication occurs in the upper airways (6). Common symptoms of a mild infection are fever, cough, shortness of breath and fatigue. Upon a lack of viral clearance by immune responses, infection can spread to the lower respiratory tract, likely by pharyngeal secretions, where SARS-CoV-2 primarily infects alveolar type II cells (7), responsible for pulmonary surfactant production. Infection in the alveoli and the lower respiratory tract can cause functional damage and progression to severe disease (6). While most of the SARS-CoV-2-exposed patients remain asymptomatic or develop mild to moderate symptoms, some develop severe to critical disease with progression to pneumonia (8). The symptoms of severe to critical disease include dyspnea (shortness of breath), hypoxaemia, systemic inflammation and acute respiratory distress syndrome that can lead to a fatal clinical outcome (9). In order to prevent the high hospitalization and mortality rate due to COVID-19-related diseases, multiple effective vaccines were developed through global scientific efforts (10). Vaccination reduced the frequency of SARS-CoV-2 transmission events (11) and lowered the disease severity of infected individuals (12). Thus, the hospitalization and mortality rates declined after the administration of vaccines (13). However, mutations of SARS-CoV-2 resulted in the emergence of new variants such as alpha (B.1.1.7), beta (B.1.351), delta (B.1.617.2) and omicron (B.1.1.529) (14). Although vaccines are highly beneficial to control SARS-CoV-2 infection, emerging variants of SARS-CoV-2, termed variants of concern (VOCs), pose a

threat because of their potentially enhanced virulence, transmissibility and immune evasion (15-17). Therefore, the need to develop a better understanding of SARS-CoV-2 pathogenicity to control the COVID-19 pandemic is ongoing.

1.1.2 SARS-CoV-2 Organization and Replication Cycle

SARS-CoV-2 belongs to *betacoronavirus* genus of *Coronaviridae* family and is an enveloped virus with a single-stranded, positive-sense RNA of 29.9 kB length (18). The virion consists of the ssRNA viral genome and the structural proteins: nucleocapsid (N), membrane (M), envelope (E) and spike (S) (19) (**Figure 1A**). The viral genome encodes for non-structural, structural and accessory proteins (**Figure 1B**). SARS-CoV-2 initially targets ciliated epithelial cells in the sinonasal airway (6, 9, 20, 21) and enters them by hijacking Angiotensin-converting enzyme 2 (ACE2) receptors using the Spike (S) glycoprotein for attachment (22, 23). After ACE2 engagement of the S1 subunit of spike, S2' cleavage site of S2 subunit becomes exposed. The cleavage of S2' site by transmembrane protease serine 2 at the plasma membrane or by cathepsin L in the endosomal compartment allows the initiation of membrane fusion (23, 24). Following entry of SARS-CoV-2 into the cell through receptor-mediated membrane fusion or endocytosis, the viral genome is released into the cytoplasm (25). The polyproteins pp1a and pp1ab are translated and subsequently cleaved by virus-encoded proteases that leads to the formation of functional non-structural proteins (Nsps) (25, 26). The host shutoff factor Nsp1 is one of the first translated proteins and interferes with the host mRNA translation by degradation of host mRNAs (27, 28). Nsp2-16 generate essential enzymes for RNA synthesis and form the viral replication-transcription complex (RTC) (29, 30). Meanwhile, Nsp3, Nsp4 and Nsp6 generate ER-derived double membrane vesicles (DMVs) (31, 32) that provide a protected environment for viral replication, shielded from host immune responses. Here, viral genome replication as well as subgenomic RNA transcription (sgRNAs) take place (33). After replication, viral RNA exits DMVs through pores created by Nsp3 for its incorporation into virions (34). The sgRNAs are translated into structural and accessory proteins. E and M proteins regulate the intracellular trafficking and processing of S (35). The structural proteins M, E and S are then transferred to ER-Golgi intermediate compartment (ERGIC) where they assemble together with the viral genome (35). N proteins encapsulate the RNA genome and generated viral particles are released from the cell through exocytosis (36, 37).

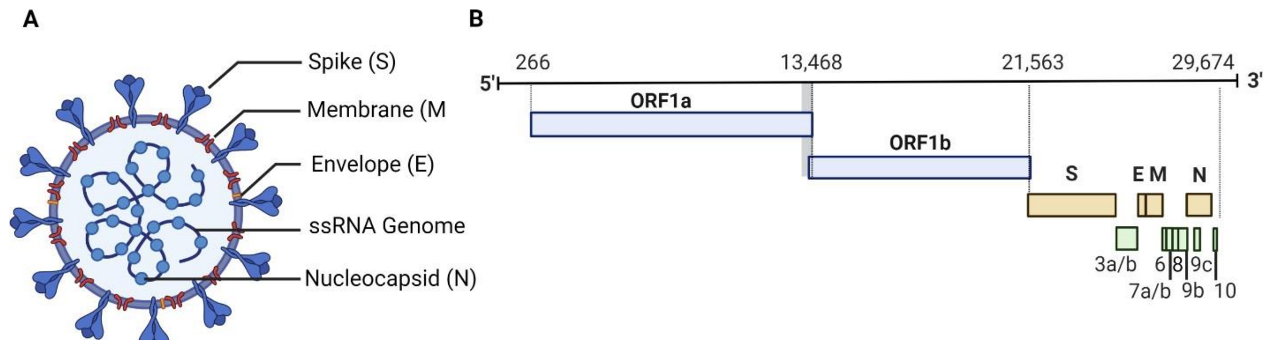

Figure 1. SARS-CoV-2 particle and genomic organization.

A. Schematic representation of SARS-CoV-2. Virion consists of a positive sense single-stranded RNA genome coated with nucleocapsid (N), enclosed by a lipid membrane of host cell origin with structural proteins spike (S), membrane (M) and envelope (E) embedded in. **B.** Genomic organization of SARS-CoV-2. The viral genome of SARS-CoV-2 has a length of about 29.9 kB and consists of ORF1a and ORF1b encoding for the 16 non-structural proteins, S, E, M, N encoding for the structural proteins, ORF3a/b, ORF6, ORF7a/b, ORF8, ORF9b/c and ORF10 (38, 39) encoding for the accessory proteins (40). Created with BioRender.com. (Source: own figure).

1.1.3 Factors Associated with Severe COVID-19

According to COVID-19 disease severity classification by WHO, disease severity classes are listed as mild, moderate, severe and critical (41). Mild cases without pneumonia or hypoxia are classified as mild disease and cases requiring hospitalization with non-severe pneumonia without a requirement of supplemental oxygen as moderate. Patients are classified to have severe disease if they suffer from severe pneumonia and need supplemental oxygen whereas patients that have acute respiratory distress syndrome, sepsis, septic shock or acute thrombosis are categorized as critically ill. Advanced age, male sex, and pre-existing comorbidities are among the clinical and demographic risk factors that contribute to the progression of COVID-19 towards a severe clinical outcome. One of the major risk factors, age, raises mortality by 7.4% per age year (42). Another major risk factor, male sex, is overrepresented among patients with critical disease and 60% of COVID-19 related deaths are reported in men (43). Both higher age and male sex are associated with weaker innate and adaptive immune responses in the course of viral infections in general (43) that might contribute to a worse clinical outcome in those groups.

Innate immune cells, such as monocytes, macrophages, and dendritic cells (DCs) are generally higher in number and more active in females than in males (44). Female cellular mosaicism due to X chromosome inactivation results in additional physiological diversity in females that can provide an immune advantage (45). Females display stronger type I IFN production by plasmacytoid dendritic cells (pDCs) (46), as well as having other immunological variations in non-classical monocyte and T cell activation. At baseline, female COVID-19 patients exhibit a stronger T cell response than male patients, with considerably higher number of activated CD8 T cells compared to male patients. The decline of T cell response also correlates with age in male but not in female patients. Moreover, number of pDCs and their ability to produce type I IFN decline with age as well as the ability of monocyte/macrophages to develop a protective immune response, including activation of NK, T, and B cells (47).

1.1.4 Treatment of Patients with Severe COVID-19

Individuals with SARS-CoV-2 infection who are at high risk of developing severe COVID-19 may be advised to receive treatment with antiviral drugs and monoclonal antibodies in the early phase of infection. Antiviral therapies are employed to prevent viral replication at different stages of the viral replication cycle through various mechanisms such as inhibiting viral entry, the activity of viral proteases or RNA-dependent RNA-polymerase. Antivirals used for the treatment of COVID-19 include Paxlovid (protease inhibitor) (48, 49) and Remdesivir (RNA-dependent RNA-polymerase inhibitor) (50, 51). Alternatively, anti-SARS-CoV-2 monoclonal antibodies targeting the spike protein, such as Bebtelovimab have been shown to provide clinical benefits in treatment of COVID-19 (52, 53). However, emergence of new variants pose a risk to the efficacy of monoclonal antibodies.

1.2 Type I Interferon

1.2.1 Type I Interferon Signaling

Type I IFN in humans consists of 13 IFN-α genes as well as single genes for IFN-β, IFN-ε, IFN-κ, and IFN-ω. The heterodimeric receptor complex made up of a single chain of interferon alpha/beta receptor 1 and 2 (IFNAR1 and IFNAR2) is shared by all 17 type I IFNs and is present on almost all nucleated cells (54).

During numerous types of viral infections, type I IFNs are crucial to mediating immune responses by induction of IFN-stimulated genes (ISGs) and establishment of an antiviral state in host cells. They contribute to both innate immunity and cell-intrinsic immunity. When type I IFNs bind to their corresponding receptors IFNAR1 and IFNAR2 on the cell surface, individual receptor chains heterodimerize (54, 55). By that, intracellular receptor-associated tyrosine kinases of Janus kinase (JAK) family are activated. Activation of the JAKs (JAK1 and TYK2) lead to the tyrosine phosphorylation of STAT2 (signal transducer and activator of transcription 2) and STAT1. This gives rise to the formation of a hetero-trimeric complex of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) that is also called IFN-stimulated gene factor 3 (ISGF3). By translocation of ISGF3 to the nucleus and its binding to the IFN-stimulated response elements (ISREs) that are promoters of ISGs, transcription of various ISGs are initiated. Alternatively, type I IFNs can induce the formation of STAT1 homodimers that directly translocate to the nucleus and bind to GAS (IFN-γ-activated site) elements to initiate ISG transcription (**Figure 2**).

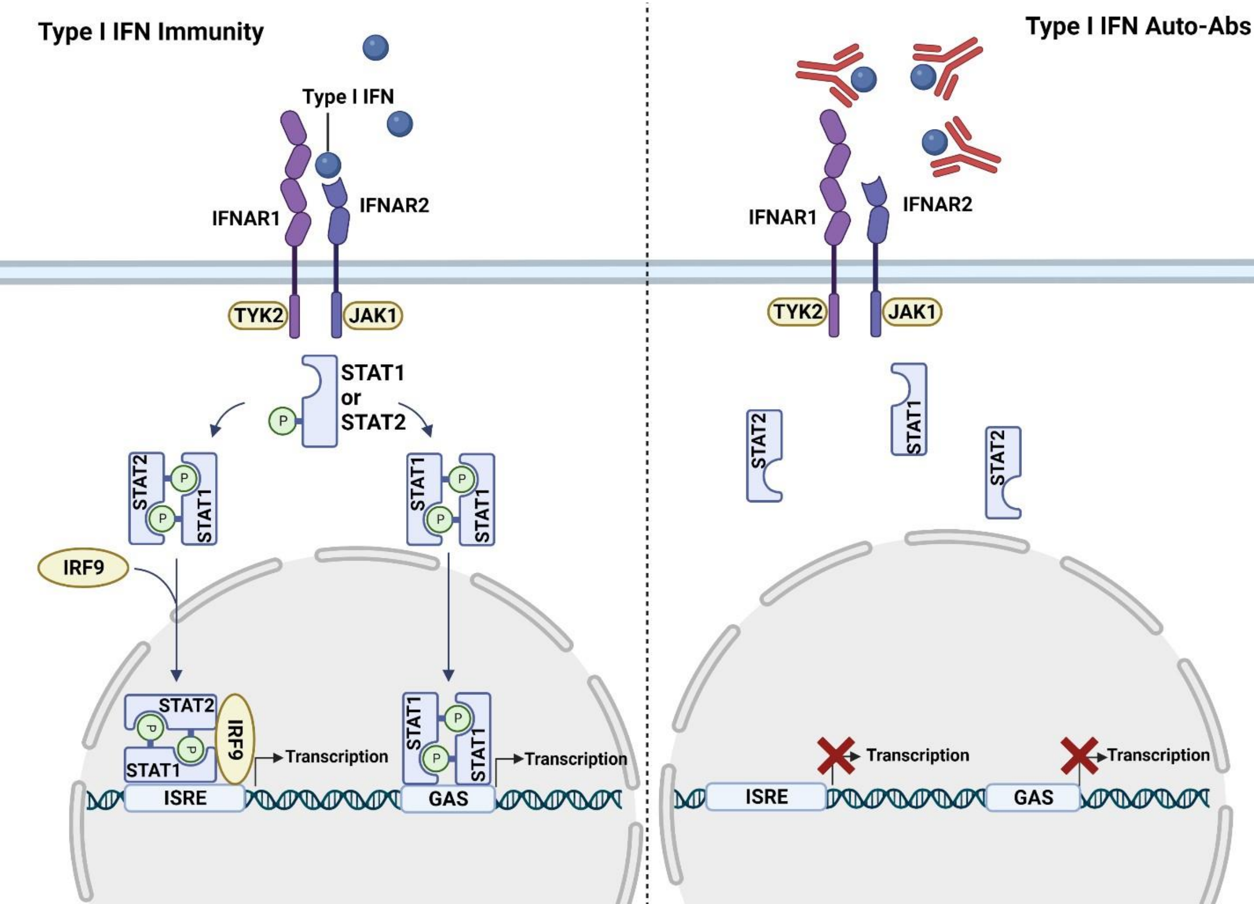

Figure 2. Type I IFN signaling and its inhibition by type I IFN-AABs.

Binding of IFN-α or IFN-ω to IFNAR receptors activate type I IFN signaling. Through activation of JAK-STAT pathway, ISREs initiate the transcription of ISGs. In the presence of neutralizing type I IFN-AABs, binding of IFNs to IFNAR receptors is prevented and this results in a lack of transcriptional activation of ISGs. Created with BioRender.com. (Source: own figure).

1.2.2 Role of Type I IFN in SARS-CoV-2 Infection

During an infection with SARS-CoV-2, viral RNA is sensed by melanoma differentiation-associated gene 5 (MDA5) and RNA helicase retinoic acid-inducible gene I (RIG-I) in the cytosol and activate mitochondrial antiviral signaling protein (MAVS) in respiratory epithelial cells (56). While sensing of viral RNA can occur by TLR3 in the endosomal receptors of respiratory epithelial cells activating IRF3, sensing by TLR7 in the endosomal receptors of plasmacytoid dendritic cells activates IRF7 via myeloid differentiation primary response 88 (MyD88). IFN-regulatory factors 3 and 7 (IRF3, IRF7), as well as NF-κB, are stimulated by the activation of these three pathways, resulting in the production of type I IFNs. Compared to some other viruses including influenza A viruses (57) and three epidemic coronaviruses, induction of type I IFNs by SARS-CoV-2 is weaker (58-61), possibly giving rise to its increased pathogenicity.

Strikingly, almost half of the viral proteins encoded by SARS-CoV-2 inhibit type I IFN signaling by targeting different proteins involved in the induction of type I IFNs. For example, ORF6 was found to inhibit type I IFN expression and stimulation of ISGs via blocking IRF3 and ISG3F nuclear translocation (58, 61-64). Nsp1 inhibits IFN signaling via blocking IRF3 phosphorylation and inducing depletion of downstream factors Tyk2 and STAT2 (65). Furthermore, Nsp6 and Nsp13 suppress IRF3 and TBK1 phosphorylation by binding TBK1 (61). ORF3a, M, ORF7a, ORF7b interfere with IFN signaling by blocking STAT1 or STAT2 phosphorylation (66). *In vitro*, pre-treatment as well as post-treatment with type I IFNs substantially attenuated SARS-CoV-2 (67, 68). The high sensitivity of SARS-CoV-2 to type I IFNs *in vitro* and the fact that so many SARS-CoV-2 viral proteins target and disrupt type I IFN signaling further supports the idea that type I IFNs play a significant role in the anti-SARS-CoV-2 immune response.

While an impaired IFN signaling at the early stages may result in severe or critical COVID-19, delayed and robust type I IFN responses at the later stages were also reported to cause severe COVID-19 due to hyperinflammation, underlining the importance of a finely balanced and timely IFN response during the course of a SARS-CoV-2 infection (69). At

the early stage of infection, IFN response was high in patients with mild to moderate disease whereas patients with severe disease had a highly impaired IFN response with a lack of IFN-β production and low IFN-α production (70). Inborn mutations causing defective type I IFN signaling (71, 72) and neutralizing type I IFN-AABs (73) were found to predispose patients to severe COVID-19. Relative to asymptomatic and mild cases, patients with life-threatening COVID-19 pneumonia had an enrichment in rare genetic variants in genes including *TLR3* (Toll-like receptor 3), *TRIF* (TIR domain-containing adaptor inducing IFN-beta), *UNC93B*, *TBK1* (TANK-Binding Kinase 1), *IRF3/7* (interferon regulatory factor 3 and 7) and *IFNAR1/2* which are all regulators of type I and III IFN signaling (72, 74). Moreover, X-linked recessive TLR7 deficiency was identified as a genetic risk factor for COVID-19 pneumonia in men (71).

1.2.3 IFN-AABs in Autoimmune Disorders

Neutralizing AABs against type I IFN are found in patients with autoimmune disorders such as autoimmune polyendocrine syndrome type-I (APS-1) (75), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), and systemic lupus erythematosus (SLE) (76). APS-1 is an inherited disease caused by mutations in the AIRE (gene encoding the protein autoimmune regulator) (77-79). Symptoms of APS-1 include autoimmunity in endocrine and non-endocrine organs, mucocutaneous candidiasis, hypoparathyroidism, Addison disease and enamel hypoplasia (80, 81). Almost every patient with APS-1 produce high titers of autoantibodies against type I IFNs (IFN-α and IFN-ω) from early childhood (75). Some of the patients also produce AABs against IFN-β and type III IFNs, although at lower titers. Although type I IFNs are potent antivirals and key players of innate and cell-intrinsic immunity, type I IFN-AABs were thought to be largely clinically silent and their contribution to viral diseases were not well studied until the emergence of COVID-19 (73, 82, 83). In the presence of neutralizing type I IFN-AABs, neutralization of IFNs blocks IFN signaling and ISGs are not activated (**Figure 2**), leading to interference with and dampening of the innate and cell-intrinsic immune responses.

1.3 Current State of the Research in the Field and Importance of this Study

1.3.1 State of the Art

In late 2020, a study involving 987 patients with severe COVID-19 showed that neutralizing autoantibodies against type I IFN were detected in 10% of patients with life-threatening COVID-19 pneumonia while they were absent in patients with asymptomatic or mild SARS-CoV-2 infection (73). Meanwhile, in the general population below the age of 70, IFN-AABs were found only in 0.17% of individuals. The prevalence of AABs showed an increase with age, reaching above 4% over the age of 70 (84).

Further studies confirmed the contribution of IFN-AABs to COVID-19 disease severity, reporting presence of IFN-AABs in 6-17% of COVID-19 patients with severe pneumonia (73, 85, 86) and in 11-19% COVID-19 patients with critical disease (73, 87, 88). Analysis of the sera from two patients showed that the AABs were present prior to an infection with SARS-CoV-2 (73).

Several case reports demonstrated patients with APS-1 developing severe COVID-19 after SARS-CoV-2 infection (73, 82, 83, 89), arguing for an increased susceptibility of these patients to severe COVID-19. The largest scale study so far has involved 22 patients with APS-1, of which 15 developed critical COVID-19. Overall, these studies showed a strong link between the presence of IFN-AABs and COVID-19 disease severity.

1.3.2 Importance

Type I IFN-AABs were long thought to be clinically silent and their role in viral illnesses was not thoroughly researched until the advent of COVID-19, despite the fact that type I IFNs are effective antiviral effectors and essential components of innate and cell-intrinsic immunity. Although several studies reported severe COVID-19 in patients with APS-1 in preselected cohorts, there have been no prospective studies on patients with APS-1 to date. Therefore, the link between the presence of IFN-AABs in patients with APS-1 and progression to severe COVID-19 remains to be elucidated.

Initially, we implemented a prospective study on a small cohort of patients with APS-1. During the course of our study, some of those patients contracted SARS-CoV-2. Our first aim was to determine if the IFN-AABs in those patients have neutralizing capacity. Secondly, we investigated the clinical penetrance of preexisting neutralizing autoantibodies

against type I IFNs for severe COVID-19. Understanding the effect of IFN-AABs to disease progression and the clinical penetrance is crucial for choosing suitable, targeted therapies for patients with APS-1 that contract SARS-CoV-2.

Despite several studies suggesting the contribution of IFN-AABs to severe disease progression of COVID-19, there are no clinical screenings or determined alternative therapies for these patients, who may greatly benefit from a more tailored diagnosis and treatment protocol.

In the second study, we hypothesized that IFN-AABs can serve as a biomarker for rapid identification of patients at risk of developing severe COVID-19. Furthermore, using clinical parameters and assays for detection of neutralizing type I IFN-AABs, we aimed to develop a clinical algorithm for early and rapid identification of patients with IFN-AABs. Alternative specific treatment strategies for the patients with type I IFN-AABs, that have a higher likelihood of developing severe COVID-19, might help to decrease the disease severity and mortality rate of this patient group.

2 Methods

2.1 Materials

2.1.1 Patient Cohorts

Table 1. Patient Cohorts (Source: own table)

<table>
<tr><th colspan="2">Cohort</th><th>Description</th></tr>
<tr><td colspan="2">APS-1</td><td>Patients with autoimmune polyendocrine syndrome type I described previously (Meisel et al., 2021)</td></tr>
<tr><td rowspan="3">Cross-sectional Cohort (CSC)</td><td>Cohort A</td><td>Patients with a max WHO score 3-8 from the Pa-COVID-19 cohort at Charité Universitaetsmedizin Berlin, Germany</td></tr>
<tr><td>Cohort B</td><td>Patients with COVID-19 with a max WHO score 3-8 from Inselspital Universitätsspital Bern, Switzerland</td></tr>
<tr><td>Cohort C</td><td>Patients with COVID-19 with a max WHO score 3-8 from Universitätsklinikum Freiburg, Germany</td></tr>
<tr><td colspan="2">Therapeutic Plasma Exchange Cohort (TPEC) = Cohort D</td><td>Patients who underwent therapeutic plasma exchange for treatment of COVID-19-associated hyperinflammatory syndrome at the Department of Internal Medicine IV of Heidelberg University Hospital, Germany (max WHO score 7-8)</td></tr>
<tr><td colspan="2">Healthy Cohort</td><td>Healthy controls recruited from a study (Covimmun) on SARS-CoV-2 exposition in healthcare workers</td></tr>
</table>

2.1.2 Cell lines

Table 2. Cell lines (Source: own table)

Cell line	Description
Calu-3 (HTB-55™)	cell line derived from human lung epithelial cells of a patient with lung adenocarcinoma (obtained from ATCC)
Vero E6 (CRL-1586™)	cell line derived from African green monkey kidney cells, exhibiting epithelial morphology (obtained from ATCC)

2.1.3 Nucleic Acids

Primers and probe used in SARS-CoV-2 RT-PCR were previously described (Corman et al. 2020).

Table 3. Primers (Source: own table)

Primers	Sequence
E Gene F primer	ACAGGTACGTTAATAGTTAATAGCGT
E Gene Probe	FAM-ACAC-TAGCCATCCTTACTGCGCTTCG-BBQ
E Gene R primer	ATATTGCAGCAGTACGCACACA

2.1.4 Proteins

Table 4. Proteins (Source: own table)

Protein	Manufacturer
Recombinant Human IFN-ω	PeproTech
Roferon®-A (IFN α-2a)	Roche

2.1.5 Media

Table 5. Media (Source: own table)

Medium	Composition
2xDMEM Medium	DMEM, high glucose supplemented with 20% fetal bovine serum, 200 U/ml penicillin, 200 mg/ml streptomycin, and 4 mM L-glutamine
DMEM Medium	DMEM, high glucose supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine
Freezing Medium	DMEM, high glucose, supplemented with 30% FCS and 10% DMSO, sterile filtered through a 0.45 μm filter
Overlay Medium	2.4 % Avicel diluted 1:2 in 2x DMEM

2.1.6 Kits

Table 6. Kits (Source: own table)

Kit	Manufacturer
MagNa Pure RNA Extraction Kit	Roche
Superscript III OneStep RT-PCR kit	Invitrogen

2.1.7 Reagents

Table 7. Reagents (Source: own table)

Reagent	Manufacturer
0.5% Trypsin-EDTA (10x)	Thermo Fisher Scientific

Avicel	Sigma-Aldrich
Crystal Violet	Sigma-Aldrich
DMEM, high glucose	Sigma-Aldrich
DMEM, high glucose, powder	Biological Industries
DPBS	Sigma-Aldrich
Ethanol	Roth
Fetal Bovine Serum (FBS)	Sigma-Aldrich
Formaldehyde	Roth
Gelatine powder	VWR
L-Glutamine	Thermo Fisher Scientific
Opti-PRO SFM	Thermo Fisher Scientific
PCR primers and probe	Biomers
Penicillin-Streptomycin	Thermo Fisher Scientific

2.1.8 Buffers and Solutions

Table 8. Buffers and Solutions (Source: own table)

Buffers and solutions	**Composition**
Crystal Violet staining solution	0.2% crystal violet, 2% ethanol and 10% formaldehyde

2.1.9 Consumables

Table 9. Consumables (Source: own table)

Consumable	**Manufacturer**
Cell counting slides	Bio-Rad
Cell culture flasks	Sarstedt
Cell culture well plates	Sarstedt

Combitips Advanced, 10 ml	Eppendorf
Dispenser Tips, 12,5ml	BRAND
Falcons	Sarstedt
Filter, 0.45µm	Thermo Scientific
Pipette tips	Starlab
Reaction tubes	Sarstedt
Reagent reservoir	Roth
RNA extraction plates (MagNa Pure Kit)	Roche
RT-PCR plates and seals	4-titude
Serological pipettes	Sarstedt

2.1.10 Equipment

Table 10. Equipment (Source: own table)

Equipment	**Manufacturer**
Bio-Rad TC20™ Automated Cell Counter	Bio-Rad
Heracell VIOS 160i Incubator	Thermo Fisher
Heraeus Megafuge 1.0 Centrifuge	Thermo Scientific
Incubator	Binder GmbH
Laminar Flow Hood	Thermo Fisher
LightCycler® 480 Real-Time PCR System	Roche
MagNa Pure 96 instrument	Roche
Microcentrifuge, Eppendorf 5430	Eppendorf
Olympus CK2 Microscope	Olympus
Primovert Microscope	ZEISS
Scales	Sartorius

Thermal Mixer	Eppendorf
VACUSAFE Suction Pump	Integra Biosciences
Vortex-mixer	VWR

2.1.11 Software

Table 11. Softwares (Source: own table)

Software	**Manufacturer**
GraphPad Prism	GraphPad
LightCycler® 480 Real-Time PCR System	Roche
Microsoft Office	Microsoft

2.2 Methods

2.2.1 Cell lines, Interferon Treatment and Viral Infection

2.2.1.1 Culture of Calu-3 and Vero E6 cells

Calu-3 and Vero E6 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma Aldrich), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine at 37 °C and 5% CO_2.

2.2.1.2 Treatment of Calu-3 cells with patient serum and IFN

For the IFN neutralization assay, Calu-3 cells were seeded in 96-well plates at a concentration of 6x10^5 cells per ml. Cells were pre-incubated with dilutions of patient serum ranging from 1:100 to 1:100000 in the presence or absence of 200-400 IU/ml IFN-α2a (Roferon®-A, Roche) or 20-50 ng/ml IFN-ω (PeproTech).

2.2.1.3 SARS-CoV-2 infection of Calu-3 cells

For infection of Calu-3 cells, a B.1 lineage SARS-CoV-2 isolate (SARS-CoV-2/human/DEU/BavPat2-ChVir984-ChVir1017/2020, NCBI GenBank Acc. No. MT270112.1) propagated in Vero E6 cells was used after verification of sequence integrity by next-

generation sequencing. One day after the treatment with IFN-α2a/IFN-ω and serum, medium was removed and cells were infected with SARS-CoV-2 at MOI 0.01. After one hour of incubation, virus inoculum was removed, cells were washed with PBS and cultured in 100µl of medium. 24 hours after infection, supernatant was collected for the quantification of viral RNA and determination of infectious virus titer. Enhanced respiratory personal protection equipment were used for all SARS-CoV-2 infection experiments under biosafety level-3 (BSL-3) conditions.

2.2.2 Quantification of viral RNA

2.2.2.1 Viral RNA harvest

For the extraction of viral RNA, 50 µl of cell culture supernatant was added into 300 µl of MagNa Pure external lysis buffer (Roche, Penzberg, Germany). Heat inactivation was performed by incubation at 70°C for 10 min. MagNa Pure 96 instrument (Roche) was used for automated pipetting for the extraction of viral RNA.

2.2.2.2 qRT-PCR

Real-time RT-PCR assay was employed to quantify SARS-CoV-2 RNA concentrations. SARS-CoV-2 E gene was targeted using the primers (Corman et al., 2020):

E_Sarbeco_F: ACAGGTACGTTAATAGTTAATAGCGT;

E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ;

E_Sarbeco_R: ATATTGCAGCAGTACGCACACA.

Superscript III OneStep RT-PCR kit (Invitrogen, Darmstadt, Germany) was used for real time RT-PCR. Reactions were conducted in 12.5 µl volume using 6.25 µl of 2x reaction buffer, 0.25 µl of 10 µM probe, 0.5 µl of 10 µM forward and reverse primers, 0.5 µl of SSIII / P. Taq enzyme mix, RNase-free water, and 2.5 µl of RNA template. Following reverse transcription at 55°C for 10 min, initial denaturation was performed at 95°C for 180 s. Afterwards, cycling and fluorescence signal acquisition was performed for 45 cycles of 95°C for 15 s and 58°C for 30 s. Experiment and data processing was performed using the LightCycler® 480 Real-Time PCR System (Roche). SARS-CoV-2-specific *in vitro*-transcribed RNA standards (Corman et al., 2020) were used for absolute quantification.

2.2.3 Quantification of viral infectivity

2.2.3.1 Infection of Vero E6 cells with virus-containing supernatant of Calu-3 cells

50 µl of cell culture supernatant was added onto 50 µl of 0.5% gelatin in Opti-Pro^{TM} to store infectious virus particles prior to infection of Vero E6 cells. Vero E6 cells were seeded in 24-wells at a density of 3.5x10^5 cells/ml. Infection was performed with serial dilutions of Calu-3 cell culture supernatants at 37°C for one hour. Virus inoculum was then removed, cells were washed with PBS and cells were cultured in overlay medium containing 2.4 % Avicel diluted 1:2 in 2x DMEM supplemented with 20 % fetal bovine serum, 200 U/ml penicillin, 200 mg/ml streptomycin, and 4 mM L-glutamine.

2.2.3.2 Staining and counting of plaques

Three days after infection, overlay medium was removed and Vero E6 cells were fixed with 6% formaldehyde, washed with PBS, followed by staining with 0.2% crystal violet, 2% ethanol and 10% formaldehyde-containing solution. Plaques were counted using the dilutions where distinct plaques were detectable and plaque forming units per ml (PFU/ml) were determined.

2.2.4 Statistical Analysis

Statistical analysis was conducted with GraphPad Prism, using Pearson’s correlation analysis.

3. Results

In this chapter, I describe the results obtained in both publications (1, 2) with a focus on the assays conducted by myself, stated in detail in the attached declaration of authorship contributions. Figures and tables mentioned in this section are referring to the original publications.

3.1 Mild COVID-19 despite AABs against type I IFNs in autoimmune polyendocrine syndrome type 1

3.1.1 Type-I IFN AABs in Sera of Patients with APS-1 Abolishes the Type-I IFN Mediated Inhibition of SARS-CoV-2 Infection

Patients with autoimmune polyendocrine syndrome type I produce AABs against type I IFNs, including IFN-α2a and IFN-ω. Recent studies had shown that patients with IFN-AABs are predisposed to severe COVID-19. Here, a cohort of six patients with APS-1 was examined for the presence of neutralizing IFN-AABs in sera. Our cohort consisted of children and young adults aged between 13 and 25 and was predominantly female (5 out of 6 patients). Initially, sera were analyzed for IFN-AABs by an ELISA-based assay in Labor Berlin (1) which confirmed the presence of high quantities of IFN-AABs in every patient. Afterwards, a SARS-CoV-2 infection based IFN neutralization assay was performed to assess the neutralization activity of the AABs.

Therefore, Calu-3 cells were treated with 1% patient serum in the presence or absence of 200 IU/ml IFN-α2a, and 5ng/ml IFN-ω, respectively, to test the IFN neutralization by IFN-AABs. After treatment, Calu-3 were infected with SARS-CoV-2 at MOI 0.01 for an hour and samples were harvested one day later to detect viral RNA in the supernatant and formation of plaques in Vero E6 cells.

Assessment of viral RNA detected in the supernatant showed an efficient infection of Calu-3 cells with SARS-CoV-2 in the absence of IFNs or patient serum (**Figure 2A, B)**. Pre-treatment with IFN-α2a and IFN-ω in the absence of patient serum or in the presence of healthy control serum showed a decrease in the viral RNA quantity detected in the supernatant, indicating inhibition of viral replication by IFN-α2a and IFN-ω. Meanwhile, cells that were treated with patient serum were efficiently infected by SARS-CoV-2 even in the presence of IFN-α2a and IFN-ω, reaching the same level of viral RNA as cells that

were infected without any IFN treatment, demonstrating an effective neutralization of IFNs by the AABs.

Secondly, supernatant of Calu-3 cells were titrated on Vero E6 cells for a further assessment of IFN neutralization. Here, an efficient virus production was observed by the titration of supernatant produced by the Calu-3 cells in the absence of IFNs or patient serum (**Fig. 2D, E, Sup. Fig. 3A, B**). Treatment with IFN-α2a or IFN-ω decreased the virus production in Vero E6 cells by almost 100-fold. In contrast, abundant virus production occured when Calu-3 cells were treated with patient serum even in the presence of IFN-α2a and IFN-ω, reaching similar levels as the conditions without IFN (**Fig. 2D, E**), further confirming the IFN neutralization by IFN AABs in patient sera. Cells that were treated with patient sera in the absence of IFNs as a control did not display any differences in the viral RNA levels or plaque formation, as expected (**Fig 2C, F**).

3.1.2 IFN Neutralization by Type-I IFN AABs is Concentration-Dependent

Next, we tested if the IFN-AABs in higher dilutions of sera still have neutralization capacity against IFN-α2a and IFN-ω to check if IFN neutralization is concentration-dependent. Calu-3 cells were treated with titrations of patient serum ranging between 1% and 0.001% in ten-fold dilution steps. Neutralization capacity was observed to be concentration-dependent. Cells treated with higher dilutions of patient serum showed a loss of IFN neutralization, reflected both by the decrease in viral RNA in the supernatant and plaque formation (**Sup. Fig. 2**). For most of the patient sera, 1% and 0.1% dilutions maintained the capacity to neutralize IFN-α2a and IFN-ω whereas the ability to neutralize was diminished or completely lost at 0.01% or 0.001%. The serum from patient 1, which maintained the ability to neutralize IFN-α2a even at the lowest concentration, 0.001%, was the serum with the highest concentration of AABs against IFN-α2a, demonstrating a correlation between the AAB concentration and neutralization capacity.

3.1.3 Patients with APS-1 Developed Mild COVID-19 Despite Neutralizing Type I IFN AABs

As described in detail in the original publication, four of the six patients in the cohort contracted SARS-CoV-2 during our study. All the patients that reported SARS-CoV-2 infection were female and their age range was 13-25. Patients reported fever, headache, cough, fatigue, rhinitis and flu-like symptoms. None of the patients developed dyspnea or

oxygen requirement and they were not hospitalized. Their symptoms remained mild and resolved within one-two weeks. Following the required isolation period, serology was performed and all samples were seropositive for SARS-CoV-2 antibodies (**Table 2**). These results showed that the presence of neutralizing IFN-AABs does not necessarily cause progression to severe COVID-19 and hint that there might be additional factors that contribute to COVID-19 severity in patients with neutralizing IFN-AABs.

3.2 Early and Rapid Identification of COVID-19 Patients with Neutralizing Type I IFN-AABs

In the second study, a total of 430 sera from four different cohorts of patients infected with SARS-CoV-2 were analyzed side by side with sera from a healthy cohort (**Table 1**). Cohorts A, B, C, (CSC, cross-sectional cohorts) were composed of patients with a maximum WHO score of 3-8. Cohort D (TPEC, therapeutic plasma exchange cohort) was composed of patients with a maximum WHO score of 7-8 who underwent therapeutic plasma exchange to treat COVID-19-associated hyperinflammation. Samples were first tested for the presence of IFN-AABs by an ELISA-based assay and for the binding specificity by a competition assay at Labor Berlin (2). For ELISA, Streptavidin plates were coated with biotinylated IFN-α2 or IFN-ω followed by incubation with serum. Afterwards, the plate was incubated with a SULFO-TAG labelled human IgG antibody to bind IFN-AABs and light signal counts (LSC) were measured by electrochemiluminescence detection (2). Although these assays are quite sensitive, there might be unspecific binding of autoantibodies in the sera to the Streptavidin plate or against proteins in the blocking agent (90). Competition assay was performed to confirm binding specificity and rule out false-positives that might be generated by the ELISA-based assay. For this, patient serum was pre-incubated with unbiotinylated IFN-α2 or IFN-ω before performing ELISA. Samples that showed a four-fold or more reduction in LSC compared to ELISA without pre-incubation scored specific. We used the 97.5th percentile of the LSC readout in our cohort of uninfected individuals to determine our ELISA cut-off line. All samples containing AABs against IFN-α2a and IFN-ω above the cut-off line in the ELISA and 102 IFN-α-AAB- and 106 IFN-ω-AAB ELISA-negative sera were further assessed for IFN neutralization in SARS-CoV-2 infection-based neutralization assay as described above.

3.2.1 Neutralizing AABs against Type I IFN are Present in 3.2% and 18.5 % of the Sera of Patients from Cross-Sectional Cohorts and Cohort D, respectively

Analysis of IFN neutralization by quantification of viral RNA in the supernatant and infectivity of the released viral particles (as described above) revealed that most of the sera with specific AABs against IFN-α2a and IFN-ω neutralized IFN-α and IFN-ω (**Sup. Fig. 1**). In total, 3.2% (13/403) and 2% (8/403) of the sera within the cross-sectional cohort (CSC) showed presence of neutralizing autoantibodies against IFN-α2a and IFN-ω, respectively **(Figure 2A, B)**. Next, sera of cohort D, composed of patients that underwent TPE, and had a max WHO score of 7-8, were analyzed for the neutralization capacity of the AABs. Here, 18.5% (5/27) and 11.1% (3/27) of the sera neutralized IFN-α2a and IFN-ω, respectively. The higher percentage of IFN-AAB-positive patients within cohort D compared to CSC was expected since cohort D is composed of patients with a higher max WHO score and previous reports showed higher rates of IFN-AAB positivity in critically ill patients. As expected, there were no neutralizing IFN-AABs detected in sera obtained from the healthy cohort.

3.2.2 Binding Specificity is a Good Indicator of Neutralizing Capacity of the Sera

Within all cohorts, 90% (18/20) and 83% (10/12) of the sera that displayed specific AABs against IFN-α2a and IFN-ω, respectively, neutralized the antiviral activity of type I IFNs in SARS-CoV-2 infection-based IFN neutralization assay that we consider as the gold standard for the analysis of IFN neutralization (**Figure 2C, D**). This displays that samples positive for specificity are highly likely to be IFN neutralizers. All the ELISA-positive samples that were unspecific were also non-neutralizers in the SARS-CoV-2-based infection assay. Among all sera tested, there were only two ELISA-negative sera (C024 and C078) that showed specific binding. They were negative in the standard IFN neutralization assay while they showed weak neutralization when tested using lower concentrations of IFN-α (**Sup. Fig. 2**). Neither of them was able to neutralize even lower concentrations of IFN-ω.

Conversely, we observed two samples that were negative in the IFN-ω-AAB ELISA that surprisingly showed neutralization capacity. Both sera had high titers of specific and neutralizing IFN-α-AAB, that might cross-react with IFN-ω. Apart from these few outliers, overall, these results suggest that the identification of specifically binding by IFN-AABs

by competition assay predicts IFN neutralization activity in the SARS-CoV-2 infection-based neutralization assay.

3.2.3 Presence of Neutralizing IFN-AABs is Associated with a Worse Clinical Outcome

While patients without neutralizing IFN-AABs developed a median max WHO score of 6 (IQR 4-7), patients with neutralizing IFN-AABs showed a median max WHO score of 8 (IQR 8-8) (**Figure 5A**). All the patients with neutralizing IFN-AABs presented fever compared to 62.6% of patients without IFN-AABs. The requirement of renal replacement therapy and/or ECMO was observed in a higher proportion of neutralizing IFN-AAB positive patients versus patients without neutralizing IFN-AABs (renal replacement therapy: 69.2%, 9/13 vs 26.6%, 98/369, ECMO: 46.2%, 6/13 vs 15.6%, 58/372). Furthermore, all patients with neutralizing IFN-AABs (13/13, 100%) required invasive mechanical ventilation (IMV) within 72 hours after hospital admission whereas 44.5% (166/372) of patients without neutralizing IFN-AABs (**Figure 5B**). In comparison to 19.1% (71/372) of patients without IFN-AABs, 92.3% (12/13) of the patients with neutralizing IFN-AABs died in hospital. The probability of survival 150 days post-symptom onset was significantly higher for patients without IFN-AABs (81.3%, 300/369) than patients with neutralizing IFN-AABs (7.7%, 1/13) (**Figure 5C**). Since this comparison did not account for the differences in disease severity, the higher disease severity in IFN-AAB positive patients might have been accountable for the lower probability of survival in these patients. In order to rule out the possibility that this effect is due to the differences in disease severity of these two groups, we compared the probability of survival of patients with and without neutralizing IFN-AABs only for the subgroup of critically ill patients with a max WHO score of 6-8. Despite similar disease severity, we observed a significantly higher probability of survival in patients without neutralizing IFN-AABs compared to the patients with neutralizing IFN-AABs within CSC (**Figure 6A**). In summary, presence of neutralizing IFN-AABs was associated with a worse clinical outcome.

3.2.4 Neutralizing Type I IFN-AABs are Present Early Post-Symptom Onset

Next, we analyzed sera collected early post-symptom onset as well as at the peak of the disease from patients in Cohort A and Cohort D (time points prior to TPE), in order to assess the temporal dynamics of IFN-AAB levels and their neutralization capacity. All the patient sera with neutralizing IFN-AABs at the peak of disease already showed presence

of IFN-AABs and neutralizing capacity early post-symptom onset (**Fig 4, Sup. Fig. 6**). Sera that tested negative for IFN-AABs at the peak of disease were also negative early post-symptom onset. These results are in line with the idea that the IFN-AABs are already present prior to infection, arguing against the induction of IFN-AABs by the infection.

3.2.5 Decrease of Serum IFN Autoantibody Levels by Therapeutic Plasma Exchange Resulted in a Loss of Neutralization Capacity

Five patients within cohort D (TPEC) that had neutralizing autoantibodies at the peak of the disease provided the chance to analyze the effect of therapeutic plasma exchange on the serum level of IFN-AABs. TPEC resulted in a decrease in the IFN-AAB levels and neutralization capacity in the sera of three (D011, D017 and D018) out of five patients involved (**Figure 6B**). Sera of all three patients positive for IFN-α2a autoantibodies showed a decrease in IFN-α2a autoantibody levels below the cut-off point of ELISA positivity and lost the ability to neutralize IFN-α. Similarly, for IFN-ω, sera of patients D017 and D018, that were positive for IFN-ω before TPE, became negative and non-neutralizing for IFN-ω-AAB after TPE. Interestingly, these sera were collected from patients that underwent repetitive TPE (5-14 times) in contrast to the other two patients (D001 and D016) that underwent two TPE sessions, suggesting a benefit from repetitive TPE for sufficient decrease in IFN-AAB levels.

Furthermore, a comparison between IFN-AAB-negative patients that displayed a max WHO score of 6-8 within the CSC and TPEC (cohort D) groups showed a similar survival rate (**Figure 6A**). Meanwhile, patients that were positive for IFN-AAB within the TPEC cohort showed a higher survival rate compared to those within CSC, who did not receive TPE (60%, 3 out of 5 patients in TPEC vs 7.7%, 1 out of 13 patients in CSC). The two groups had similar basic demographic characteristics such as median age, sex distribution, BMI and comorbidities, indicating the potential of TPE as an alternative treatment for IFN-AAB positive patients.

4. Discussion

4.1 Short summary of results

Sera from a cohort of six patients with APS-1 were analyzed for the quantity and the quality of AABs against type I IFNs (IFN-α2a and IFN-ω). As the presence of IFN-AABs weakens the IFN-mediated immunity and based on several previous reports on patients with APS-1 suffering from severe COVID-19, we initially hypothesized that patients with APS-1 are predisposed to severe COVID-19. The patients in our cohort were in a regular follow-up and not specifically recruited for having contracted COVID-19, however, during the course of the study, four out of six patients reported SARS-CoV-2 infection. Although we confirmed the presence of neutralizing autoantibodies against type I IFN, contrary to our hypothesis, none of the SARS-CoV-2-positive patients required hospitalization. Rather, they were either asymptomatic or showed mild symptoms that resolved within two weeks, displaying an incomplete clinical penetrance of neutralizing AABs against type I IFNs for severe COVID-19.

Since several studies highlighted the contribution of IFN-AABs to severe to critical COVID-19, we hypothesized that IFN-AABs can be used as biomarkers to identify patients that are vulnerable to develop life-threatening COVID-19. This large scale, cross-sectional, multi-cohort study confirmed that IFN-AABs are present in critically ill COVID-19 patients and associate with lower probability of survival. All the patients with neutralizing IFN-AABs had fever and required supplemental oxygen within first 72 hours after hospital admission. Type I IFN-AABs were likely already present prior to SARS-CoV-2 infection and repetitive TPE showed a trend towards reduction of serum levels of the AABs. Using our findings, we were able to develop a clinically applicable algorithm for identification of IFN-AAB-positive patients so that they can be referred to alternative treatments at an early stage of SARS-CoV-2 infection to prevent severe COVID-19.

4.2 Interpretation of the results within current state of research

Our observation of mild COVID-19 in patients with APS-1 despite the presence of neutralizing type I IFN autoantibodies may appear to be in conflict with the previous findings of individuals with APS-1 who experienced severe COVID-19. However, most of the pa-

tients with APS-1 in previous studies were pre-selected for severe COVID-19 and therefore do not provide a complete view on the likelihood of COVID-19 disease progression in patients with APS-1. Thus, the prospective nature of our study might be a potential explanation for our different observation regarding COVID-19 severity of patients with APS-1.

Despite the high prevalence of severe COVID-19 in patients with APS-1, one study showed that 7 out of 22 patients with APS-1 developed only mild to moderate COVID-19 (91). Two of those patients who were of ages 8 and 13, that were found to be SARS-CoV-2 positive during testing for screening purposes, remained asymptomatic. Interestingly, all four SARS-CoV-2-infected patients in our cohort were of a younger age (13, 14, 22 and 25 years old) and they were all female. While 15 of 22 patients that developed severe COVID-19 in the aforementioned study also included some young female patients with severe to critical COVID-19, most patients with AABs against type I IFNs and severe COVID-19 are male patients (95%) of an age older than 65 (73). Together with previous studies, our findings point towards a model where additional factors such as age and sex might co-contribute to severe COVID-19 in patients with IFN-AABs. Younger females might have additional protective factors that are absent or less frequent in elderly patients and/or males. Vice versa, older males may exhibit additional risk factors for severe COVID-19 that are absent or less frequent in the majority of young patients and/or females. For example, rare X-linked recessive TLR7 deficiency due to deleterious variants was found in about 1.8% of male patients under the age of 60 that suffered from life-threatening COVID-19 (71) which might be a co-contributing risk factor for males. On the other hand, plasmacytoid dendritic cells (pDCs) that are the major type I IFN producing cells in viral infections including SARS-CoV-2 (92, 93), produce a stronger type I IFN response in response to TLR7 activation in females than males (94-97), and, thus, might be an additional protective factor in females. In addition, both the number and function of pDCs decline with age that might contribute to impaired type I IFN immune response (98, 99) and be an additional risk factor in elderly patients. Furthermore, defects in the pathways that induce IFN production contribute to a weakened early IFN production in elderly. In contrast, children display a stronger local IFN response during a SARS-CoV-2 infection compared to adults (100).

In agreement with previous studies that showed presence of neutralizing AABs against IFN-α and/or IFN-ω in 6–17% of severely (73, 84-86, 88) and 11–19% of critically ill patients (73, 84, 87, 88) with COVID-19, we detected IFN-AAB prevalences of 3.2% (13/403) in our cross-sectional patient cohort (median max. WHO score 6) and 18.5% (5/27) in critically affected patients (median max. WHO-Score 7). Despite being ELISA-negative for IFN-ω, two serum samples (B004 and B044) showed neutralizing activity against IFN-ω in the SARS-CoV-2 infection-based neutralization assay (**Figure 2,** (2)). A possible explanation for neutralizing activity in the absence of detectable IFN might be a technical error with the detection of the AABs due to their concealment by the binding of the cytokine to the plate or cytokine biotinylation. Alternatively, cross-reactivity of IFN-α-AABs with IFN-ω could be a reason of detecting a false neutralizing activity and would be a possible explanation since both sera displayed high titers of IFN-α-AABs.

Higher disease severity and lower probability of survival in our cohorts reflect earlier findings that IFN-AAB positivity is linked to a worse clinical outcome (73, 83-87, 91, 101). On the other hand, while previous studies reported a link between the presence of IFN-AABs and demographical characteristics such as male sex and advanced age, our cohort did not exhibit such an association. This might be merely due to the small size of our cohort. However, in our cohorts, the presence of neutralizing IFN-AABs was linked to elevated levels of soluble and cellular markers of acute-phase reactions, such as CRP, procalcitonin, LDH, ferritin, and total neutrophil and leukocyte counts, as well as fever and the need for supplemental oxygen within 72 hours post hospital admission. Therefore, we concluded that fever and need for supplemental oxygen might serve as suitable clinical criteria for detection of IFN-AAB positive patients. In line with previous studies (84, 101), our detection of neutralizing IFN-AABs early after symptom onset argue for their presence prior to infection and support our hypothesis that they can be used as clinical biomarkers for early identification of patients predisposed to severe COVID-19. Combining the information we gained from all these results, we developed a clinical algorithm to rapidly identify IFN-AAB positive patients early after symptom onset so that they can be potentially directed to specific treatments. Our proposed algorithm involves a screening of hospitalized COVID-19 patients by using pre-selection criteria that would decrease the number of patients needed to screen for detection of one IFN-AAB positive patient by 50%, according to the analysis we conducted on our cohort. We propose to apply a pre-selection based on fever and the need for supplemental oxygen within 72 hours after admission

that both co-presented with IFN-AAB positivity. Next, we suggest selection of the patients that score above ELISA cut-off for further screening by competition assay. Patients that score highly positive, above the LSC (light signal count) value of 35,639, are recommended to be considered positive for neutralizing IFN-AABs, since all the samples exceeding this value in our study showed neutralizing activity against IFN-α (Figure 2c). Samples that score positive in ELISA but below the LSC value of 35,639 are suggested to be screened by competition assay that served as a good indicator of IFN neutralizing capacity. This clinical algorithm, that requires further assessment and verification by clinical studies, can allow the early identification of IFN-AAB-positive COVID-19 patients that can be directed to alternative therapies.

4.3 Strengths and weaknesses of the studies, implications for practice and future research, open questions and outlook

Up to now, to our best knowledge, our study is the only prospective study on patients with APS-1, providing an important view by displaying an incomplete clinical penetrance of IFN-AABs to severe COVID-19. However, the small size of our cohort makes it difficult to generalize our findings. Large-scale studies involving prospectively followed patients with APS-1 are required to estimate the likelihood of patients with autoantibodies developing severe or critical COVID-19. Similarly, large-scale studies on patients with IFN-AABs can also reveal a possible association between disease severity and age and sex of patients with IFN-AAB, that we could not observe in our study involving 430 patients.

ELISA and multiplex particle-based assays result in a small percentage of false-positive results despite their great sensitivity and high throughput (90, 102), underlying the need for functional assays to confirm the neutralizing activity. The most commonly employed functional assays include luciferase-based reporter assays (86, 87), analyses of STAT phosphorylation by flow cytometric analysis (1, 73, 87), and SARS-CoV-2 infection-based assays (1, 101). Especially the infection-based assays are time-consuming but they allow examination of the IFN-AABs capacity to neutralize infection-inhibitory concentrations of IFN-α and IFN-ω. Therefore, the SARS-CoV-2 infection-based assay can be considered as the gold-standard assay to validate IFN neutralization. In order to ensure the accuracy of our results, we performed an ELISA to identify AABs with high sensitivity, a competition assay to validate the specificity of AAB binding and a SARS-CoV-2 infection based assay

to confirm the functional neutralization. We were able to show that the competition assay results were consistent with the results of the neutralization assay.

Within the uninfected population, there is a sharp increase in the prevalence of type I IFN-AABs between the ages 70-85 (84). Type I IFN-AABs are identified in 0.17% of individuals below the age of 70 and in more than 4% of individuals between the ages of 80 and 85. The prevalence of AABs decrease to 2.6% after 85 years of age. The increased prevalence of type I IFN-AABs in older age groups is in agreement with many previous studies that reported a higher prevalence of AABs such as rheumatoid factor, antinuclear and anti-cardiolipin antibodies, antibodies against gastric parietal cells and thyroid epithelial cells in elderly population (103-105). Although the mechanism of AAB induction in elderly is not well understood, increased tissue damage and apoptosis due to aging is suggested to be responsible rather than autoimmunity (106, 107). Undiagnosed diseases appearing with age, asymptomatic chronic diseases or unrecognized environmental factors such as intercurrent viral infections might also be inducers of AAB production in elderly (108, 109). Moreover, changes in properties and proportion of helper/inducer T-cells, decrease in membrane fluidity of peripheral blood lymphocytes and increase in somatic mutations have been suggested as other potential reasons of AAB overproduction (108, 110). Yet, further research is required to understand the exact mechanism of increased type I IFN-AAB production in elderly.

The mechanism of IFN-AAB-associated severe COVID-19 outcome still remains to be clarified. Our study on patients with APS-1 and another study on hypoxemic breakthrough COVID-19 pneumonia cases show that IFN-AABs do not interfere with antibody production elicited by SARS-CoV-2 infection or a SARS-CoV-2 vaccine (1, 111). Following SARS-CoV-2 infection, IFNAR1-deficient mice showed similar levels of viral replication but lower levels of immune cell infiltration in the lungs compared to WT mice (112). However, another study with SARS-CoV-2-infected mice showed an increase in immune cell infiltration with no effect on viral load when given anti-IFNAR1 monoclonal antibodies (113). Critically ill patients with IFN-AABs were shown to have a compromised nasal type I IFN immunity (114). Based on current literature, it is unclear if the lack of intact type I IFN response leads to increased viral replication and higher viral load or a more efficient spread of the virus to the lungs despite similar viral load in IFN-AAB-positive patients. Nevertheless, a two-step model of pathogenesis has been suggested involving an early

inadequate type I IFN response allowing viral dissemination to the lungs and blood, followed by a hyper-inflammatory response through recruitment of immune cells and production of excessive amounts of cytokines (115, 116).

Therapeutic options for COVID-19 patients with IFN-AABs comprise the administration of monoclonal anti-spike antibodies and antiviral drugs that are recommended at the early stages of infection for patients that have a high risk of developing severe disease. Additionally, therapeutic plasma exchange (TPE) might serve as an alternative treatment. TPE was previously reported to decrease circulating IFN-AAB and showed benefits in four severely ill COVID-19 patients (101) and in a child with APS-1 that had severe COVID-19 (83). In agreement with those reports, we observed reduced levels of circulating IFN-AAB and higher probability of survival in patients that underwent TPE in our study. Overall, these results suggest that TPE might be a promising treatment for patients with IFN-AAB. However, we had few patients where serum samples were collected over the course of TPE sessions. Therefore, we cannot generalize our findings on TPE and large-scale clinical studies are needed to investigate the potential benefits of TPE.

After the findings on predisposition of patients with type I IFN-AABs to severe COVID-19, more studies have focused on investigating the disease outcome in type I IFN-AAB-positive patients during other viral infections. Some recent studies reported that in addition to SARS-CoV-2, type I IFN-AABs increase the patient susceptibility to severe disease in influenza A and herpesvirus infections (117). A higher incidence rate and a more severe clinical course of herpes zoster due to varicella zoster virus (VZV) reactivation was observed in a cohort of 44 patients with APS-1. Another study demonstrated that type I IFN-AABs were present in about 5% of patients with critical influenza pneumonia and this prevalence increased to 8% over the age of 70 in male patients (118). Moreover, type I IFN-AABs were detected in patients who suffered from life-threatening vaccine reactions after receiving yellow fever live attenuated vaccine (119). Breakthrough cases of critical COVID-19 are considered to occur due to poor or waning anti-SARS-CoV-2 spike antibodies in vaccinated individuals. A high proportion of vaccine breakthrough hypoxemic COVID-19 pneumonia cases were reported despite a normal antibody response and IFN-AABs were found in 20% (10/42) of those cases (111). Together, these studies suggested that IFN-AAB-positive patients are vulnerable to various viral diseases in addition to COVID-19.

In our cohort of 430 patients, we detected IFN-AABs in 13 patients and created our suggested clinical algorithm based on those results. Therefore, this algorithm would need to be tested in a clinical setting for further assessment and confirmation. The simple pre-clinical criteria and the chosen assays to rapidly and accurately identify IFN-AAB presence in the patients make this algorithm easily applicable in the clinical setting. Furthermore, the fact that IFN-AABs can also underlie critical influenza pneumonia (118), life-threatening yellow fever live vaccine-associated disease (119) and severe herpes zoster due to VZV reactivation (117) further supports the importance of identifying IFN-AAB positive patients as early as possible. Studies on early detection of IFN-AABs might not only benefit patients that are infected with SARS-CoV-2 but also patients with other viral infections who might otherwise suffer from severe disease.

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Statutory Declaration

"I, Bengisu Akbil, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Autoantibodies Against Type I Interferons in SARS-CoV-2 Infection, Autoantikörper gegen Typ-I-Interferone bei SARS-CoV-2 Infektion" independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to the publications

Bengisu Akbil contributed the following to the below listed publications:

Publication 1: Meisel C*, Akbil B*, Meyer T, Lankes E, Corman VM, Staudacher O, Unterwalder N, Kölsch U, Drosten C, Mall MA, Kallinich T, Schnabel D, Goffinet C#, von Bernuth H#, Mild COVID-19 despite AABs against type I IFNs in autoimmune polyendocrine syndrome type 1, The Journal of Clinical Investigation, 2021
*Shared first-authorship, #Shared last-authorship

Contribution: I designed the experimental work for infection-based neutralization assays which is considered as the gold standard for the analysis of IFN neutralization. I established, optimized and validated this functional assay and conducted all corresponding experiments including biosafety level 3 work with SARS-CoV-2 infection of the human lung cell line Calu-3, in the presence and absence of human sera and type I IFN, viral RNA quantification and plaque assays in VeroE6 cells to quantify the amount of virus released from serum-treated cells. Furthermore, I analyzed and interpreted all the corresponding data. Figure 2, Supplemental figures 2 and 3 and the graphical abstract of the paper were prepared by me. In addition, I contributed to the reviewing and editing of the manuscript at the final stages.

Publication 2: Akbil B*, Meyer T*, Stubbemann P*, Thibeault C*, Staudacher O, Niemeyer D, Jansen J, Mühlemann B, Doehn J, Tabeling C, Nusshag C, Hirzel C, Sanchez DS, Nieters A, Lother A, Duerschmied D, Schallner N, Lieberum JN, August D, Rieg S, Falcone V, Hengel H, Kölsch U, Unterwalder N, Hübner RH, Jones TC, Suttorp N, Drosten C, Warnatz K, Spinetti T, Schefold JC, Dörner T, Sander LE, Corman VM, Merle U; Pa-COVID study Group, Kurth F#, von Bernuth H#, Meisel C#, Goffinet C#, Early and Rapid Identification of COVID-19 Patients with Neutralizing Type I Interferon Auto-antibodies, Journal of Clinical Immunology, 2022
*Shared first-authorship, #Shared last-authorship

Contribution: I designed and conducted the experimental work for infection-based neutralization assays, analyzed and interpreted all the corresponding data. Figure 2, 4, 6, supplemental figures 1, 2, 6, 8 and 9 were prepared by me and were largely based on the data I generated. In addition, I contributed to the reviewing and editing of the manuscript at the final stages.

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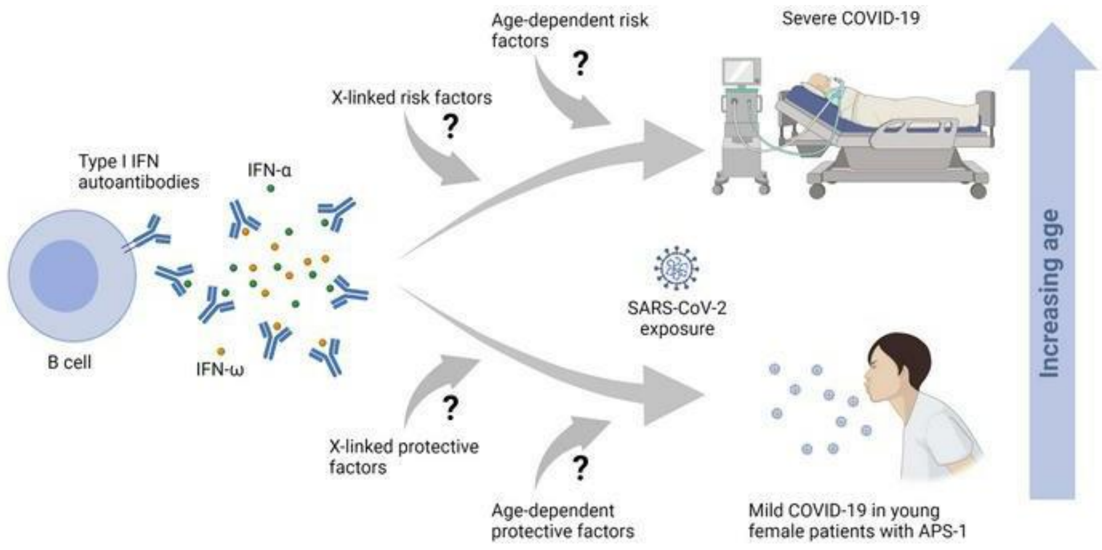
Mild COVID-19 despite autoantibodies against type I IFNs in autoimmune polyendocrine syndrome type 1

Christian Meisel, … , Christine Goffinet, Horst von Bernuth

J Clin Invest. 2021;131(14):e150867. https://doi.org/10.1172/JCI150867.

Concise Communication COVID-19 Immunology

Graphical abstract



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Mild COVID-19 despite autoantibodies against type I IFNs in autoimmune polyendocrine syndrome type 1

Christian Meisel,[1,2] Bengisu Akbil,[3,4] Tim Meyer,[1] Erwin Lankes,[5] Victor M. Corman,[3,4] Olga Staudacher,[1,4,6,7] Nadine Unterwalder,[1] Uwe Kölsch,[1] Christian Drosten,[3,4] Marcus A. Mall,[4,6,8] Tilmann Kallinich,[4,6,9] Dirk Schnabel,[5] Christine Goffinet,[3,4] and Horst von Bernuth[1,4,6,7]

[1]Department of Immunology, Labor Berlin GmbH, Berlin, Germany. [2]Institute of Medical Immunology, Charité — Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany. [3]Institute of Virology, and German Centre for Infection Research (DZIF), associated partner, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany. [4]Berlin Institute of Health at Charité — Universitätsmedizin Berlin, Germany. [5]Berlin Institute of Health, Center for Chronically Sick Children, Pediatric Endocrinology, Charité — Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin, Germany. [6]Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité — Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany. [7] Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité — Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health (BIH), Berlin, Germany. [8]German Center for Lung Research (DZL), associated partner, Berlin, Germany. [9]Deutsches Rheuma-Forschungszentrum (DRFZ), an Institute of the Leibniz association, Germany.

Autoantibodies against IFN-α and IFN-ω (type I IFNs) were recently reported as causative for severe COVID-19 in the general population. Autoantibodies against IFN-α and IFN-ω are present in almost all patients with autoimmune polyendocrine syndrome type 1 (APS-1) caused by biallelic deleterious or heterozygous dominant mutations in *AIRE*. We therefore hypothesized that autoantibodies against type I IFNs also predispose patients with APS-1 to severe COVID-19. We prospectively studied 6 patients with APS-1 between April 1, 2020 and April 1, 2021. Biobanked pre–COVID-19 sera of APS-1 subjects were tested for neutralizing autoantibodies against IFN-α and IFN-ω. The ability of the patients' sera to block recombinant human IFN-α and IFN-ω was assessed by assays quantifying phosphorylation of signal transducer and activator of transcription 1 (STAT1) as well as infection-based IFN-neutralization assays. We describe 4 patients with APS-1 and preexisting high titers of neutralizing autoantibodies against IFN-α and IFN-ω who contracted SARS-CoV-2, yet developed only mild symptoms of COVID-19. None of the patients developed dyspnea, oxygen requirement, or high temperature. All infected patients with APS-1 were females and younger than 26 years of age. Clinical penetrance of neutralizing autoantibodies against type I IFNs for severe COVID-19 is not complete.

Introduction

Mutations in *AIRE* (gene encoding the protein autoimmune regulator) cause autoimmune polyendocrine syndrome type 1 (APS-1) (1–3). *AIRE* is expressed in thymic epithelium and secondary lymphoid organs (4). AIRE regulates promiscuous gene expression of tissue-specific self-antigens in the thymus, a prerequisite for central negative selection of autoreactive T cells. Further, AIRE contributes to the generation of naturally occurring, $CD4^+CD25^+$ $CD127^{lo/–}FOXP3^+$ regulatory T cells (5). Patients with APS-1 develop autoimmunity in endocrine and nonendocrine organs, chronic mucocutaneous candidiasis (CMC), and enamel hypoplasia (6, 7). Patients with APS-1 produce autoantibodies against the Th17 cytokines, IFN-α and IFN-ω (type I IFNs) (8). The role of autoantibodies against IL-17 for CMC in patients with APS-1 is well defined (9). In contrast, a role of autoantibodies against type I IFNs for infectious diseases has only recently been suspected, as patients with APS-1 developed severe coronavirus disease 2019 (COVID-19) caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (10–12). However, to date there has been no prospective follow-up of patients with APS-1 who contracted SARS-CoV-2.

By blocking a cytokine's biological function, patients with neutralizing anti-cytokine autoantibodies may present with a clinical phenotype resembling corresponding genetic disorders (13). Autoantibodies against type I IFNs were reported in patients with severe COVID-19 (11), among whom a strong bias toward males (95%) and patients older than 65 years (>50%) was also noted (11). Autoantibodies against type I IFNs in severe COVID-19 were confirmed in additional cohorts (14–17). However, to date, only cohorts collected for severe COVID-19 have been analyzed (11, 15–18). We are not aware of a prospective follow-up of patients with preexisting autoantibodies against type I IFNs. Even if preexisting autoantibodies against type I IFNs are a strong risk factor for severe COVID-19 in preselected cohorts, the clinical penetrance of preexisting neutralizing autoantibodies against type I IFNs for severe COVID-19 is unknown on the individual level as well as on the population level.

Authorship note: CM and BA contributed equally to this work. TM and EL contributed equally to this work. CG and HvB contributed equally to this work.

Conflict of interest: VMC together with Euroimmun GmbH holds a patent regarding SARS-CoV-2 diagnostics via antibody testing (patent application number EP 20158626.0 — 1118/3715847 "A method and reagents for the diagnosis of SARS-CoV-2").

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Submitted: April 26, 2021; **Accepted:** May 25, 2021; **Published:** July 15, 2021.

Reference information: *J Clin Invest*. 2021;131(14):e150867. https://doi.org/10.1172/JCI150867.

Table 1. Basic characteristics and clinical and immunological phenotype of patients with APS-1

Basic characteristics					Clinical phenotype			Immunological phenotype				
Patient	Family	Age	Sex	Mutations in *AIRE*	AI in endocrine organs	AI in non-endocrine organs	Enamel hypoplasia	Anti–IFN-α autoantibody titer (before/after infection)	Anti–IFN-ω autoantibody titer (before/after infection)	Inhibition of STAT1 phosphorylation upon IFN-α2	Neutralization of the ability of IFN-α2 to block replication of SARS-CoV-2	Neutralization of the ability of IFN-ω to block replication of SARS-CoV-2
1	1	13 yr, 11 mo	F	c.967_979del13/ c.784delC	Parathyroid, adrenal cortex, gonads	Retina	+	1:100,000	1:1000	+	+	+
2	2	13 yr, 8 mo	F	c.62C>T/ c.1096-1G>A	Parathyroid, gonads, pituitary gland	Gastritis, anti–GABA receptor encephalitis	+	1:1000/1:1000	1:1000/1:1000	+	+	+
3	3	15 yr, 6 mo	M	c.769C>T homozygous	Parathyroid, adrenal cortex	Alopecia totalis	+	1:1000	1:10,000	+	+	+
4	4	25 yr, 9 mo	F	c.1096-1G>A homozygous	Parathyroid, adrenal cortex, gonads	Systemic onset juvenile idiopathic arthritis	+	1:10,000/1:10,000	1:10,000/1:10,000	Not done	+	+
5		14 yr, 2 mo	F	c.1096-1G>A homozygous	Adrenal cortex	Parotitis	+	1:1000/1:10,000	1:1000/1:10,000	+	+	+
6	5	22 yr, 2 mo	F	c.247A>G/ c.607C>T	Parathyroid	Calcification of basal ganglia	+	1:10,000	1:10,000/1:10,000	Not done	+	+

AI, autoimmunity.

As greater than 95% of patients with APS-1 develop high titers of neutralizing autoantibodies against type I IFNs (8), APS-1 is a model disease to prospectively study the role of preexisting autoantibodies against type I IFNs for severe COVID-19. To date, 3 patients with APS-1 and severe COIVD-19 (10, 12, 19), as well as severe COVID-19 in 15 of 22 patients in a series of APS-1 patients, have been described (18). We therefore hypothesized that autoantibodies against type I IFNs predispose patients with APS-1 to severe COVID-19. Here, we report on 6 patients with APS-1 and high titers of preexisting neutralizing autoantibodies against IFN-α and IFN-ω, of whom 4 contracted SARS-CoV-2, yet developed mild COVID-19. Our study consists of only patients in regular follow-up for APS-1 who were not recruited due to COVID-19.

Results and Discussion

Patients with APS-1 develop autoimmunity. Prior to the COVID-19 pandemic, all patients had been followed up at Charité-Universitätsmedizin Berlin for more than 70 patient years (Table 1). Patient 1 is a 13-year-old girl of European descent who developed hypoparathyroidism at 1 year and 4 months of age and adrenal insufficiency at 4 years of age. Compound heterozygous mutations in *AIRE* were diagnosed. She further developed CMC, retinal degeneration with optical atrophy, and hypergonadotropic hypogonadism. She is treated with hydrocortisone, fludrocortisone, recombinant parathyroid hormone (rPTH), calcium, magnesium, and sex hormone substitution. She irregularly takes liposomal amphotericin B. Patient 2 is a 13-year-old girl of Arabic origin who presented with hypoparathyroidism at 2 years of age. She experienced an enteroviral meningoencephalitis at 3 years, followed by autoimmune encephalitis at 7 years of age (20). Upon encephalitis, she was treated with plasmapheresis and received mycophenolate mofetil for 36 months. Compound heterozygous mutations in *AIRE* were diagnosed at 11 years of age. She also developed atrophic gastritis, growth hormone deficiency, and hypergonadotropic hypogonadism. She is treated with rPTH, calcium, vitamin D, and recombinant human growth hormone. Patient 3 is a 15-year-old boy of European descent who presented with hypoparathyroidism at 8 years of age, when adrenal insufficiency was also noticed and a homozygous mutation in *AIRE* was identified. At 10 years of age he developed alopecia totalis. He is treated with calcium, calcitriol, hydrocortisone, and fludrocortisone. Patient 4 is a 25-year-old woman of Arabic origin who had been treated for systemic onset juvenile idiopathic arthritis before being diagnosed with hypoparathyroidism at 11 years and adrenal insufficiency at 13 years of age. The diagnosis of APS-1 became evident at 22 years of age. APS-1 is most likely caused by the same homozygous mutation in *AIRE* as in her younger sister (patient 5). Patient 4 is treated with calcitriol, calcium, hydrocortisone, fludrocortisone, and estradiol for ovarian insufficiency. Patient 5, the younger sister of patient 4, is a 14-year-old girl. At 2.5 years of age she presented with unilateral parotitis and adrenal insufficiency at 8 years of age. A homozygous mutation in *AIRE* was found at 11 years of age. She is treated with hydrocortisone and fludrocortisone. Patient 6 is a 22-year-old woman of Turkish origin who developed hypoparathyroidism at 4 years of age. Compound heterozygous mutations in *AIRE* were diagnosed at 4 years of age. She receives calcitriol. All patients show enamel hypoplasia.

Table 2. Serology for SARS-CoV-2 in patients with APS-1

Patient	Time point relative to SARS-CoV-2 infection	S1-IgG ELISA		S1-IgA ELISA		SARS-CoV-2 IgG SeraSpot				
		S1-IgG ratio	S1-IgG result	S1-IgA ratio	S1-IgA result	N	RBD	S1	Complete spike	Result
1	No infection reported	0.1	Neg	0.6	Neg	0	0	0	0	Neg
2	Before	0.13	Neg	0.31	Neg	0	0	0	0	Neg
2	After	3.89	Pos	5.6	Pos	0.6	2.6	1.1	1.5	Pos
3	No infection reported	0.07	Neg	0.55	Neg	0	0	0	0	Neg
4	Before	0.07	Neg	0.54	Neg	0	0	0	0	Neg
4	After	4.2	Pos	2.43	Pos	1.3	2.7	1.4	1.9	Pos
5	Before	0.09	Neg	0.36	Neg	0.1	0	0.1	0	Neg
5	After	8.24	Pos	>12	Pos	3.1	5.9	4.6	5.1	Pos
6	Before	0.06	Neg	0.37	Neg	0	0	0	0	Neg
6	After	3.08	Pos	1.7	Pos	0.3	2.0	0.6	1.0	Pos

S1, subunit S1 of spike protein; N, nucleocapsid; RBD, S1 receptor-binding domain. S1 ratios were calculated by dividing the measured optical density of the specific serum by that of a cutoff control tested in parallel on each ELISA plate.

Infections with SARS-CoV-2 caused mild COVID-19 in 4 patients with APS-1. Patient 2 presented with vomiting, headache, and rhinitis. SARS-CoV-2 smear was positive. Three days later, smell and taste sense were absent. Fatigue, temperatures up to 38.5°C, slight pain in both knees, as well as headaches for 10 days were reported. Smell and taste returned 10 days after onset of symptoms. Patient 4 presented with up to 39°C, flu-like symptoms, and cough. SARS-CoV-2 smear was positive. Symptoms resolved after 7 days. Patient 5, living in the same household as patient 4, reported mild rhinitis, cough for 5 days, and normal body temperature. In patient 6, SARS-CoV-2 was suspected because of a positive test in the household. The patient reported cough, rhinitis, headaches, myalgia, a sore throat, normal body temperature, and loss of taste for 4 days. After 7 days all symptoms resolved apart from fatigue for 1 more week. As patients developed neither high fever nor dyspnea, all were seen by their local physician and adhered to quarantine measures. None of the patients was admitted to the hospital. When quarantine measures were lifted, serology for SARS-CoV-2 was performed. All patients who reported SARS-CoV-2 infection–compatible symptoms proved seropositive for antibodies specific for SARS-CoV-2 (Table 2). In summary, 4 patients with APS-1 contracted SARS-CoV-2 but all presented with mild COVID-19.

Patients with APS-1 have high titers of preexisting neutralizing autoantibodies against type I IFNs. We assessed preexisting sera of all APS-1 patients for autoantibodies against IFN-α, IFN-ω, IFN-β, IL-6, IFN-γ, and GM-CSF. All were positive for autoantibodies against IFN-α and IFN-ω, none for autoantibodies against IFN-β, IL-6, IFN-γ, or GM-CSF (Figure 1A). Dilution experiments showed high titers of autoantibodies against IFN-α and IFN-ω, as a serum dilution of up to 1:100,000 was necessary to reach background levels of healthy, autoantibody-negative controls (Figure 1, B and C). Titers of autoantibodies against type I IFNs rose slightly in APS-1 patients upon infection with SARS-CoV-2 (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI150867DS1). Neutralizing activity of autoantibodies against IFN-α was assessed by comparing STAT1 phosphorylation in monocytes upon ex vivo stimulation with recombinant IFN-α2 in whole blood of a healthy control and in patients. Although 1 ng/mL IFN-α2 was sufficient to induce maximum STAT1 phosphorylation in monocytes in whole blood from a healthy donor, the phospho-STAT1 signal in samples from APS-1 patients was suppressed even after stimulation with 10 ng/mL IFN-α2. In contrast, IFN-γ–induced STAT1 phosphorylation was similar between patients and the control sample (Figure 1D).

Type I IFN–mediated inhibition of SARS-CoV-2 replication is abolished by autoantibodies in patients' plasma in vitro. Neutralizing activity of autoantibodies against IFN-α and IFN-ω was further assessed by quantifying their ability to nullify the antiviral effect of exogenous IFN in a SARS-CoV-2 infection model of respiratory epithelial Calu-3 cells. As expected, treatment of cells with recombinant IFN-α2a and IFN-ω in the absence of serum or in the presence of a healthy individual's serum reduced their susceptibility to SARS-CoV-2 infection, as assessed by quantification of viral RNA in culture supernatant (Figure 2, A and B). In contrast, SARS-CoV-2 efficiently infected Calu-3 cells that were inoculated with the patients' sera, even in the presence of fixed doses of IFN-α2a (Figure 2A) and IFN-ω (Figure 2B), respectively. In general, IFN neutralization was serum concentration dependent. Specifically, for most sera, virus replication in the presence of a fixed dose of type I IFN was strongest when Calu-3 cells were incubated with 1% patient sera and weakest when incubated with 0.001% patient sera (Supplemental Figure 2, A and B). Interestingly, we failed to out-titrate the serum of patient 1 in the presence of IFN-α2a, indicating high anti–IFN-α2a neutralization capacity, which is in line with the highest titer of autoantibodies in this serum (Figure 1). The neutralizing activity of autoantibodies against IFN was further confirmed by assessing the infectivity of released virions (Figure 2, D–F, and Supplemental Figure 3). In the absence of IFNs and serum, inoculation of cells with SARS-CoV-2 gave rise to abundant de novo virus production. Addition of exogenous IFNs efficiently prevented virus production, both in the absence of serum and in the presence of serum, of an autoantibody-negative individual. However, incubation of cells with individual patient sera allowed efficient production of infectious virions even in the presence of IFN-α2a (Figure 2D) and IFN-ω (Figure 2E), confirming efficient neutralization of antiviral IFNs, mirroring our results obtained by

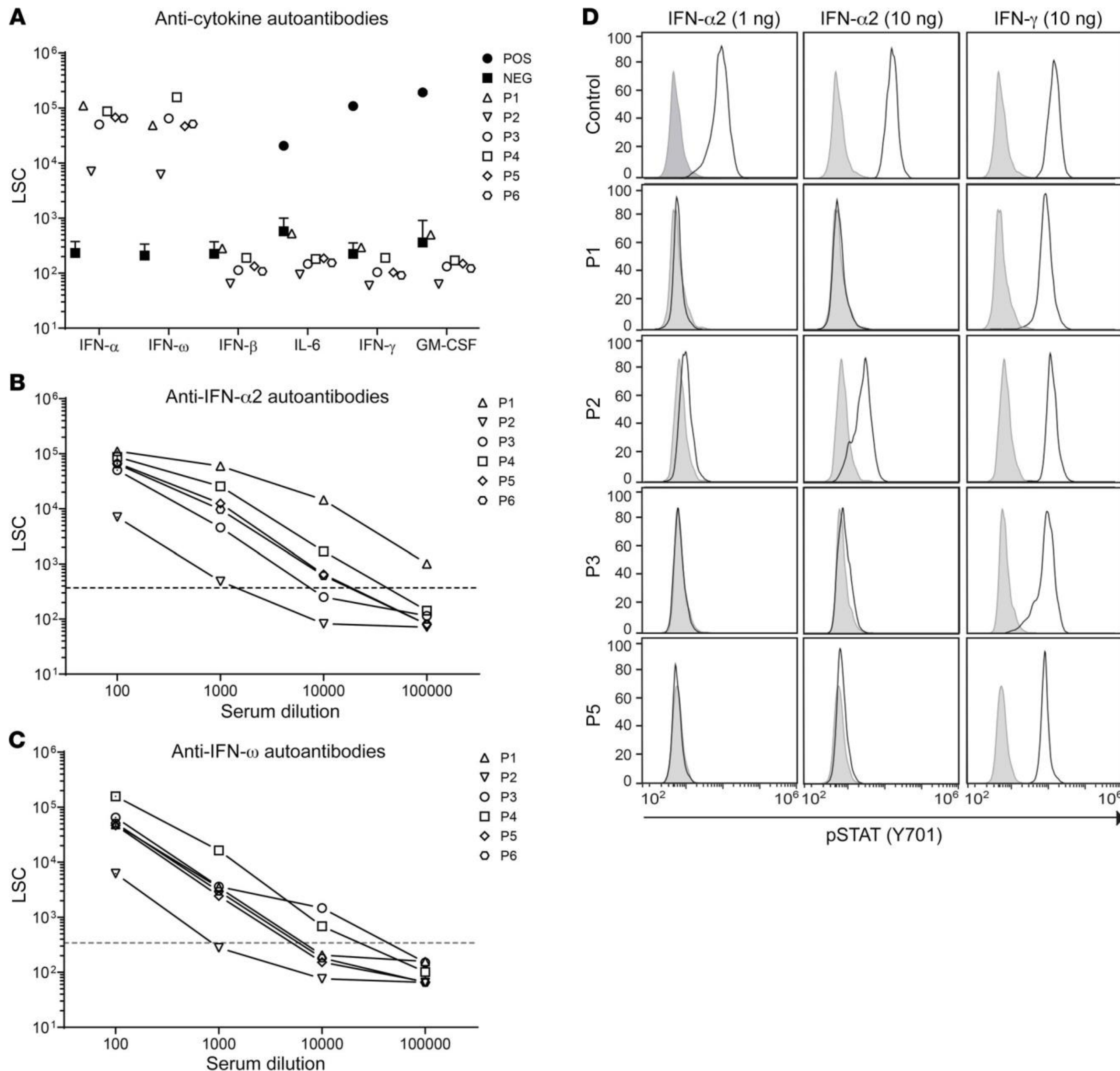

Figure 1. Neutralizing auto-Abs against IFN-α2 and IFN-ω in patients with APS-1. (**A**) Detection of IgG auto-Abs against IFN-α2, IFN-ω, IFN-β, IFN-γ, IL-6, and GM-CSF in sera (1:100 dilution) from patients with APS-1 (P1–P6), healthy controls (NEG, n = 17), and patients with known auto-Abs against IFN-γ, IL-6, and GM-CSF (POS, n = 1). Detection of auto-Abs against (**B**) IFN-α2 and (**C**) IFN-ω in serially diluted patient sera. Dotted lines indicate the maximum light signal counts (LSC) in the anti–IFN-α2 and anti–IFN-ω assay in the cohort of healthy controls. (**D**) FACS histograms depicting STAT1 phosphorylation (p-STAT1) in whole-blood monocytes from a healthy control and 4 APS-1 patients stimulated with IFN-α2 (1 and 10 ng/mL) or IFN-γ (10 ng/mL).

real-time reverse transcription PCR (qRT-PCR) (Figure 2, A and B). IFN neutralization was generally serum concentration dependent, again with the exception of the serum of patient 1 in the presence of IFN-α2a (Supplemental Figure 2, C and D). Importantly, in the absence of IFNs, healthy individuals' and patients' sera did not modulate infection efficiency as compared with the condition without serum addition (Figure 2, C and F), arguing for a specific proviral effect exerted by the patients' sera that manifests itself specifically in the presence of IFNs. In summary, all patients with APS-1 in our cohort exhibited autoantibodies at titers that are sufficient for functional neutralization of type I IFNs in an IFN-sensitive SARS-CoV-2 infection assay.

Mild COVID-19 despite high titers of neutralizing autoantibodies against type I IFNs in 4 patients with APS-1. Here, we describe 4 patients with APS-1 and high titers of preexisting, neutralizing autoantibodies against type I IFNs who experienced only mild COVID-19. Our observation may seem difficult to reconcile with reports of 3 patients with APS-1 who developed severe COVID-19 (10, 12, 19). Further, autoantibodies against type I IFNs were described as a risk factor for severe COVID-19 in at least 10% of patients with severe COVID-19 (11). Recently, a study described severe COVID-19 in 15 of 22 patients in a cross-sectional case series of patients with APS-1; however, 7 patients of the same cohort of 22 developed mild to moderate COVID-19, of whom 3 were not even

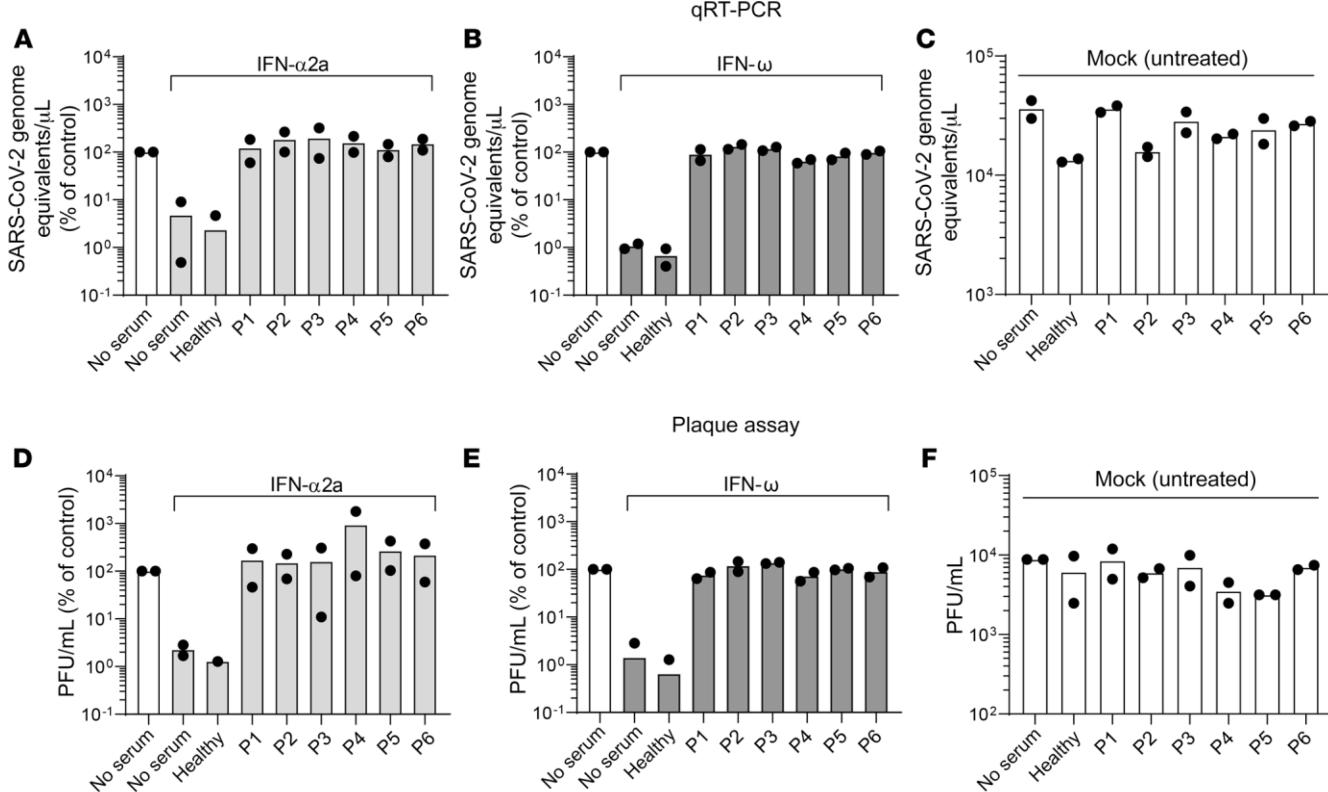

Figure 2. Auto-Abs in patients with APS-1 neutralize the ability of type I IFNs to inhibit SARS-CoV-2 infection. Calu-3 cells were mock treated (no serum) or pretreated with indicated concentrations of human serum in the presence or absence of 200 IU/mL IFN-α2a (**A** and **D**) or 5 ng/mL IFN-ω (**B** and **E**) for 16 hours before infection. IFN and serum were removed, and cells were infected with SARS-CoV-2 at an MOI of 0.01 for 1 hour, washed, and fresh medium was applied to the cells. Twenty-four hours after infection, supernatant was harvested for viral RNA extraction and plaque assays. (**A**–**C**) Viral RNA was extracted from supernatant and SARS-CoV-2 genome equivalents/μL were quantified by qRT-PCR using primers targeting the E gene region. (**D**–**F**) Supernatants were titrated on Vero E6 cells and incubated for plaque formation for 3 days. Plaques were counted and PFU/mL was determined. Data were generated in 2 independent assays. Values obtained in the absence of serum and IFN were set to 100%.

hospitalized (18). SARS-CoV-2 is sensitive to the antiviral properties of type I IFNs, as has been shown extensively in vitro, ex vivo, and in vivo (21). Therefore, it appears intuitive that interference with these cytokines results in a worsened outcome of SARS-CoV-2 infection. Strikingly, all individuals with high titers of preexisting and neutralizing autoantibodies against type I IFNs yet mild COVID-19 in our study were young females (13, 14, 22, and 25 years of age), whereas a pronounced excess of males older than 65 years was noted among most patients with autoantibodies against type I IFNs and severe COVID-19 (11). We were not able to verify to what extent autoantibodies against IFN-α and IFN-ω block the respective IFNs in our patients in vivo. So, our surprising observation of mild COVID-19 despite high titers of neutralizing autoantibodies against both IFN-α and IFN-ω in young females may be explained by the assumption that these autoantibodies do not fully neutralize either type I IFN in vivo. Consequently, if autoantibodies against IFN-α and IFN-ω do not completely block, but only dampen the biological activity of, IFN-α and IFN-ω in vivo, older males may exhibit additional risk factors for severe COVID-19 that are yet absent or less frequent/less present in most young patients and/or females.

Rescue treatment in patients with APS-1 only in severe COVID-19. In conclusion, even if preexisting autoantibodies against type I IFNs increase the risk for severe COVID-19, penetrance for severe COVID-19 is not complete. Importantly, and in contrast to previous studies (10, 12, 18, 19), our report is the first to our knowledge based on a prospective follow-up of patients with preexisting autoantibodies against type I IFNs. Large prospective studies may help to estimate the true risk of patients with preexisting autoantibodies against type I IFNs, such as in patients with APS-1 for severe COVID-19. As clinical penetrance for severe COVID-19 in the presence of preexisting autoantibodies against type I IFNs is unclear at the population and individual level, we do not advise admitting all patients with APS-1 who contracted SARS-CoV-2 to the hospital for upfront therapies (e.g., monoclonal antibodies, IFN-β, plasmapheresis). Nevertheless, we strongly advise informing all patients with autoantibodies against type I IFNs about their increased risk for severe COVID-19. As severe COVID-19 has been described also in young and in female patients with APS-1, all patients with APS-1 who contracted SARS-CoV-2 must be followed up closely.

Methods

A complete description of the materials and methods can be found in the supplemental material.

Study approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Charité

— Universitätsmedizin Berlin, Germany, EA2/132/11) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study.

Author contributions

CM, CG, and HVB planned the study. TM and OS assessed autoantibodies and STAT1 phosphorylation. BA assessed IFN neutralization in Calu-3 cells. TK, EL, and DS recruited patients. CD provided the SARS-CoV-2 isolate. VMC generated serology data. MAM, UK, and NU critically discussed the manuscript. HVB wrote the initial version of the manuscript, and CM, CG, and HVB completed the final version. All authors read and approved the final version of the manuscript.

Acknowledgments

We thank the patients and their families for ongoing trust and collaboration. We thank Mahtab Maleki and Jenny Jansen for excellent technical support. This work was supported by the Innovationsfond of Labor Berlin (to CM, CG, and HVB); by funding from the Deutsche Forschungsgemeinschaft (DFG) Collaborative Research Centre CRC900 "Microbial Persistence and its Control," project number 158989968, project C8 (awarded to CG); by funding from the Berlin Institute of Health (BIH) (to CG); and by the German Federal Ministry for Education and Research (82DZL0098B1 and 01IK20337 to MAM). We thank Jean-Laurent Casanova for discussions on our manuscript.

Address correspondence to: Christine Goffinet, Institute of Virology, Charité — Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany. Phone: 49.30.450.525489; Email: christine.goffinet@charite.de. Or to: Horst von Bernuth, Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, D-13353 Berlin, Germany. Phone: 49.30.450.566693; Email: horst.von-bernuth@charite.de.

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Journal of Clinical Immunology
https://doi.org/10.1007/s10875-022-01252-2

ORIGINAL ARTICLE

Early and Rapid Identification of COVID-19 Patients with Neutralizing Type I Interferon Auto-antibodies

Bengisu Akbil[1,2] · Tim Meyer[3] · Paula Stubbemann[4] · Charlotte Thibeault[4] · Olga Staudacher[3,5] · Daniela Niemeyer[1,6] · Jenny Jansen[1,2] · Barbara Mühlemann[1,6] · Jan Doehn[4] · Christoph Tabeling[2,4] · Christian Nusshag[7] · Cédric Hirzel[8] · David Sökler Sanchez[9,10] · Alexandra Nieters[11] · Achim Lother[12] · Daniel Duerschmied[12] · Nils Schallner[13,14] · Jan Nikolaus Lieberum[13,14] · Dietrich August[15] · Siegbert Rieg[15] · Valeria Falcone[16] · Hartmut Hengel[16] · Uwe Kölsch[3] · Nadine Unterwalder[3] · Ralf- Harto Hübner[2,4] · Terry C. Jones[1,6,17] · Norbert Suttorp[4] · Christian Drosten[1,6] · Klaus Warnatz[9,10] · Thibaud Spinetti[18] · Joerg C. Schefold[18] · Thomas Dörner[19,20] · Leif Erik Sander[4] · Victor M. Corman[1,6,21] · Uta Merle[22] · Pa- COVID study Group · Florian Kurth[4,23] · Horst von Bernuth[2,3,5,24] · Christian Meisel[3,25] · Christine Goffinet[1,2]

Received: 21 November 2021 / Accepted: 14 March 2022
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Abstract

Purpose Six to 19% of critically ill COVID-19 patients display circulating auto-antibodies against type I interferons (IFN- AABs). Here, we establish a clinically applicable strategy for early identification of IFN-AAB-positive patients for potential subsequent clinical interventions.

Methods We analyzed sera of 430 COVID-19 patients from four hospitals for presence of IFN-AABs by ELISA. Binding specificity and neutralizing activity were evaluated via competition assay and virus-infection-based neutralization assay. We defined clinical parameters associated with IFN-AAB positivity. In a subgroup of critically ill patients, we analyzed effects of therapeutic plasma exchange (TPE) on the levels of IFN-AABs, SARS-CoV-2 antibodies and clinical outcome.

Results The prevalence of neutralizing AABs to IFN-α and IFN-ω in COVID-19 patients from all cohorts was 4.2% (18/430), while being undetectable in an uninfected control cohort. Neutralizing IFN-AABs were detectable exclusively in critically affected (max. WHO score 6–8), predominantly male (83%) patients (7.6%, 18/237 for IFN-α-AABs and 4.6%, 11/237 for IFN-ω-AABs in 237 patients with critical COVID-19). IFN-AABs were present early post-symptom onset and at the peak of disease. Fever and oxygen requirement at hospital admission co-presented with neutralizing IFN-AAB positivity. IFN- AABs were associated with lower probability of survival (7.7% versus 80.9% in patients without IFN-AABs). TPE reduced levels of IFN-AABs in three of five patients and may increase survival of IFN-AAB-positive patients compared to those not undergoing TPE.

Conclusion IFN-AABs may serve as early biomarker for the development of severe COVID-19. We propose to implement routine screening of hospitalized COVID-19 patients for rapid identification of patients with IFN-AABs who most likely benefit from specific therapies.

Keywords COVID-19 · SARS-CoV-2 · Type I interferon · Autoantibodies

Introduction

Since its first detection in Wuhan, China, in 2019, severe-acute-respiratory syndrome coronavirus 2 (SARS-CoV-2) has placed an unprecedented burden on health care systems worldwide. The clinical spectrum of the associated disease, COVID-19, ranges from asymptomatic infection to severe disease with hypoxemia, acute respiratory distress syndrome (ARDS), multiorgan failure, and death [1]. Approximately

Bengisu Akbil, Tim Meyer, Paula Stubbemann and Charlotte Thibeault and Kurth, von Bernuth, Meisel, Goffinet. These authors contributed equally.

Collaborators listed at the end of the article

Extended author information available on the last page of the article

Published online: 05 May 2022

35% of patients remain asymptomatic, 55% develop upper respiratory tract infections, whereas 15% develop severe pneumonia (defined as SpO2 < 90% at room air) and 5% critical pneumonia (defined as acute respiratory distress syndrome (ARDS), requiring mechanical ventilation or extra-corporeal membrane oxygenation (ECMO) [2].

Scores containing clinical and laboratory parameters support risk stratification and resource allocation in clinical practice worldwide [3]. Demographic and clinical risk factors for a severe disease course include advanced age, male sex, and pre-existing comorbidities [4]. Moreover, genetic polymorphisms are associated with progression to severe disease [5]. Cell-intrinsic innate viral sensors and antiviral cytokines, including type I and type III interferons (IFNs), orchestrate the control of SARS-CoV-2 infection [6]. Inherited mutations of genes involved in IFN induction and signaling and circulating auto-antibodies (AABs) that neutralize type I IFNs have been found to predispose infected individuals to severe COVID-19 [7, 8], presumably by contributing to an ineffective immune response with delayed or abolished type I IFN signaling. Neutralizing type I IFN-AABs are present in 6–17% of hospitalized COVID-19 patients with severe pneumonia [7, 9, 10] and 11–19% in critically ill COVID-19 patients [7, 11, 12], greatly exceeding estimated prevalences of around 0.33% [7] in uninfected individuals. Intriguingly, while neutralizing IFN-AABs in patients with autoimmune polyendocrine syndrome type 1 (APS-1) can associate with a severe course of SARS-CoV-2 infection [13–17], their mere presence does not inevitably lead to severe disease [18]. A recent global multi-cohort study reports prevalence of neutralizing IFN-AABs in 4% of uninfected individuals over 70 years of age, suggesting that IFN-AABs may pre-exist in some individuals that develop a critical course of COVID-19 [19]. Thus, we reasoned that IFN-AABs may serve as biomarkers that could, in conjunction with other clinical parameters, help to predict risk for developing severe COVID-19 and to stratify patients for specific therapies.

Specific therapies may comprise the administration of recombinant IFN-β or therapeutic plasma exchange (TPE). However, the clinical benefit of TPE and other approaches remains to be defined and requires studies involving large numbers of patients. With IFN-AABs present in up to 18% of deceased COVID-19 patients [19] and given the limited therapeutic options for severely affected COVID-19 patients, testing specific therapeutic approaches is of high urgency, yet clinically implementable strategies for rapid and early identification of IFN-AAB-positive patients upfront are missing.

Methods

Study Cohorts and Data Collection

Patients were recruited and data and sample collection was performed within one of four prospective observational studies conducted at Charité—Universitätsmedizin Berlin, Germany (Cohort A, [20]), Inselspital Universitätsspital Bern, Switzerland (Cohort B), Universitätsklinikum Freiburg, Germany (Cohort C), and Universitätsklinikum Heidelberg, Germany (Cohort D). For this analysis, all patients with a maximum WHO score of 3–8 (see supplementary methods) were included from Cohorts A–C (henceforth summarized as cross-sectional cohorts, CSC). For cohort D (therapeutic plasma exchange cohort, TPEC), only patients who underwent therapeutic plasma exchange for treatment of COVID-19-associated hyperinflammatory syndrome at the Department of Internal Medicine IV of Heidelberg University Hospital, Germany ([21] and supplementary methods), were retrospectively selected. All TPE procedures were performed in accordance with the German Medical Devices Act ("Medizinproduktegesetz"). Healthy controls were recruited from a study on SARS-CoV-2 exposition in health care workers (HC cohort). Samples from APS-1 patients were obtained from a published study [18] and published values are shown here for reference. All studies were conducted according to the Declaration of Helsinki and Good Clinical Practice principles.

Detection of IFN-AABs by Reverse ELISA

IFN-AABs were detected using an electrochemiluminescence immunoassay (ECLIA)-platform (MSD, Rockville, USA), as described recently [18]. Briefly, MSD GOLD 96-well small spot streptavidin SECTOR Plates (MSD) were washed with wash buffer (MSD) and blocked with 150 µl blocking buffer (Thermo Fisher, Waltham, USA) per well at 4 °C overnight. All further incubations were performed for 60 min at room temperature. After blocking, plates were incubated with IFN-α2 (Merck Sharp & Dohme, Kenilworth, USA) or IFN-ω (Peprotech, Rocky Hill, USA) linked to biotin (Thermo Scientific, Waltham, USA). Next, plates were incubated with patients' sera following dilution at 1:100 in blocking buffer. Cytokine AABs were detected using a monoclonal mouse antibody to human IgG (D20JL-6, MSD). After incubation and washing, 150 µl of read buffer (ReadBufferT (4x), MSD) was added, incubated for 10 min at room temperature, and plates were analyzed using the MESO QuickPlex SQ 120 analyzer (MSD). Data are shown as light signal counts (LSC).

Competition Assays

All sera whose IFN-α2-AABs and/or IFN-ω-AABs levels exceeded the 97.5th percentile of AAB levels of the analyzed health-care workers' sera and samples that scored close to, but below this cut-off were assessed by competition assay using unbiotinylated IFN-α2 or IFN-ω. The sera of interest were diluted 1:100 with blocking buffer and incubated overnight at 4 °C with 2.5 mg/ml, 0.025 mg/ml, and 0.00025 mg/ml unbiotinylated IFN-α2 or IFN-ω. After incubation, reverse ELISA was performed, as described above. IFN-AABs in a given serum scored specific when preincubation with the highest concentration of IFN-α2 or IFN-ω resulted in an at least four-fold reduction of LSC in comparison to analysis of the identical serum without IFN-α2 or IFN-ω pre-incubation.

Virus Infection-Based Neutralization Assays

Calu-3 cells were pre-incubated with 1% human serum in the presence or absence of 200–400 IU/ml IFN-α2a (Roferon®-A, Roche) or 20–50 ng/ml IFN-ω (PeproTech). After 24 h, IFN and serum were removed and cells were infected with SARS-CoV-2 at a multiplicity of infection 0.01. Virus inoculum was removed after 1 h, cells were washed with PBS, and 100 µl medium was added per well. Twenty-four hours post-infection, cell culture supernatant was collected for viral RNA quantification by RT-PCR and infectious titer determination by plaque assay.

Cytokine and Chemokine Measurements

Cytokines and chemokines from a subset of patients from cohort A were analyzed using Quanterix' single molecule array technology or multiplex ECLIA.

Results

We analyzed 430 serum samples collected within four independent observational clinical studies on COVID-19 for IFN-AAB positivity (Table 1), comprising 237 patients with critical COVID-19 (max. WHO score 6–8). Median age of patients in the CSC (cohorts A–C, 403 patients) was 61 years (IQR 52–71) and 72.2% (291/403) were male. Median Charlson Comorbidity Index (CCI) was 3 (IQR 1–4). Twenty-seven patients with critical disease course (median max. WHO score 7 (IQR 7–8)) who underwent TPE as compassionate use were selected retrospectively from center D (TPEC). Median age of patients in the TPEC was 65 years (IQR 56–72), 74.1% (20/27) were male, and median CCI was 4 (IQR 3–5). All patients from the TPEC required invasive mechanical ventilation (IMV), 77.8% (21/27) renal replacement therapy, one patient was treated with ECMO, and 13 out of 27 (48.2%) patients died despite maximum care. Six hundred sixty-seven serum samples from a healthy cohort (HC) consisting of health-care workers (Table S1) were screened for the presence of neutralizing IFN-AABs to set the cut-off for IFN-AAB positivity.

Prevalence of AABs Against IFN-α2 and IFN-ω in Patients with COVID-19

We first aimed to establish a sensitive screening assay for type I IFN-AABs. To this end, we first screened samples of our HC for prevalence of AABs against IFN-ɑ and/or IFN-ω by ELISA. Samples were considered positive when the respective LSC value exceeded the 97.5th percentile of AAB levels of the analyzed sera from the HC (cut-off for IFN-ɑ = 1980 LSC, IFN-ω = 1961 LSC). We then screened sera obtained at the peak of the disease (i.e., during the hospitalization period with highest individual WHO score) from patients of cohorts A-C (CSC) and cohort D (TPEC). The proportion of ELISA-positive patients in the CSC was 5.0% (20/403) for IFN-ɑ AABs and 4.2% (17/403) for IFN-ω AABs. It was significantly higher in cohort D (TPEC) (IFN-ɑ AABs 18.5%, 5/27, $p = 0.0035$ and IFN-ω AABs 14.8%, 4/27, $p = 0.0132$), as expected (Fig. 1a, Fig. S1, Table S2). Some sera displayed values approaching or equaling those detected in sera from patients with autoimmune polyendocrine syndrome type 1 (APS-1), a genetic disease involving the generation of high titer neutralizing type I IFN-AABs (Fig. 1a, [18]).

Nonspecific binding is a common phenomenon in immunoassays for the detection of AABs, and high levels of inflammatory parameters such as C-reactive protein (CRP) correlate with non-specific binding of (auto-) antibodies [22]. Therefore, we probed the specificity of all samples exceeding the 97.5th percentile of the HC sera in the IFN-AAB ELISAs and 117 and 118 samples that scored below this cut-off, respectively, from all five cohorts in a competition assay (Fig. 1b, Table S2). As expected, sera that scored below the 97.5th percentile of the ELISA had a low chance of scoring positive in the competition assay (2/117 for IFN-ɑ: C024, C078; 2/118 for IFN-ω: C024, B044). A substantial part, but not all, ELISA-positive samples of the five cohorts scored positive in the competition assay (18/34 alpha; 10/34 omega), indicating specific binding of IFN in those. Overall, we established a prevalence of specific IFN-ɑ-AAB of 3.7% (15/403) and of specific IFN-ω-AAB of 2% (8/403) in the CSC (Fig. 1a, b). Cohort D (TPEC) showed 18.5% of sera specifically binding IFN-ɑ (5/27) and 14.8% for IFN-ω (4/27) or both (14.8%, 4/27) (Fig. 1a, b). Importantly, none of the tested sera from the HC displayed antibodies that specifically bound IFN-ɑ or IFN-ω.

Table 1 Baseline patient characteristics

	Individual cohorts				All patients of cross-sectional cohort		
	Center A, Berlin cohort	Center B, Bern cohort	Center C, Freiburg cohort	Center D, Heidelberg cohort (TPEC)	IFN-AAB Neutralizing	IFN-AAB Non-neutralizing	*p*-value
Number of patients	266	50	87	27	13	390	/
IFN-AAB	2.6 (7/266)	6.0 (3/50)	.5 (3/87)	18.5 (5/27)	100 (13/13)	/	/
Neutralizing IFN-alpha	2.6 (7/266)	6.0 (3/50)	3.5 (3/87)	18.5 (5/27)	100 (13/13)		
Neutralizing IFN-omega	1.1 (3/266)	6.0 (3/50)	2.3 (2/87)	11.1 (3/27)	61.5 (8/13)		
Age (Median, IQR, available n)	61 (50–71), 266	67.3 (56.8–74.5), 50	59 (53–67), 87	65 (56–72), 27	69.4 (52.5–75.6), 13	61.0 (52–70.2), 390	0.19
Sex							Male:
Female	27.8 (74/266)	18.0 (9/50)	33.3 (29/87)	25.9 (7/27)	15.4 (2/13)	28.2 (110/390)	0.31
Male	72.2 (193/266)	82.0 (41/50)	66.7 (58/87)	74.1 (20/27)	84.6 (11/13)	71.8 (280/390)	OR = 2.16 (0.47–9.91)
BMI (kg/m^2, Median, IQR, available n)	28.4 (24,9–32,5), 245	27 (26–31), 47	27.7 (25.2–32.2), 56	31.5 (25.8–40.1), 27	27.4 (25.5–29.5), 10	28 (24.9–32.4), 338	0.73
Comorbidities							
CCI (Median, IQR, available n)	2 (1–3.75), 265	4 (2–6.5), 33	3 (2–5), 83	4 (3–5), 27	3 (1.5–4), 13	3 (1–4), 367	0.54
Chronic heart disease (%)	58.9 (155/263)	30.3 (10/33)	23.0 (20/87)	77.8 (21/27)	46.2 (6/13)	48.4 (179/370)	0.74, OR = 0.83 (0.27–2.53)
Chronic pulmonary disease (%)	18.5 (47/254)	27.3 (9/33)	10.4 (9/87)	3.7 (1/27)	23.1 (3/13)	17.2 (62/361)	0.58, OR = 1.45 (0.39–5.41)
Diabetes (%)	26.7 (70/262)	42.4 (14/33)	26.4 (23/87)	44.4 (12/27)	23.1 (3/13)	28.2 (104/369)	0.54, OR = 0.70 (0.19–2.58)
Obesity (%)	39.2 (96/245)	31.9 (15/47)	28.6 (16/56)	55.6 (15/27)	20.0 (2/10)	37.0 (125/338)	0.27, OR = 0.43 (0.08–2.04)
Autoimmune disease (%)	2.8 (7/251)	2.0 (1/50)	5.7 (5/87)	3.7 (1/27)	0 (0/13)	3.5 (13/375)	0.72
Symptoms: Fever	57.0 (151/265)	80.0 (40/50)	77.5 (55/71)	85.2 (23/27)	100 (12/12)	62.6 (234/374)	0.0079
Days between symptom onset and admission (median, IQR, available *n*)	6 (2–9), 233	4 (2–8), 50	6 (3–9), 66	6 (3–8), 27	4 (3–8), 11	5 (2–9), 338	0.81
Need for supplementary oxygen within first 72 h after admission	78.4 (189/241)	74.0 (37/50)	72 (36/50)	100 (12/12)	100 (12/12)	76.0 (250/329)	0.0528
IMV	46.2 (117/253)	60,0 (30/50)	39,0 (32/82)	100 (27/27)	100 (13/13)	44.5 (166/372)	0.0001
Length of ventilation in days (median, IQR, available n)	32,5 (18,25–56,5), 116	10,5 (5–18,5), 30	16 (6–21), 23	24 (14–37), 27	20 (10.75–29.25), 12	24 (11–47,5), 157	0.40
Length of hospital stay in days (median, IQR, available n)	20 (10–44), 262	13,5 (5–23,25), 50	16 (7–33), 85	41 (24–63), 27	25 (16–47), 13	17 (9–37,8), 384	0.045

Table 1 (Continued)

	Individual cohorts				All patients of cross-sectional cohort		
	Center A, Berlin cohort	Center B, Bern cohort	Center C, Freiburg cohort	Center D, Heidelberg cohort (TPEC)	IFN-AAB Neutralizing	IFN-AAB Non-neutralizing	*p*-value
Medication/treatment							
Dexamethasone	46.9 (123/262)	48.0 (24/50)	21.0 (17/87)	74,1 (20/27)	46.2 (6/13)	41.6 (158/380)	0.74
Remdesivir	9,2 (16/174)	0 (0/50)	12.6 (11/87)	29.6 (8/27)	0 (0/13)	9.0 (27/300)	0.52
Renal replacement therapy	29.3 (74/253)	26.0 (13/50)	25.3 (20/79)	77.8 (21/27)	69.2 (9/13)	26.6 (98/369)	0.0008
ECMO	17.4 (44/253)	0 (0/50)	24.4 (20/82)	3.7 (1/27)	46.2 (6/13)	15.6 (58/372)	0.0036
Plasmapheresis	0 (0/266)	0 (0/50)	0 (0/87)	100 (27/27)	0 (0/13)	0 (0/390)	/
max. WHO score (Median, IQR, available n)	5 (4–7), 266	7 (4–8), 50	6 (4–8), 83	7 (7–8), 27	8 (8–8), 13	6 (4–7), 386	0.0001
Outcome							
Discharged or transferred	81.0 (205/253)	72.0 (36/50)	72.0 (59/82)	51.8 (14/27)	7.7 (1/13)	80.4 (299/372)	
Deceased	19.0 (48/253)	28.0 (14/50)	25.6 (21/82)	48.2 (13/27)	92.3 (12/13)	19.1 (71/372)	0.0001
Unknown	/	/	2.4 (2/82)	/	/	0.5 (2/372)	

Data are shown in % (*N*/*n*) unless otherwise indicated. *IMV* invasive mechanical ventilation, *IQR* interquartile range, *CCI* Charlson's comorbidity index. Patients with DNI/DNR were excluded for IMV, RRT, ECMO, and Outcome (*N* = 13 Center A, *N* = 0 Center B, *N* = 5 Center C, and *N* = 0 Center D)

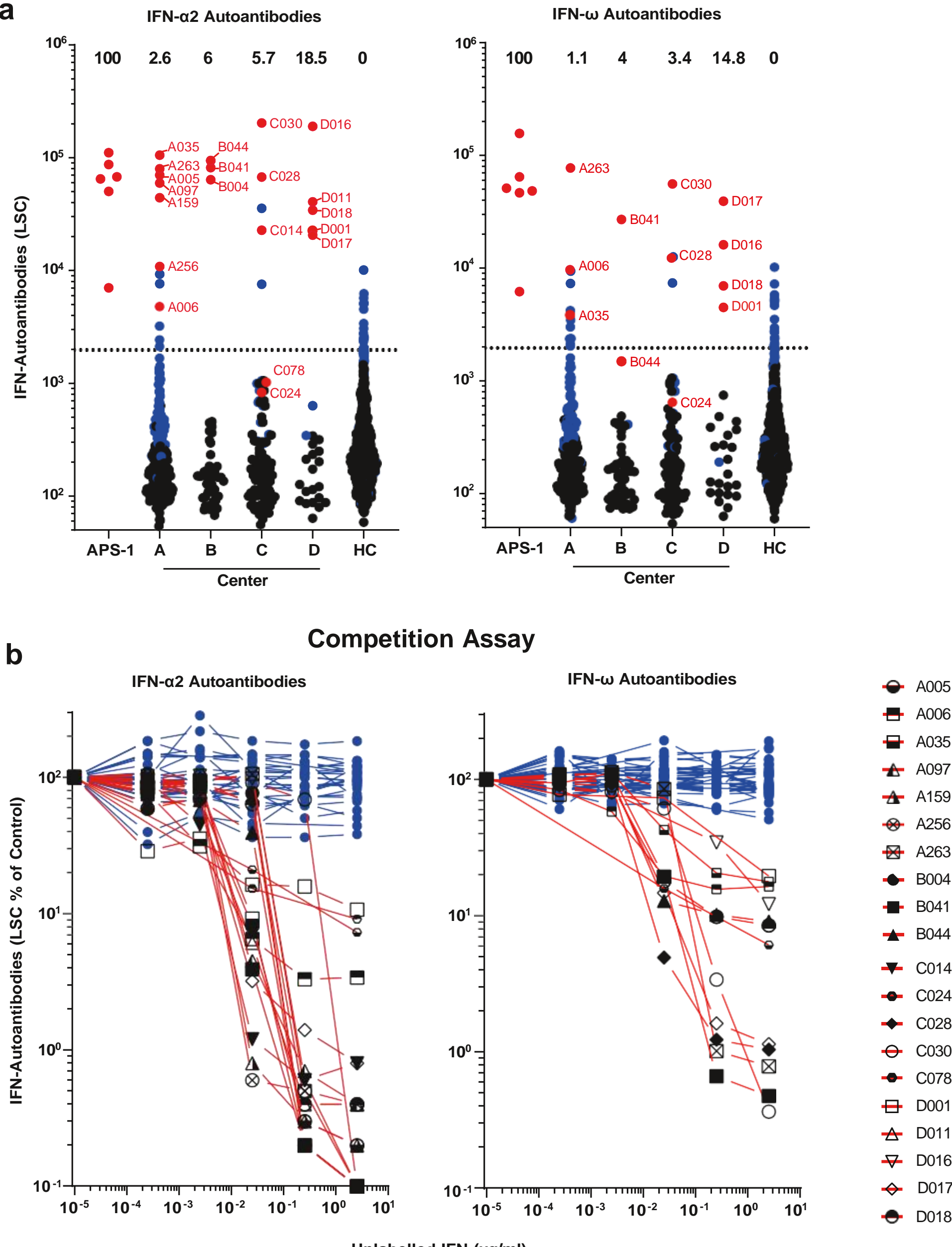


Fig. 1 Prevalence of AABs against IFN-α2 and IFN-ω in patients with COVID-19. **a** ECLIA-based assay for detection of IgG AABs against IFN-α2 and IFN-ω in sera from hospitalized patients with COVID-19 from four different university hospital cohorts (Center A, $n=266$; Center B, $n=50$; Center C, $n=87$; Center D, $n=27$), in patients with APS-1 ($n=6$), and healthy health care workers (HC) without documented SARS-CoV-2 infection ($n=667$). Dotted lines indicate the 97.5th percentile of the ECLIA assay LSC in sera from the HC cohort. Dots indicate samples containing AABs scoring specific (red) or unspecific (blue) for IFN-α2 and IFN-ω binding in the competition assay (see **b**), respectively. Samples depicted as black dots were not tested in the competition assay. The prevalence of sera with specifically binding type I IFN-AABs in each cohort is given in percent. **b** Specificity of the ECLIA assay signal for IFN-α2- and IFN-ω-AABs was tested in an competition assay by preincubation of sera with increasing concentrations of unlabeled IFN-α2 and IFN-ω protein (0–2.5 µg/ml) before analysis. Samples showing a decrease in assay signal by at least 75% in the presence of the highest competitor concentration were defined as specific for type I IFN antibody reactivity and are indicated with red lines (IFN-α2 $n=20$, IFN-ω $n=12$). Samples showing no decrease in the presence of excess unlabeled type I IFN protein were regarded as unspecific for type I IFN antibody reactivity and are indicated with blue lines (IFN-α2 $n=62$, IFN-ω $n=39$)

IFN- AABs Neutralize Exogenous IFN in a Virus Infection- Based Assay

We next analyzed whether the presence of detectable and specifically IFN-binding AABs corresponded to a functional neutralization of IFN during infection. To this end,

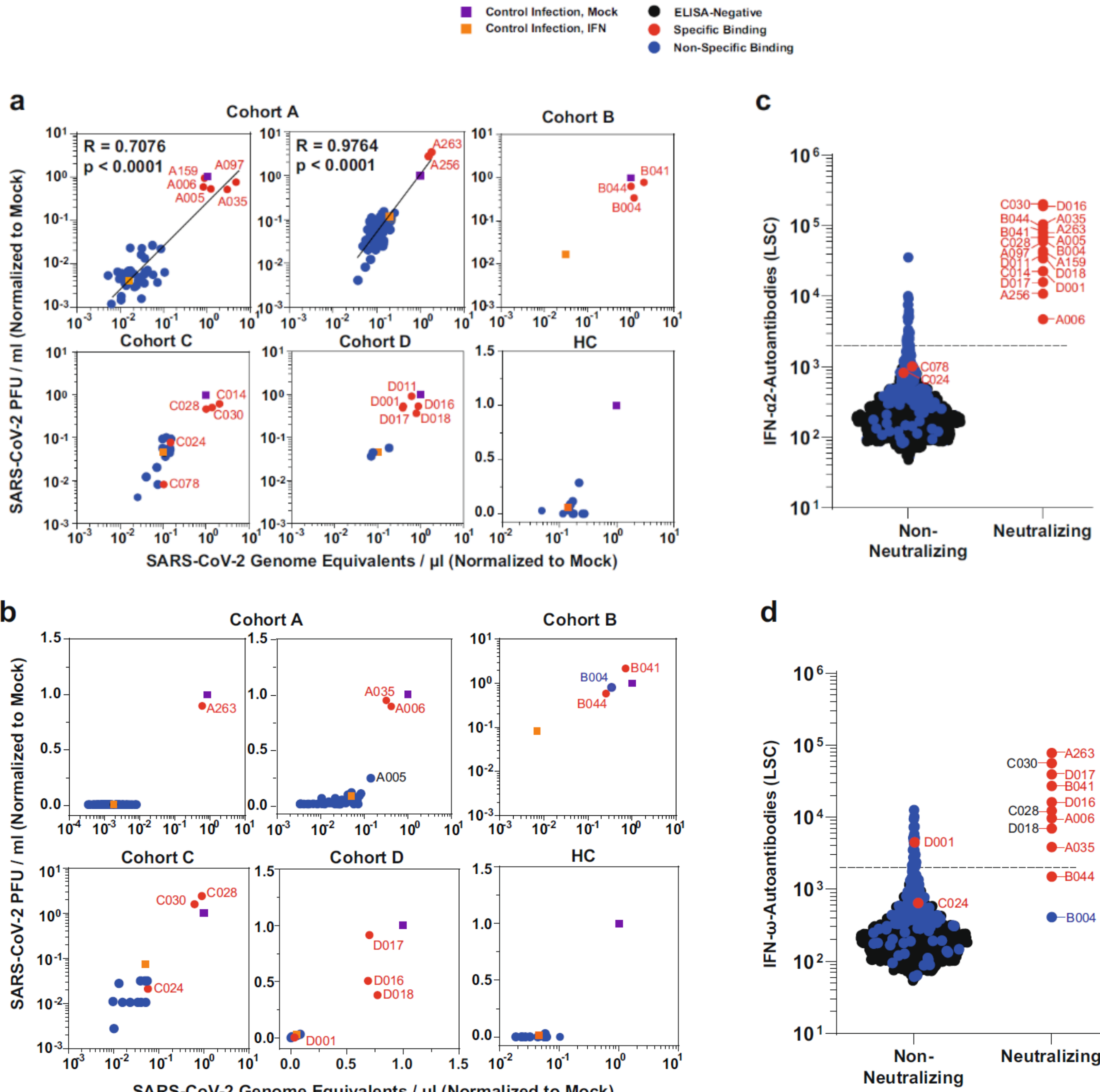


Fig. 2 IFN-AABs neutralize exogenous IFN in a virus infection-based assay. **a**, **b** Selected sera were analyzed for IFN neutralization activity in a SARS-CoV-2 infection-based assay. The ability of individual sera to neutralize exogenous IFN-α2 (**a**) and IFN-ω (**b**) is shown by the rescue of susceptibility to infection as judged by quantification of viral RNA (*x*-axis) and infectivity (*y*-axis) in the supernatant. The infection condition in the absence of serum and IFN is set to 1. **c**, **d** The LSC value for individual sera, grouped into non-neutralizing and neutralizing sera, for the four COVID-19 cohorts. Dots indicate sera containing AABs scoring specific (red) or unspecific (blue) for IFN-α2 and IFN-ω binding in the competition assay (see **b**), respectively. Black dots indicate samples that scored below the threshold of the ELISA. Black dotted lines indicate the 97.5th percentile of the ECLIA assay LSC in sera from the healthy health care workers (HC) cohort (see Fig. 1). Neutralization ability of IFN-α and IFN-ω can be predicted at 100% for sera displaying LSCs above the respective red dotted lines (IFN-α: 35,639; IFN-ω: 12,603)

we applied a previously established assay of IFN-based inhibition of SARS-CoV-2 infection of the immortalized lung cell line Calu-3 [18], which we consider the gold standard for analysis of IFN neutralization. We tested the extent to which sera neutralize the antiviral activity of type I IFNs, resulting in efficient infection despite presence of IFNs. We tested all ELISA-positive sera as well as 102 IFN-α-AAB- and 106 IFN-ω-AAB ELISA-negative sera as a reference. 3.2% (13/403) and 2% (8/403) of the sera of the CSC specifically neutralized exogenous IFN-α and IFN-ω, respectively, as judged by PCR-based quantification of SARS-CoV-2 genomic RNA in the supernatant and plaque assays that quantify infectivity of virus progeny (Fig. 2a, b, Fig. S1, Table S2). In cohort D (TPEC), 18.5% (5/27) and 11.1% (3/27) sera neutralized IFN-α and IFN-ω activity, respectively (Fig. 2a, b, Fig. S1).

Strikingly, among all competition assay-positive samples of the four COVID-19 cohorts, 90% (18/20) and 83% (10/12) sera displayed IFN-α and IFN-ω-neutralizing activity, respectively, indicating that a positive result in the competition assay associates with neutralization activity with a high likelihood (Fig. 1c, d). Examples for sera potentially containing low quantities of binding-competent, but non-neutralizing sera were derived from patients C078 (IFN-α) and C024 (IFN-ω). Conversely, a negative result in the competition assay was predictive of absence of neutralization ability. Specifically, among ELISA-positive, but competition assay-negative sera, 0 (0%) of 23 and 0 (0%) of 29 sera were able to neutralize IFN-α and IFN-ω, respectively. Among 102 IFN-α-AAB-ELISA-negative sera, we observed two sera (C024 and C078) which scored negative in our standard IFN neutralization assay but that may weakly neutralize lower amounts of IFN-α (Fig. S2). Interestingly, we identified two samples that neutralized IFN-ω despite scoring negative in the IFN-ω-AAB ELISA (i.e., having LSC counts below the 97.5th percentile cutoff, B004 and B044) (Fig. 2b, d). The pronounced ability of these exact two sera to neutralize IFN-α (Fig. 2a, c) was the reason why we included them in the IFN-ω test, and suggests a potential cross-reactivity of IFN-α-AAB with IFN-ω.

Merging results from all three assays (Fig. 2c, d) revealed that an LSC value in the screening ELISA of > 35.639 (IFN-α) and > 12.603 (IFN-ω) predicted specific binding in the competition assay and neutralization ability in the functional assay. Finally, the prevalence of ten individual antiphospholipid-ABs did not differ between patients with and without neutralizing IFN-AABs from cohort A (Fig. S3), suggesting that the presence of AABs is not generally increased in IFN-AAB-positive patients.

Laboratory Parameters of COVID-19 Patients Displaying Type I IFN-AABs

We next aimed to characterize the clinical phenotype of IFN-neutralizing AAB-positive COVID-19 patients in the CSC at hospital admission and to identify discriminatory markers that may serve as pre-selection criteria for their early identification and stratification. Interestingly, there were no statistically significant differences regarding clinical baseline characteristics, including demographic criteria and preexisting comorbidities between patients with and without IFN-neutralizing AABs in the CSC using univariate analyses (Table 1). Yet, of all patients with available symptom records (cohorts A and C), the proportion of patients who reported fever and required supplemental oxygen therapy within 72 h from admission was higher in patients with neutralizing IFN-AABs than in those without (fever: 100%, 12/12 versus 62.6% (234/374), $p = 0.0079$ and oxygen: 100% (12/12) versus 76.0% (250/329), $p = 0.0528$).

Furthermore, patients with IFN-AABs for which respective data were available displayed higher median values of C-reactive protein (CRP), procalcitonin, lactate dehydrogenase (LDH), ferritin, total leukocyte and neutrophil count, and neutrophil-to-lymphocyte ratio within the first 3 days of hospital admission compared to patients without IFN-AABs (Fig. 3, Fig. S4). In addition, patients with neutralizing IFN-AABs showed low levels of CD169/Siglec-1 expression on monocytes, a well-known type I IFN-response marker (Fig. S5). Interestingly, there was a tendency toward a negative correlation between CRP levels and CD169/Siglec-1 within 72 h from hospital admission.

In IFN-AAB-Positive Patients, High Quantities of Neutralizing IFN-α2-AABs Were Present Both Soon Post-symptom Onset and at the Peak of Disease

Next, we evaluated the temporal dynamics of IFN-AAB levels in sera from COVID-19 patients soon after symptom onset as compared to the peak of the disease. Available samples obtained in cohort A and in cohort D (TPEC) prior to TPE were analyzed (Fig. 4, Fig. S6). In all patient sera with detectable neutralizing IFN-AABs at the peak of the disease, early sera corresponding to ten (min. 4 to max. 20) days post-symptom onset contained abundant and neutralizing (Fig. 4, Fig. S5) IFN-AABs, suggesting that IFN-AABs either existed prior to the infection or were generated very early post-symptom onset. In contrast, sera collected early post symptom onset from patients that were IFN-AAB-negative at the peak of disease were negative, arguing against a transient induction of IFN-AABs.

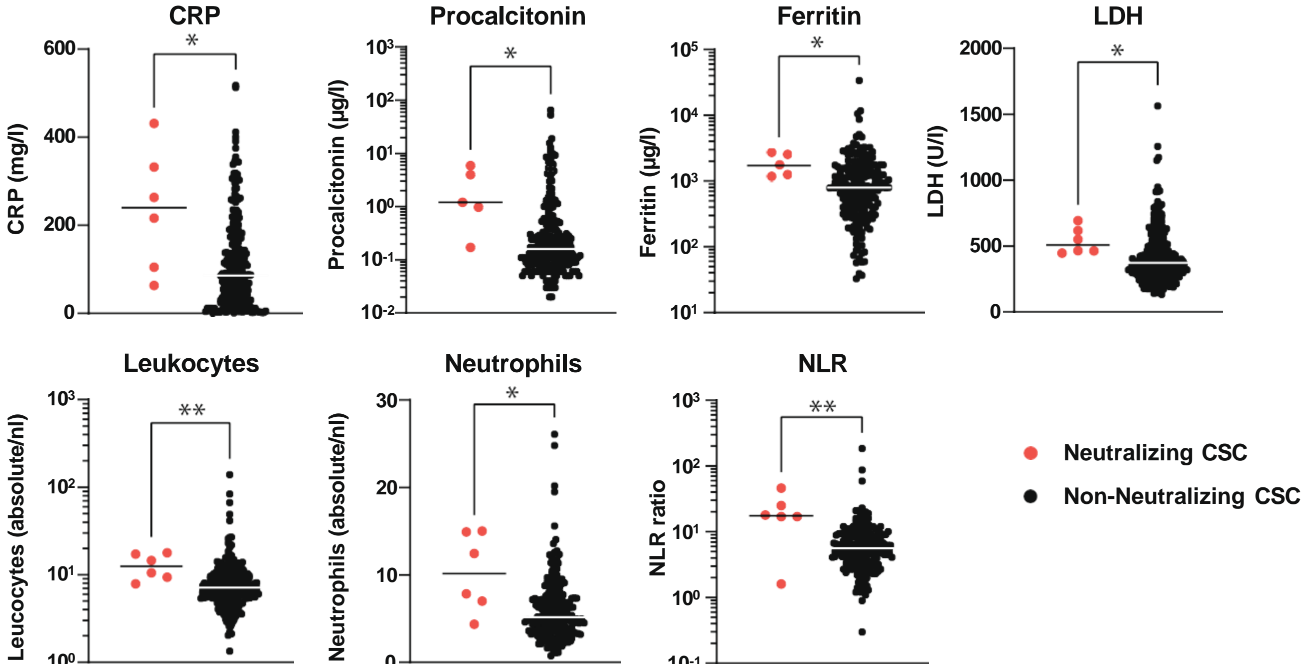


Fig. 3 Laboratory parameters of COVID-19 patients displaying type I IFN-AABs. Values of C-reactive protein (CRP), procalcitonin, fer- ritin, lactate dehydrogenase (LDH), absolute leukocyte and neutrophil count, and neutrophil-to-lymphocyte ratio (NLR) of patients with ($N = 5$–6) and without neutralizing IFN-AABs ($N = 200$–265) from the cross-sectional cohort (CSC, all WHO scores). For each patient, the first available parameter within 72 h of hospital admission is shown. Statistical testing was performed with Mann–Whitney *U* test

Cytokine and Humoral Responses to SARS-CoV-2 Infection in IFN-AAB-Positive Patients

We next aimed to identify potential quantitative and/or qualitative differences in cytokine responses, viral load, and seroconversion kinetics in IFN-AAB-positive as opposed to IFN-AAB-negative patients. We analyzed serum cytokine levels in a subset of critical patients (WHO max. 6–8) from cohort A. Patients with neutralizing IFN-AABs demonstrated significantly higher levels of IFN-γ, and IFN-γ-induced protein 10 (IP-10) 1 to 2 weeks post-symptom onset while monocyte chemoattractant protein-1 (MCP-1) and TNF-α concentrations were similar compared to sera from patients without neutralizing IFN-AABs (Fig. S7). However, levels equalized among the two groups at 3 to 4 weeks post-symptom onset. As expected, patients with IFN-AABs had undetectable serum IFN-ɑ levels. Of note, by comparing upper-respiratory tract swabs and sera from patients with and without IFN-AABs from all infected cohorts, we failed to identify detectable differences in viral load level or decay over time (Fig. S8a) and we found no evidence for a difference in duration until seroconversion post-symptom onset (Fig. S8b). In conclusion, some cytokine responses were aberrantly elevated in patients with IFN-AABs within the first 2 weeks post-symptom onset. However, they normalized at weeks 3 and 4, and viral RNA production and time to seroconversion remained indistinguishable from patients without IFN-AABs.

Clinical Outcome of COVID-19 Patients with Neutralizing IFN-AABs

Neutralizing IFN-AAB-positive patients developed significantly higher max. WHO scores than patients without neutralizing IFN-AABs (median max. WHO score 8 (IQR 8–8) vs 6 (IQR 4–7), respectively; $p < 0.0001$, Fig. 5a). All patients with neutralizing IFN-AABs in the CSC required IMV (13/13, 100%), compared to 44.5% (166/372) in patients without IFN-AABs ($p < 0.0001$, Fig. 5b). Similarly, the proportion of neutralizing IFN-AAB-positive patients requiring renal replacement therapy and/or ECMO was markedly higher than in those without IFN-AABs (renal replacement therapy: 69.2%, 9/13 versus 26.6%, 98/369, $p = 0.0008$, ECMO: 46.2%, 6/13 versus 15.6%, 58/372, $p = 0.0036$, Table 1). Twelve out of thirteen neutralizing IFN-AAB-positive patients (92.3%) died in hospital compared to 19.1% (71/372) of patients without IFN-AABs ($p < 0.0001$) in the CSC (Table 1). Median survival of patients with neutralizing IFN-AABs was 28 days (IQR 22–65 days). Irrespective of the disease severity, the probability of surviving to 150 days post-symptom onset is 81.3% (300/369) for the patients from the non-neutralizing group,

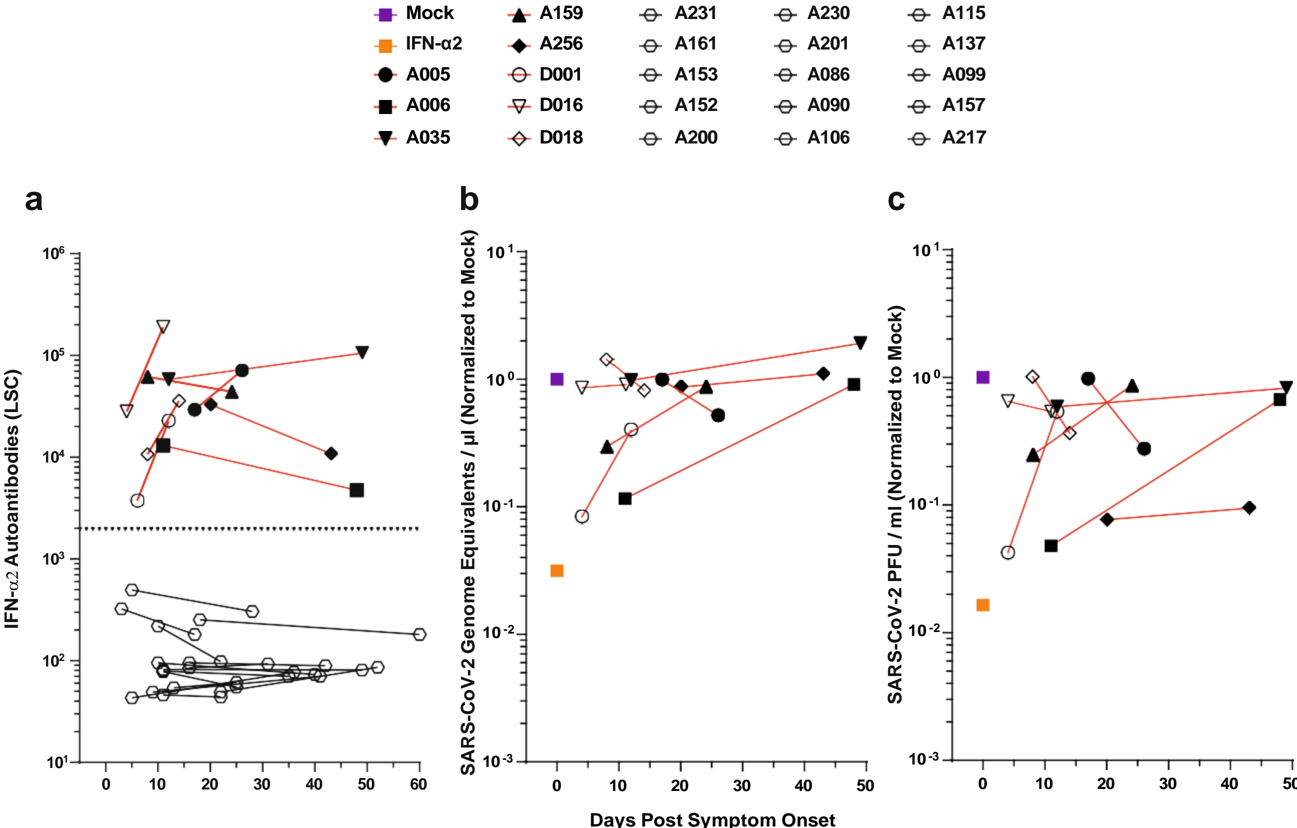


Fig. 4 In IFN-AAB-positive patients, high quantities of neutralizing IFN-α2-AABs were present both soon post-symptom onset and at the peak of disease. **a** Time course of antibody quantities in patient sera that scored IFN-AAB-positive at the peak of disease ($N = 8$, red lines). Additionally, time course of antibody quantities in patient sera that scored IFN-AAB-negative of the peak of disease is plotted ($N = 15$, black lines). The dotted line indicates the 97.5th percentile of the ECLIA assay LSC in sera from the HC cohort (see Fig. 1). **b**, **c** The ability of the sera to neutralize exogenous IFN-α2 is shown by the rescue of susceptibility to infection as judged by quantification of viral RNA (**b**) and infectivity (**c**) in the supernatant. The infection condition in the absence of serum and IFN is set to 1

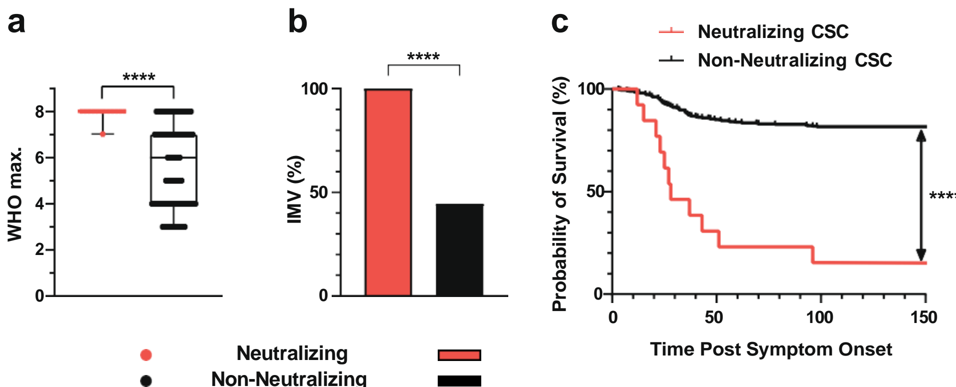


Fig. 5 Clinical outcome of COVID-19 patients with neutralizing IFN- AABs. **a** Median max. WHO score in hospital. Statistical testing was performed using the Mann–Whitney *U* test. **b** Proportion of patients requiring invasive mechanical ventilation (IMV) after hospital admis- sion. Statistical testing was performed using the chi-square test. **c** Probability of survival of patients with and without neutralizing IFN-AABs from the cross-sectional cohort (CSC) from symptom onset until discharge (up to 150 days), death or transferral ($p < 0.0001$). Statistical testing was performed using a log-rank test. Neutralizing ($N = 13$), non-neutralizing (panels a and b: $N = 372$, panel c: $N = 369$)

as opposed to 7.7% (1/13) for the patients of the neutralizing IFN-AAB-positive group (Fig. 5c). Conclusively, IFN-AAB positivity was associated with severe disease trajectories of COVID-19 and a worse clinical outcome in our cohorts.

Inter-individual Effect of Therapeutic Plasma Exchange on IFN-AABs and SARS-CoV-2 Antibodies

Cohort D (TPEC) allowed us to compare trajectories of IFN-AAB-positive patients undergoing TPE to those not undergoing TPE. Criteria for initiation of TPE were presence of ARDS requiring IMV and/or vasopressor-dependent circulatory shock, clinical and laboratory features of a COVID-19-associated immunopathology with elevated D-dimers and ferritin levels, and persistent and refractory fever ≥ 38.5 °C without conclusive pathogenic evidence and despite anti-infectious treatment. TPE was initiated without prior screening for IFN-AABs within a median of 6 days (IQR 1–10) after hospital admission and the median number of TPE sessions per patient was 3 (IQR 2–5). TPE was performed using a continuous-flow centrifugation blood cell separator. Plasma with enclosed cytokines and immunoglobulins are separated from blood cells by gravity due to different densities of the respective blood components [21].

Focusing on severely ill patients (WHO group 6–8), survival of IFN-AAB-negative patients in the CSC cohorts and TPEC was similar ($p = 0.34$). Importantly, the proportion of neutralizing IFN-AAB-positive patients from the TPEC that survived in hospital was higher than of those patients from the CSC who did not undergo TPE (60%, 3/5 patients from the TPEC survived versus 7.7%, 1/13 patients from the CSC, $p = 0.0412$) (Fig. 6a), despite similar disease severity. The two groups (IFN-AAB-positive, CSC with $N = 13$ vs. TPEC, $N = 5$) displayed no differences regarding basic demographic characteristics and share similar median age, sex distribution, BMI, and comorbidities (not significant, Table S3). The five patients from the TPEC showed a longer median length of ventilation and a longer median stay in hospital compared to the 13 patients in the CSC. In both groups, patients were treated with dexamethasone but only the IFN-AAB-positive TPEC patients partly received remdesivir (3/5 patients, 60.0% vs. 0/13, 0%, $p = 0.0044$). No ECMO treatment was used in the IFN-AAB-positive TPEC patients. Regarding both groups, the four survivors in both groups (one in the CSC vs. three in the TPEC) showed no distinct demographic characteristics, comorbidities, or treatments in hospital (except TPE for the TPEC). They were older than 50 years and predominantly male (3/4, 75%). One had a BMI above 40 kg/m^2 and CCI ranged from 1–3. Three out of 4 patients (75%) received renal replacement therapy and none of the patients was undergoing ECMO. Three out of 4 patients (75%) received dexamethasone and two patients (2/4, 50%) remdesivir.

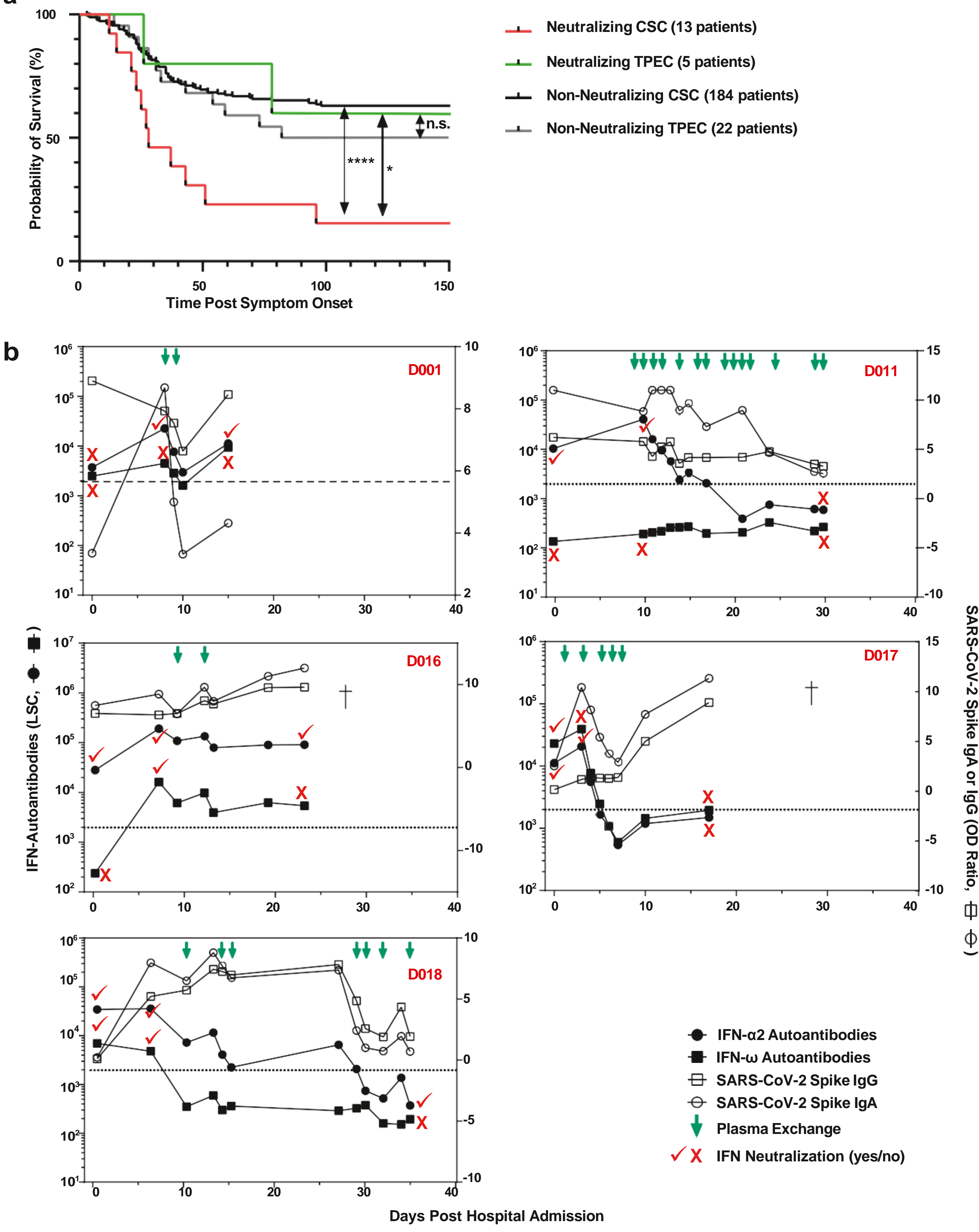
Longitudinal analysis of sera revealed that three (D011, D017, D018) out of five patients responded to TPE with decreasing IFN-AAB levels below the cut-off and to a level that coincided with absence of neutralizing activity. In addition, a sustained reduction of IFN-AAB quantities was achieved only by repetitive TPE (Fig. 5b, Fig. S9). In contrast to IFN-AABs, quantities of SARS-CoV-2 Spike IgG and IgA were less, if at all, affected by TPE (note the logarithmic scale for IFN-AABs versus the linear scale for SARS-CoV-2-IgG/IgA). Overall, our findings in a limited number of patients suggest that TPE could positively affect the survival of critically ill IFN-AAB-positive patients. This needs to be corroborated in future, adequately powered clinical investigations. Potentially, a sustained and significant reduction of peripheral IFN-AAB levels must be achieved to prevent death.

Discussion

IFN-AABs strongly associate with adverse clinical outcome of SARS-CoV-2 infection [7, 9–11, 13, 17, 23].

In several studies, detection and quantification of IFN-AABs in sera from COVID-19 patients relies on ELISA and multiplex particle-based assay. While these assays are amenable to high-throughput and are highly sensitive, they result in a small proportion of false-positive results [22, 24], highlighting the ongoing need to reanalyze positive-tested patient material in functional assays demonstrating the neutralization activity. However, such assays are sophisticated and time-consuming. They include luciferase-based interferon-stimulated response element (ISRE) promoter reporter assays [10, 11], flow cytometry-based analyses of STAT phosphorylation [7, 11, 18], and virus infection-based assays [18, 23]. The latter allows probing the activity of the IFN-AABs in the context of infection-inhibitory concentrations of IFN-α and IFN-ω. Here, we applied and cross-validated previously established assays comprising an ELISA for sensitive identification, a specificity-validating competition assay, and a functional neutralization assay [18] using a large collection of serum samples obtained from three cross-sectional cohorts.

Surprisingly, sera from two patients were found to neutralize exogenous IFN-ω despite negative ELISA results. Presence of neutralization activity in the absence of detectable IFN-AABs has been reported [19]. Explanations for this phenomenon could include technical aspects of the detection method, including the possibility that IFN-AABs may be concealed by the binding of the cytokine to the plate or biotinylation of the cytokine [25].


a
Probability of Survival (%)
Time Post Symptom Onset
Neutralizing CSC (13 patients)
Neutralizing TPEC (5 patients)
Non-Neutralizing CSC (184 patients)
Non-Neutralizing TPEC (22 patients)
n.s.
****
*
b
D001
D011
D016
D017
D018
IFN-Autoantibodies (LSC, ●, ■)
SARS-CoV-2 Spike IgA or IgG (OD Ratio, □, ○)
IFN-α2 Autoantibodies
IFN-ω Autoantibodies
SARS-CoV-2 Spike IgG
SARS-CoV-2 Spike IgA
Plasma Exchange
IFN Neutralization (yes/no)
Days Post Hospital Admission

◂ **Fig. 6** Inter-individual effect of therapeutic plasma exchange on IFN-AABs and SARS-CoV-2 antibodies. **a** Probability of survival of neutralizing IFN-AAB-positive and -negative patients with critical COVID-19 (max. WHO score 6–8) with and without plasma exchange (CSC and TPEC) from symptom onset until discharge, death or transferral ($p = 0.04$, neutralizing CSC versus neutralizing TPEC; $p < 0.0001$, neutralizing CSC versus non-neutralizing CSC). Statistical testing was performed using a log-rank test. Neutralizing CSC ($N = 13$), non-neutralizing CSC ($N = 184$), neutralizing TEPC ($N = 5$), and non-neutralizing TPEC ($N = 22$). **b** Antibody profile in serum from individual COVID-19 patients of the TPEC subjected to plasma exchange. The quantity of IFN-α2- and IFN-ω-AABs, SARS-CoV-2-IgG and -IgA, and the IFN-α2 and IFN-ω neutralization status are given for various time points. The patient identifier is given in red. Viral load profiles were only available for patients D011 and D018 and are shown in Supplementary Fig. 7

Here, we calibrated our ELISA cut-off based on the 97.5th percentile in a cohort of uninfected individuals. Although this strategy may be inexact, the absence of prevalence IFN-AABs in a cohort of younger and predominantly female healthcare workers supports an age-dependent increase of IFN-AAB prevalence in uninfected individuals [19]. Furthermore, the prevalence of 3.2% (13/403) of patients with neutralizing AABs against IFN-α and/or IFN-ω in our cross-sectional patient cohort (median max. WHO score 6) and 18.5% (5/27) in critically affected patients (median max. WHO-Score 7) is in line with reported prevalences of 6–17% in severely [7, 9, 10, 12, 19] and 11–19% in critically ill [7, 11, 12, 19] individuals with COVID-19. Moreover, IFN-AAB positivity is associated with a worse clinical outcome and a decreased survival probability of hospitalized patients in our cohorts, confirming previous reports [7, 9–11, 13, 17, 19, 23]. Interestingly, a single study to date [26] suggested that survival was not adversely affected by the presence of type I IFN-AABs, while confirming the widely accepted association with an increased risk of admission to the intensive care unit.

We failed to identify a clear association of IFN-AABs with previously described demographic parameters in our cross-sectional cohort, including male sex or advanced age [7, 13], probably due to the relatively limited sample size of our cohorts. However, the presence of neutralizing IFN-AABs was associated with fever and need for supplementary oxygen within 72 h post hospital admission, as well as with elevated soluble and cellular markers of acute-phase reaction including elevated levels of CRP, procalcitonin, LDH and ferritin, and elevated total neutrophil and leukocyte counts within the first 3 days of admission in our CSC. Higher CRP values constitute a biomarker for a severe disease course, are included in a widely-used clinical risk score for mortality of COVID-19 [3], and associate with neutralizing IFN-AABs along with lower lymphocyte counts in severely affected patients [10]. As hospital admission and thus clinical deterioration occurred at a median of 5 days post-symptom onset in the CSC, fever and need for supplemental oxygen therapy up to 72 h post hospital admission may serve as suitable and simple clinical criteria to identify patients at risk for a severe disease course.

Our ability to detect IFN-AABs as early as 4 days post-symptom onset in sera from most patients that present with IFN-AABs at the peak of their disease suggest that they were present prior to the infection, or alternatively, but less likely, were induced very early post infection. Our data are in agreement with recently demonstrated presence of IFN-AABs at the day of hospital admission [23] and in 4% of uninfected individuals > 70 years old [19], underlining the idea that they can serve as biomarkers for predisposition for a severe course of SARS-CoV-2 infection. Future studies are required to elucidate the biological mechanisms that lead to elicitation of IFN-AABs in an age-dependent manner.

Given that IFN-AABs are risk factors for a worse clinical outcome in hospitalized patients with COVID-19, future rapid identification of IFN-AAB-positive patients after hospital admission seems key for the potential implementation and success of specific interventions such as antivirals and/or monoclonal antibodies and/or TPE. Mass screening of all hospitalized COVID-19 patients may be the ideal goal. However, in the context of limited resources, combination of clinical parameters and targeted diagnostic testing may serve to facilitate early, sensitive, and specific identification of IFN-AAB-positive patients. In the CSC cohort, the patient number needed to screen (NNS) without preselection in order to identify one patient with neutralizing IFN-AAB was 31.0 (403/13). We hypothesized that applying clinical pre-selection criteria which co-present with the neutralizing IFN-AAB positivity diminishes the NNS. Due to the limited number of IFN-AAB-positive patients (13), multiple testing correction was not feasible. Using univariate analyses, we established that temperature (> 38.5 °C or self-reported fever) before or upon hospital admission and the need for supplemental oxygen within the first 72 h after admission correlated best with presence of IFN-AAB positivity in the screening assay in all hospitalized COVID-19 patients (fever: $p = 0.0079$; supplemental oxygen $p = 0.0528$). In order to prevent early exclusion of IFN-AAB-positive patients by mere pre-selection on statistically significant parameters, we included fever and the need for supplemental oxygen which nominally associated with IFN-AAB positivity. Importantly for clinical implementation, both parameters are easily measurable and clinically reasonable and reduce the NNS to 15.6 (172/11). Selection of patients exceeding the cut-off for ELISA positivity (in our cohort 97.5th percentile of the HC) for further testing by competition assay would adjust the NNS in the competition assay to 1.4 (15/11). Therefore, in order to increase sensitivity, we propose to consider the need for supplemental oxygen within

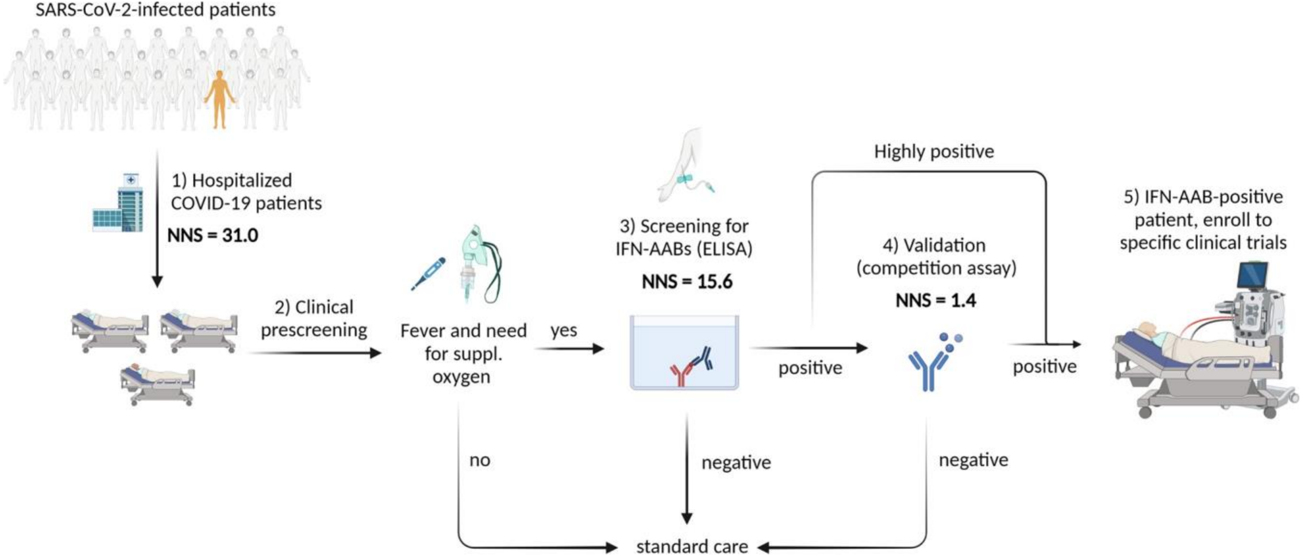


Fig. 7 Proposed diagnostic algorithm for rapid identification of neutral- izing IFN-AAB-positive patients. The number needed to screen (NNS) is based on results from the cross-sectional cohort (CSC). ELISA for IFN-AAB detection was considered to be positive if it exceeded the
97.5th percentile of the healthy control cohort. (1) NNS of all hospitalized COVID-19 patients without preselection was 31.0 (403 patients in total, 13 patients with neutralizing IFN-AABs). (2) Prescreening of patients using the clinical criteria of fever at admission and need for supplemental oxygen within the first 72 h after hospitalization diminished the NNS in the IFN-AAB ELISA (3) by half, to 15.6 (172/11). For patients identified as positive in the screening ELISA, the NNS in the competition assay to confirm the presence of IFN-specific AABs is reduced to 1.4 (15/11) (4). For patients with high-titer IFN-AABs (light signal count > 35.639), the competition assay can be omitted. Patients highly positive in the IFN-AAB ELISA and those with specific results in the competition assay may be included in clinical studies that aim testing specific therapies, including therapeutic plasma exchange (5). Figure created with BioRender.com

72 h after admission and fever as pre-selection criteria for patients that undergo ELISA screening (Fig. 7).

Sera from all patients exceeding the LSC value of 35,639 (13/13) in the screening ELISA assay demonstrated neutralizing activity against IFN-α (Fig. 2c). We therefore propose to conduct the IFN neutralization assay only in case of an LSC value lower than 35,639, whereas patients with sera exceeding this value can be considered positive for neutralizing IFN-AABs without further testing (Fig. 7). Taken together, we identified clinical parameters that co-present with IFN-AAB positivity at hospital admission, which may serve as preselection in a yet-to-be-verified diagnostic algorithm. Due to the low number of IFN-AAB-positive patients in our study, the usefulness of these parameters and their statistical robustness require assessment and verification in prospective clinical studies.

Treatment with antiviral compounds and monoclonal antibodies is recommended in the early phase of SARS-CoV-2 infection for patients at high risk for progression to severe disease and may therefore also serve as therapeutic options for IFN-AAB-positive COVID-19 patients in addition to removal of autoantibodies by TPE and substitution of type I IFN by IFN-β administration. TPE in the context of COVID-19 has been analyzed in individual case reports and case–control studies, including IFN-AAB-positive and negative patients [17, 21, 23, 27] and might efficiently remove soluble circulating FcγReceptor-activating immune complexes [28]. TPE effectively decreased circulating IFN-AAB, but not SARS-CoV-2 antibody concentrations in four IFN-AAB-positive, severely ill patients [23] and in a child with APS-1 suffering from severe COVID-19 [17]. In our study, TPE was offered to patients in one center. Here, it reduced circulating IFN-AABs with patient-specific efficiency and appeared to increase the chances of in-hospital survival. Although clinical characteristics in both groups were similar, we cannot rule out confounding factors due to different clinical settings between centers contributing to different survival rates, such as the differences regarding administration of remdesivir described above. However, our data underline the rationale to initiate large-scale, adequately powered clinical trials in order to corroborate the potential benefit of TPE in a general cohort of adult, critically ill COVID-19 patients. Interestingly, SARS-CoV-2-IgG and IgA quantities were less affected by TPE for unknown reasons, which may include their rapid replenishment by highly abundant plasmablasts or an extravascular-to-intravascular rebound since immunoglobulins have a substantial extravascular distribution.

Given the low prevalence of detectable IFN-β-AABs (up to 1.3% in patients with critical COVID-19 [19]), IFN-β administration may substitute for neutralized IFN-α and -ω. While IFN-β therapy failed to result in a detectable clinical benefit in the SOLIDARITY trial [29], specifically

IFN-AAB-positive patients may benefit from IFN-β therapy, a patient group that might have been under-represented in this study. Furthermore, the benefit of IFN-β administered by different routes should be systematically explored in this patient group.

Conclusions

Rapid and early identification of COVID-19 patients with circulating IFN-AABs at hospital admission is key to provide them with yet-to-be-established specific therapies before they clinically deteriorate. A high-throughput-amenable assay pipeline, composed of an ELISA-based assay for IFN-AABs in serum and a consecutive ELISA-based validation assay, can substitute methodologically complex gold-standard assays that quantify functional neutralization of IFNs. Future, large-scale prospective observational studies are required to verify if this pipeline may be stratified to a preselected group of patients based on clinical parameters that appeared to associate with IFN-AAB positivity, including presentation with fever and need for supplemental oxygen therapy within 72 h after admission. Identification of at-risk patients will enable clinicians to directly allocate them to larger clinical trials which are urgently required to determine clinical effectiveness of targeted therapies in this particularly vulnerable patient group.

Pa-COVID study Group (Consortium representative: Florian Kurth)

Set up study platform: Stefan Hippenstiel[4], Martin Witzenrath[4], Elisa T. Helbig[4], Lena J. Lippert[4], Pinkus Tober-Lau[4], David Hillus[4], Sarah Steinbrecher[4], Sascha S. Haenel[4], Alexandra Horn[4], Willi M[4]. Koch, Nadine Olk[4], Rosa C. Schuhmacher[4], Katrin K. Stoyanova[4], Lisa Ruby[4], Claudia Zensen[4], Mirja Mittermaier[4], Fridolin Steinbeis[4], Tilman Lingscheid[4], Bettina Temmesfeld-Wollbrück[4], Thomas Zoller[4], Holger Müller-Redetzky[4], Alexander Uhrig[4], Daniel Grund[4], Christoph Ruwwe-Glösenkamp[4], Miriam S. Stegemann[4], Katrin M. Heim[4], Bastian Opitz[4], Kai-Uwe Eckardt[26], Martin Möckel[27], Felix Balzer[28], Claudia Spies[28], Steffen Weber-Carstens[28], Frank Tacke[29], Chantip Dang-Heine[2], Michael Hummel[30], Georg Schwanitz[31], Uwe D. Behrens[31], Maria Rönnefarth[2], Sein Schmidt[2], Alexander Krannich[2], Saskia Zvorc[2], Jenny Kollek[2] and Christof von Kalle[2].

Inclusion of patients and clinical data curation: Linda Jürgens[4], Malte Kleinschmidt[4], Sophy Denker[32], Moritz Pfeiffer[4], Belén Millet Pascual-Leone[4], Luisa Mrziglod[4], Felix Machleidt[4], Sebastian Albus[4], Felix Bremer[4], Tim Andermann[4], Carmen Garcia[4], Philipp Knape[4], Philipp M. Krause[4], Liron Lechtenberg[4], Yaosi Li[4], Panagiotis Pergantis[4], Till Jacobi[4], Teresa Ritter[26], Berna Yedikat[4], Lennart Pfannkuch[4], Ute Kellermann[4], Susanne Fieberg[4], Laure Bosquillon de Jarcy[1], Anne Wetzel[4], Markus C. Brack[4], Moritz Müller-Plathe[4], Jan M. Kruse[26], Daniel Zickler[26], Andreas Edel[28], Britta Stier[26], Roland Körner[26], Nils B. Müller[26], Philipp Enghard[26], Lucie Kretzler[2], Lil A. Meyer-Arndt[33], Linna Li[2], and Isabelle Wirsching[2].

Biobanking and -sampling: Denise Treue[30], Dana Briesemeister[30], Jenny Schlesinger[30], Birgit Sawitzki[25], Lara Bardtke[4], Kai Pohl[4], Philipp Georg[4], Daniel Wendisch[4], Anna L. Hiller[4], Sophie Brumhard[4], Marie Luisa Schmidt[1], Leonie Meiners[1], and Patricia Tscheak[1].

Study Group-Specific Affiliations

[26]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Nephrology and Internal Intensive Care Medicine, Berlin, Germany.

[27]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Division of Emergency Medicine and Department of Cardiology, Berlin, Germany.

[28]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Anesthesiology and Intensive Care Medicine, Berlin, Germany.

[29]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Hepatology and Gastroenterology, Berlin, Germany.

[30]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Central Biobank Charité (ZeBanC), Institute of Pathology, Berlin, Germany.

[31]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Clinical Study Center, Berlin, Germany.

[32]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Hematology, Oncology and Tumor Immunology, Berlin, Germany.

[33]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Department of Neurology, Berlin, Germany.

Supplementary Information The online version contains supplemen- tary material available at https://doi.org/10.1007/s10875-022-01252-2.

Acknowledgements We thank Mahtab Maleki for support with IFN- AAB ELISAs and competition assays. We thank Patricia Tscheak, Marie Luisa Schmidt, and Tatjana Schwarz for support with sample logistics and SARS-CoV-2 antibody testing. We thank Leif Hanitsch

for advice on patient recruitment and evaluation. CT and VMC are participants of the Charité Clinician Scientist program funded by Charité – Universitätsmedizin Berlin and the Berlin Institute of Health. We thank Karin Reiter for the anti-phospholipid antibody measurements. We thank the patients for their ongoing trust and collaboration. We thank the involved biobanks (ZeBanC Berlin, FREEZE-Biobank Freiburg) for their support. DD and AL are members of SFB1425, funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Project #422681845. NS is partly funded by the Deutsche Forschungsgemeinschaft (DFG, Research Grant No. SCHA1838/4-2).

Author Contribution *Conceptualization*: FK; HvB; CM; CG
Methodology: BA; TM; OS
Formal analysis and investigation: BA; TM; PS; Charlotte T; OS; JJ; BM; J-MD; Christoph T; CN, CH; DSS; DD, AL; NS; JNL; DA; SR; VF; UK; NU; TS; JS; TD; VMC; UM
Writing—original draft preparation: PS; Charlotte T; CG
Writing—review and editing: BA; TM; PS; Charlotte T; OS; DN; BM; CN; DD, AL; TCJ; KW; TS; JS; LS; VMC; UM; FK; HvB; CM; CG
Funding acquisition: JS; KW; UM; FK; HvB; CM; CG
Resources: DN, AN; CD; LES; FK; HvB; CM; CG
Supervision: HH; R-HH; NS; KW; VMC; FK; HvB; CM; CG

Funding Open Access funding enabled and organized by Projekt DEAL. This work was supported by the Innovationsfond of Labor Berlin (to H.V.B, C.M., and C.G.); by funding from the Deutsche Forschungsgemeinschaft (DFG) Collaborative Research Centre CRC900 "Microbial Persistence and its Control," project number 158989968, project C8 (awarded to C.G.); and by funding from the Berlin Institute of Health (BIH) (to C.G.). T.C.J. is in part funded through NIAID-NIH CEIRS contract HHSN272201400008C. Parts of the work were funded by the European Union's Horizon 2020 research and innovation program through project RECOVER (GA101003589) to C.D.; the German Ministry of Research through the projects VARIPath (01KI2021) to V.M.C., and NaFoUniMedCovid19—COVIM, FKZ: 01KX2021 to C.D., V.M.C., L.E.S., F.K. and H.H. The Pa-COVID-19 Study is funded by BIH and the German Ministry of Research NaFoUniMedCovid19 NUM-NAPKON FKZ: 01KX2021. The COV-IMMUN study is supported by the Berlin University Alliance and the BIH Clinical Study Center (BIH-CSC). KW has received funding by the Deutsche Forschungsgemeinschaft (DFG, Research Grant No. WA 1597/6–1 and WA 1597/7–1).

Data Availability The datasets and materials generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Code Availability Not applicable. No data were coded in this study.

Declarations

Research Involving Human Participants All studies were conducted according to the Declaration of Helsinki and Good Clinical Practice principles.

The Pa-COVID-19 study was approved by the ethics committee of Charité – Universitätsmedizin Berlin (EA2/066/20) and is registered in the German and WHO international clinical trials registry (DRKS00021688).
The Covimmun-study was approved by the ethics committee of Charité – Universitätsmedizin Berlin (EA1/068/20).
The study "Evaluation der humoralen und zellulären Immunantwort und der thromboembolischen Komplikationen bei Infektion mit dem neuartigen Coronavirus (nCoV19)" was approved by the Kantonale Ethikkommission KEK, Bern, Switzerland, Nr. 2020–00877, and is registered under the name "Characterizing the Immune Response and Neuronal Damage in COVID-19" at http://clinicaltrials.gov (NCT04510012).
The three studies (COVID-19 Register Freiburg, FREEZE-Covid19 cohort, and Biomarker COVID-19) were approved by the ethics committee of the University of Freiburg (EK-FR 153/20; FREEZE-Biobank EK-FR 383/19; EK-FR 225/20) and are registered in the German and WHO international clinical trials registry (DRKS00021522 for Biomarker COVID-19).
The Covid-Immun study was approved by the local Ethics Committee of the Medical Faculty of Heidelberg (S148/2020) and is registered in the German clinical trials registry (DRKS00021810).

Informed Consent to Participate and to Publish Written informed consent to participate was obtained from all individual participants included in the studies. Furthermore, the authors affirm that human research participants provided written informed consent for publication of their demographic, clinical and laboratory data.

Conflict of Interest V.M.C is named together with Euroimmun GmbH on a patent application filed recently regarding the diagnostic of SARS- CoV-2 by antibody testing. Technische Universität Berlin, Freie Uni- versität Berlin, and Charité—Universitätsmedizin have filed a patent application for siRNAs inhibiting SARS-CoV-2 replication with D.N. as co-author. J.C.S, T.S. (full departmental disclosure): the department of Intensive Care Medicine has/had research and/or development/ consulting contracts with (full disclosure) Orion Corporation, Abbott Nutrition International, B. Braun Medical AG, CSEM SA, Edwards Lifesciences Services GmbH/SA, Kenta Biotech Ltd, Maquet Critical Care AB, Omnicare Clinical Research AG, Phagenesis Ltd, Cytel, and Nestlé. No personal financial gains resulted from respective develop- ment/consulting contracts and/or educational grants.

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Authors and Affiliations

Bengisu Akbil[1,2] · Tim Meyer[3] · Paula Stubbemann[4] · Charlotte Thibeault[4] · Olga Staudacher[3,5] · Daniela Niemeyer[1,6] · Jenny Jansen[1,2] · Barbara Mühlemann[1,6] · Jan Doehn[4] · Christoph Tabeling[2,4] · Christian Nusshag[7] · Cédric Hirzel[8] · David Sökler Sanchez[9,10] · Alexandra Nieters[11] · Achim Lother[12] · Daniel Duerschmied[12] · Nils Schallner[13,14] · Jan Nikolaus Lieberum[13,14] · Dietrich August[15] · Siegbert Rieg[15] · Valeria Falcone[16] · Hartmut Hengel[16] · Uwe Kölsch[3] · Nadine Unterwalder[3] · Ralf- Harto Hübner[2,4] · Terry C. Jones[1,6,17] · Norbert Suttorp[4] · Christian Drosten[1,6] · Klaus Warnatz[9,10] · Thibaud Spinetti[18] · Joerg C. Schefold[18] · Thomas Dörner[19,20] · Leif Erik Sander[4] · Victor M. Corman[1,6,21] · Uta Merle[22] · Pa- COVID study Group · Florian Kurth[4,23] · Horst von Bernuth[2,3,5,24] · Christian Meisel[3,25] · Christine Goffinet[1,2]

✉ Florian Kurth
florian.kurth@charite.de

✉ Horst von Bernuth
horst.von-bernuth@charite.de

✉ Christian Meisel
christian.meisel@laborberlin.com

✉ Christine Goffinet
christine.goffinet@charite.de

1 Institute of Virology, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität Zu Berlin, Charitéplatz 1, 10117 Berlin, Germany

2 Berlin Institute of Health at Charité - Universitätsmedizin Berlin, Berlin, Germany

3 Labor Berlin GmbH, Department of Immunology, Charité - Universitätsmedizin Berlin, Sylter Str. 2, 13353 Berlin, Germany

4 Department of Infectious Diseases and Respiratory Medicine, Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität Zu Berlin, Augustenburger Platz 1, 13353 Berlin, Germany

5 Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität Zu Berlin, and Berlin Institute of Health, Augustenburger Platz 1, 13353 Berlin, Germany

6 DZIF German Centre for Infection Research (DZIF), Partner Site Charité, 10117 Berlin, Germany

7 Department of Nephrology, Heidelberg University Hospital, Heidelberg, Germany

8 Department of Infectious Diseases, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

9 Department of Rheumatology and Clinical Immunology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

10 Center for Chronic Immunodeficiency (CCI), Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

11 University Medical Center Freiburg and Faculty of Medicine, University of Freiburg, Center for Biobanking, FREEZE-Biobank, Freiburg, Germany

12 Cardiology and Medical Intensive Care, Heart Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany

13 Department of Anesthesiology and Critical Care, Medical Center - University of Freiburg, Freiburg, Germany

14 Faculty of Medicine, University of Freiburg, Freiburg, Germany

15 Division of Infectious Diseases, Department of Medicine II, Medical Centre – University of Freiburg, Faculty of Medicine, University of Freiburg, 79106 Freiburg, Germany

16 Institute of Virology, Freiburg University Medical Center, Faculty of Medicine, Albert-Ludwigs-University of Freiburg, Freiburg, Germany

17 Centre for Pathogen Evolution, Department of Zoology, University of Cambridge, Downing St, Cambridge CB2 3EJ, UK

18 Department of Intensive Care Medicine, Inselspital, Bern University Hospital, University of Bern, Freiburgstrasse, CH-3010 Bern, Switzerland

[19] Department of Medicine/Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität Zu Berlin, Berlin, Germany

[20] DRFZ, Berlin, Germany

[21] Labor Berlin GmbH, Berlin, Germany

[22] Department of Gastroenterology, Heidelberg University Hospital, Heidelberg, Germany

[23] Department of Tropical Medicine, Bernhard Nocht Institute for Tropical Medicine and Department of Medicine I, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany

[24] Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität Zu Berlin, and Berlin Institute of Health (BIH), Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Berlin, Germany

[25] Institute of Medical Immunology, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität Zu Berlin, Berlin, Germany

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publication list

Publications:

1. Bosquillon de Jarcy, L., **<u>Akbil, B.</u>**, Mhlekude, B., Leyens, J., Postmus, D., Harnisch, G., Jansen, J., Schmidt, M.L., Aigner, A., Pott, F. and Chua, R.L. (2023). **90K/LGALS3BP expression is upregulated in COVID-19 but may not restrict SARS-CoV-2 infection.** Clinical and Experimental Medicine, pp.1-12. Nov;23(7):3689-3700. https://doi.org/10.1007/s10238-023-01077-2. **IF: 5.057**

2. **<u>Akbil B*</u>**, Meyer T*, Stubbemann P*, Thibeault C*, Staudacher O, Niemeyer D, Jansen J, Mühlemann B, Doehn J, Tabeling C, Nusshag C, Hirzel C, Sanchez DS, Nieters A, Lother A, Duerschmied D, Schallner N, Lieberum JN, August D, Rieg S, Falcone V, Hengel H, Kölsch U, Unterwalder N, Hübner RH, Jones TC, Suttorp N, Drosten C, Warnatz K, Spinetti T, Schefold JC, Dörner T, Sander LE, Corman VM, Merle U; Pa-COVID study Group, Kurth F#, von Bernuth H#, Meisel C#, Goffinet C#. (2022). **Early and Rapid Identification of COVID-19 Patients with Neu- tralizing Type I Interferon Auto-antibodies**. *Journal of Clinical Immunology*, 42(6), 1111–1129. https://doi.org/10.1007/s10875-022-01252-2. **IF: 8.542**

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*Shared first-authorship, #Shared last-authorship

Acknowledgements

First of all, I thank Prof. Christian Drosten for creating the opportunity for me to conduct my doctoral research at the Institute of Virology at Charité University Hospital Berlin.

I am so grateful to have had Prof. Christine Goffinet as my PhD supervisor. Through these years, she helped me grow as a scientist and a critical thinker and always encouraged me to reach the potential she saw in me. She has been an amazing mentor beyond my imagination and a true role model and inspiration in many ways! The perspective she gave me will keep guiding me in my future journey.

I thank my co-supervisors, Prof. Andreas Hocke and Prof. Thomas Krey, who provided valuable feedback on my progress and suggestions to improve my projects.

I thank our technical assistant, Jenny Jansen, who has always been ready to help and made things work with her excellent planning and teamwork skills when I accidentally planned way more experiments than one can handle at a time!

I thank my former master student, Johanna Leyens, for being so helpful, supportive, dedicated and motivated in all the work we did together during her time in our lab!

Many thanks to Dylan for proofreading my dissertation. Furthermore, I thank all members of my group, Christiane, Dylan, Fabian, Julia, Jenny, Baxolele, Saskia, Laure, Anna Julia, Daan for creating such a nice, warm and supportive atmosphere. I also want to thank all the members of the Institute of Virology, I feel really grateful for having worked in such a collaborative and supportive environment.

Finally, special thanks to my family and friends that were always there to support me throughout the years, especially my mother, Barak and Cigdem.