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Patients with severe COVID-19 do not have elevated autoantibodies against common diagnostic autoantigens

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Abstract

Objectives: Infection by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative pathogen of coronavirus disease 2019 (COVID-19) presents occasionally with an aberrant autoinflammatory response, including the presence of elevated circulating autoantibodies in some individuals. Whether the development of autoantibodies against self-antigens affects COVID-19 outcomes remains unclear. To better understand the prognostic role of autoantibodies in COVID-19, we quantified autoantibodies against 23 markers that are used for diagnosis of autoimmune disease. To this end, we used serum samples from patients with severe [intensive care unit (ICU)] and moderate (ward) COVID-19, across two to six consecutive time points, and compared autoantibody levels to uninfected healthy and ICU controls.

Methods: Acute and post-acute serum (from 1 to 26 ICU days) was collected from 18 ICU COVID-19-positive patients at three to six time points; 18 ICU COVID-19-negative patients (sampled on ICU day 1 and 3); 21 ward COVID-19-positive patients (sampled on hospital day 1 and 3); and from 59 healthy uninfected controls deriving from two cohorts. Levels of IgG autoantibodies against 23 autoantigens, commonly used for autoimmune disease diagnosis, were measured in serum samples using MSD® U-PLEX electrochemiluminescence technology (MSD division Meso Scale Discovery®), and results were compared between groups.

Results: There were no significant elevations of autoantibodies for any of the markers tested in patients with severe COVID-19.

Conclusions: Sample collections at longer time points should be considered in future studies, for assessing the possible development of autoantibody responses following infection with SARS-CoV-2.

Keywords: autoantibodies; autoimmunity; COVID-19; electrochemiluminescence; Intensive care unit (ICU); prognostic markers; SARS-CoV-2; severe disease.

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Introduction

Infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) most commonly results in a mild or asymptomatic disease. When symptoms are severe it often presents with an excessive inflammatory response

characterized by the induction of a systemic cytokine storm, dominated primarily by interleukin-6 (IL-6) and tumor-necrosis factor α (TNF-α) [1], and associated inflammation in various organs such as the lungs, brain, heart and kidney (reviewed by [2]). In fact, such an aberrant immune response appears to contribute to disease severity along with the tissue damage caused directly by the virus, which has substantiated the clinical application of anti-inflammatory and immunomodulatory agents to manage the disease [3, 4].

In addition to this excessive response of the innate immune system, recent findings suggest that the adaptive immune response is also hyperactivated in patients with critical disease, resulting in acute extrafollicular expansion of autoimmune-like B cells [5] and elevated circulating autoantibodies [6]. More specifically, neutralizing autoantibodies against type I interferons (IFNs) were found in ~10% of patients with life-threatening COVID-19 [7], but were absent in individuals with mild or asymptomatic infection, while 0.3% of healthy uninfected individuals presented with such autoantibodies. Given their early detection (within 10 days of infection), these autoantibodies appear to be pre-existing rather than induced autoantibodies, and were not linked with worse COVID-19-related outcomes such as increased hospitalization or death when compared to critically ill patients without such autoantibodies [8]. Thus, while the anti-type I IFN autoantibodies and inborn errors in the type I IFN immunity [9] may be useful for understanding disease pathogenesis, their prognostic value in COVID-19 outcomes appears to be limited. There is a need to continue searching for valuable prognostic biomarkers for COVID-19, to better guide patient cohorting and healthcare resource management.

Previous studies have shown higher levels of autoantibodies predominantly in patients with severe COVID-19 which were detected within the first two weeks of disease onset [6, 7]. In this study, we asked whether patients with severe COVID-19 had elevated autoantibodies against self-antigens during ICU care. To this end, we measured levels of serum autoantibodies against 23 autoantigens that are clinically used for diagnosis of autoimmune diseases such as Type 1 diabetes (T1D), mixed connective tissue disease (MCTD), vasculitis, and systemic lupus erythematosus (SLE). We reasoned that the autoantibodies against the selected 23 antigens would likely be elevated in patients with severe SARS-CoV-2 infection, thus explaining the widespread autoimmune-like tissue damage often seen in severe COVID-19 [10]. To this end, we examined samples from 18 patients with critical COVID-19, and 21 patients with moderate symptoms who received ward care, both at the Lawson Health Research Institute in Ontario, Canada. Additionally, we included samples from 18 SARS-CoV-2-negative patients who received ICU care for sepsis at the same center. Lastly, we included 59 single time point samples collected prior to the onset of COVID-19 pandemic from uninfected healthy individuals (healthy negative group).

Serum samples were analyzed on the MSD U-PLEX® electrochemiluminescence platform (see Methods), which allows for the rapid and simultaneous detection of multiple autoantibodies (up to 10), thus minimizing the sample volume needed and providing readouts with meaningful value for decision making in an urgent clinical setting. The panel of selected autoantigens includes proteins such as nuclear antigens [e.g. Ro/SSA-52, Ro/SSA-60, Scl 70 (Topoisomerase I), Jo-1, U1 ribonucleoprotein (snRNP) A, U1 snRNP68/70, U1 snRNP C], vasculitis-associated antigens [myeloperoxidase (MPO), and Antineutrophil cytoplasmic antibodies – proteinase 3 (ANCA-PR3)], T1D-related antigens [Insulin, Proinsulin, Zinc transporter 8 protein (ZnT8), Glutamic acid decarboxylase 65 (GAD65), Insulinoma 2(IA-2)], thyroiditis-associated-antigens (thyroid peroxidase [TPO], thyroglobulin), and celiac disease-related antigens (transglutaminase 2 [TGM2], deamidated forms of gliadin peptides [DGP]), among others. Supplementary Table 1 shows the full list of autoantigens tested in the study.

Materials and methods

Study design and patients

The study consists of four groups. 1) ICU COVID-19 positive patients (18 subjects, sampled between ICU day 1–26, three-six time points/patient), 2) Ward-treated COVID-19 positive patients (21 subjects, sampled on hospital day 1 and 3), 3) ICU (Sepsis) COVID-19 negative patients (18 subjects, sampled on ICU day 1 and 3), 4) Uninfected healthy controls (59 subjects, single samples). The healthy negative group consists of two cohorts; one (20 subjects) deriving from healthy blood donors in our region (Ontario, Canada; here referred to as Ontario Negative), and the second (39 subjects) commercially purchased by MSD, USA (referred to as MSD Negative), both collected prior to the COVID-19 pandemic. All COVID-19-infected and uninfected patients were confirmed by nasopharyngeal sampling and RT-PCR analysis. Samples from COVID-19 positive patients and COVID-19 negative patients were collected from March – May 2020, during the first wave of the COVID-19 pandemic, when the wild-type SARS-CoV-2 was prevalent.

Demographic and clinical characteristics of participants in the study are shown in Table 1.

This is a retrospective study. Our study was approved by the Institutional Review Boards of the Lawson Health Research Institute and Mount Sinai Hospital and was conducted according to the Helsinki declaration.

Table 1: Study subject demographic and clinical characteristics.

Variable	ICU COVID-19 positive (n = 18)	ICU COVID-19 negative (n = 18)	Ward COVID-19 positive (n = 21)	Healthy (n = 59)	p-Value	Sig. p-Value (post-hoc tests)
Age, years (mean ± StDev)	61.56 ± 8.99	61.78 ± 8.72	62.86 ± 14.89	48.54 ± 15.97	<0.0001	Healthy vs. ward-positive (0.0006) Healthy vs. ICU-positive (0.0043) Healthy vs. ICU-negative (0.0035)
Sex (% female)	61%	61%	33.3%	44%	0.1998	
Weight, kg (mean ± StDev)	88.26 ± 17.96	73.57 ± 20.52	85.81 ± 14.81		0.0361	ICU-positive vs. ICU-negative (0.046)
BMI, kg/m^2 (mean ± StDev)	31.86 ± 7.15	25.40 ± 5.91	28.82 ± 4.57		0.013	ICU-positive vs. ICU-negative (0.0094)
Diabetes, %	28%	33%	24%		0.3092	
Hypertension, %	50%	61%	38%		0.3566	
Steroids, %	72%	33%	100%		<0.0001	ICU-positive vs. ICU-negative (0.0437) ICU-positive vs. ward-positive (0.0149) ICU-negative vs. ward-positive (<0.0001)
Vasoactive medications, %	89%	67%	10%		<0.0001	ICU-positive vs. ward-positive (<0.001) ICU-negative vs. ward-positive (0.0005)
High-flow nasal cannula, %	56%	28%	62%		0.0841	
Antiplatelet treatment, %	22%	50%	14%		0.0375	ICU-negative vs. ward-positive (0.0346)
Non-invasive mechanical ventilation, %	33%	22%	10%		0.1894	
Invasive mechanical ventilation, %	89%	78%	14%		<0.0001	ICU-positive vs. ward-positive (<0.0001) ICU-negative vs. ward-positive (<0.0001)
ICU hospital, days [median (IQR)]	17 (17.5)	5 (2.25)	11 (8)		<0.0001	ICU-positive vs. ICU-negative (<0.0001) ICU-negative vs. ward-positive (0.0032)
Death, %	39%	6%	14%		0.0308	ICU-positive vs. ICU-negative (0.0408)
WBC, $\times 10^9$/L (mean ± StDev)	11.26 ± 6.04	15.87 ± 7.25	7.90 ± 2.99		0.0002	ICU-positive vs. ICU-negative (0.0434) ICU-negative vs. ward-positive (0.0001)
Neutrophils, $\times 10^9$/L (mean ± StDev)	9.36 ± 5.43	13.33 ± 6.88	6.43 ± 2.89		0.0006	ICU-negative vs. ward-positive (0.0004)
Lymphocytes, $\times 10^9$/L (mean ± StDev)	0.74 ± 0.44	1.61 ± 1.40	0.96 ± 0.69		0.0208	ICU-positive vs. ICU-negative (0.0214)
Platelets, $\times 10^9$/L (mean ± StDev)	224.89 ± 92.81	269.06 ± 121.00	214.81 ± 61.10		0.1756	

p-Value denotes differences between all groups, whereas sig. p-value shows only significant differences between two groups, as revealed by post-hoc pairwise comparisons. StDev, standard deviation; ICU, intensive care unit.

Quantitation of autoantibody levels

Autoantibody measurements were carried out using MSD's bridging or classical serology approaches. The assay format (bridging simultaneous, bridging sequential, or classical serology; all using MSD's U-PLEX technology) as used per type of marker is referenced in Supplementary Table 1. Samples for the bridging simultaneous assays were acid-treated. All samples were diluted six or 30-fold and tested in duplicate on each assay plate. To quantitate the autoantibody responses for each autoantigen and assess assay reproducibility, samples were tested along with MSD human serum-derived positive and negative controls and calibrators on each assay plate. Autoantibody concentrations were derived from their respective calibration curves and presented as arbitrary units (Units/mL). Samples with values at or below the limits of detection (LOD) were assigned LOD values.

Determination of cut-off levels

Cut-off values for autoantibodies against each autoantigen were determined based on their values in the healthy cohort. Initially, cut-off values were determined for each healthy cohort (MSD Negative and Ontario Negative) using their respective median concentrations of healthy subjects + (2.2 × Interquartile Range) for most assays, and median + (0.4 × LOD) for GAD65, insulin and proinsulin antibody assays as most samples presented with values at or below the LOD and hence were assigned the LOD concentration. These estimations yielded overall comparable results between the two healthy groups (Supplementary Table 2). A technical problem precluded determination of cut-offs using MSD negative samples for four assays (DGP, TGM2, thyroglobulin, Smith A [bridging]). Therefore, for consistency, the final cut-offs for all markers were determined using the Ontario Negative cohort. The markers in which at least 80% of samples in every group were below the detection limit (GAD-65, insulin, proinsulin), were excluded from further analysis (Supplementary Figure 1).

Statistical analysis

Statistical analyses were conducted using Prism 9 (GraphPad) software. Graphs were done using Excel (Office 360). Autoreactivity against each analyte was assessed based on the cut-off values established for every analyte, as described above, and analyses were performed in three ways, a) across the study time points for every subject with severe COVID-19 (ICU COVID-19 positive group), b) averaged for all time points for every subject in all patient groups, c) only the first time point per subject was considered. Statistical analyses to compare differences in autoreactivity between the four study groups were conducted using either the average or first values. Since the healthy control group consisted of two cohorts (Ontario Negative and MSD Negative) we performed five group comparisons first. If there were no significant differences between the two healthy cohorts, then their values were combined in one group and four group comparisons were carried out. If there were differences between the healthy control cohorts, the data were not analyzed further. Assays for which one control group was missing due to technical problems (DGP, TGM2, thyroglobulin, Smith A [bridging]) were excluded from further analysis (Supplementary Figure 1), to ensure that only markers for which the two control cohorts were statistically similar were considered. To this end, a Kruskal–Wallis test was performed, as at least one group would fit the criteria for non-parametric data. Post-hoc tests for selected pairwise comparisons were carried out corrected for false discovery rate (FDR). Continuous variables in the clinical data (e.g. age, blood cell counts) were analyzed by either ANOVA or Kruskal–Wallis, depending on whether the data fit the criteria for parametric testing – followed by appropriate multiple pairwise comparisons. Categorical variables in the clinical dataset (e.g. sex, pre-existing conditions, treatment) were analyzed by a Fisher's exact test (for two groups comparisons) or Chi-square test (more than two groups comparisons). For all analyses, statistical significance was set to 0.05, after correcting for multiple comparisons where appropriate. Graphs show mean (Units/mL) ± standard error of mean (SEM).

Results

Study group characteristics

The COVID-19-positive cohort consisted of 39 patients with an RT-PCR confirmed diagnosis of COVID-19, of whom 18 (46%) were treated in ICU at the time of sample collection, whereas the remaining 21 (54%) patients received ward care. The COVID-19-negative cohort consisted of 77 individuals, of whom 18 (24%) received care in the ICU for sepsis [ICU (Sepsis) COVID-19 negative group], and the remaining 59 (76%) were healthy individuals who donated blood prior to COVID-19 pandemic onset. Female/male ratio was similar across all four groups. Age differed significantly between the healthy control group and the three patient groups, although it was similar when compared between the patient groups alone. Of note, age was similar between the Ontario Negative cohort (which is the cohort used for the final cut-off values in the study; see methods for details) and all three patient groups. However, individuals in the MSD cohort were younger, thus significantly lowering the mean age of the healthy group (consisting of Ontario Negative and MSD Negative) compared to the patient groups. Demographic and clinical characteristics of participants in the study are shown in Table 1.

All the ICU-COVID-19 positive patients received antibiotic therapy, and 72% of them received corticosteroids, compared to 33% in the ICU (Sepsis) COVID-19 negative group, and 100% in the ward-COVID-19 positive group. High-flow oxygen was administered in 56% of the ICU-COVID-19 positive patients, compared to 28% of ICU (Sepsis) COVID-19 negative and 62% of the ward-COVID-19 positive patients. Invasive mechanical ventilation was provided in 89% and non-invasive mechanical ventilation in 33% of ICU-COVID-19-positive patients compared to 22 and 78% in ICU (sepsis) COVID-19 negative, and 10 and 14% in ward-COVID-19 positive patients, respectively. The

median number of ICU days for patients in the ICU-COVID-19 positive group was 17 and 39% of patients in this group died, whereas in the ICU (sepsis) COVID-19 negative group ICU length (median) was 5 days, and death rate was 6%. These differences were statistically significant (Table 1). In the ward-COVID-19 positive group, hospitalization length (median) was 11 days and death rate was 14%. Taken together, the ICU-COVID-19 positive group was matched to the ICU (Sepsis) COVID-19 negative group and ward-COVID-19 positive for age and sex but variables such as steroid use and ICU duration were different.

Statistical differences in demographic and clinical data between groups of the study are detailed in Table 1.

Autoantibody quantitation

There was no significant elevation of autoantibodies against any of the markers tested in the ICU-COVID-19 positive groups compared to the rest of the groups. On average, for all markers assessed, the majority (84%) of patients with severe COVID-19 had autoantibody levels lower than the specified cut-off values at any given time point (Figure 1, ICU COVID-19 positive). Similarly, 83% of patients in the ward COVID-19 positive group and 92% in the ICU (Sepsis) COVID-19 negative group had autoantibodies below cut-off values (Figure 1, controls). Overall, the percent changes in autoantibody reactivity between the first and second time point of assessment –which were common time points among the patient groups– were similar between the ICU-COVID-19 positive, ICU (sepsis) COVID-19 negative and ward COVID-19 positive patients. In the ICU-COVID-19 positive group, the highest change in autoantibody reactivity was between the first (ICU day 1) and the third (ICU day 10) time point of assessment for most markers (Figure 1, ICU COVID-19 positive), but we did not have data from a third time point in the control patient groups to evaluate whether the rate of change in autoreactivity differed between groups during the first 10 days of ICU/hospitalization. Taken together, we found no significant increase in autoantibody levels against our panel of autoantigens in patients with severe COVID-19, compared to the rest of the groups.

Discussion

In this study, we measured the levels of autoantibodies in patients with COVID-19 against 23 self-antigens used for diagnosis of common autoimmune diseases, to determine whether patients with severe COVID-19 had significantly elevated levels of such autoantibodies. Our findings show that patients with severe COVID-19 do not have significantly elevated levels of autoantibodies against such markers during the first month of ICU care.

It typically takes 2 weeks to develop new antibodies [11]. In individuals with COVID-19 it takes 2–3 weeks to develop anti-SARS-CoV-2 antibodies following disease onset, irrespective of disease severity [12–14]. Thus, given the time course of this study, the autoantibodies measured here most likely represent pre-existing rather than newly-induced autoantibodies. Indeed, our data corroborate this notion, as we did not see a significant increase in autoantibody levels for any of the markers in the ICU-COVID-19 positive cohort – which we followed for up to 26 ICU days. Given that on average (median) ICU admission for COVID-19 occurs 10 days after symptom onset [8], the time course of our study corresponds to approximately 10–36 days post-symptom onset. Other studies have shown elevated autoantibodies in patients with COVID-19 during similar time courses as ours. In particular, the study by Wong et al., conducted within 1–35 days post-symptom onset found increased autoantibody reactivity against immune-related proteins such as interferons (IFNs), cytokines and complement proteins in individuals infected with SARS-CoV-2, compared to uninfected controls, with highest autoreactivities observed in patients with severe disease [6]. Other studies showed that about 10% of patients with critical COVID-19 disease present with elevated anti-type I IFN autoantibodies and confirmed their pre-existing nature [7, 8].

Numerous studies have found increased levels of autoantibodies against various other autoantigens in critically ill patients with COVID-19 [summarized in articles [15, 16]], however a common limitation in these studies is the lack of matched contemporaneous uninfected healthy and/or uninfected critically ill controls [17]. To circumvent this limitation here we included samples from ICU(Sepsis) COVID-19 negative patients and ward COVID-19 positive patients, all collected during the same period as the samples for the ICU COVID-19 positive group. While the patient groups [i.e. ICU COVID-19 positive, ICU(Sepsis) COVID-19 negative and ward-COVID-19 positive] were matched for age, sex and pre-existing conditions (diabetes and hypertension) there were significant differences in important variables such as BMI, use of steroids, death rate etc. As an additional control, patient samples were benchmarked against sex-matched uninfected healthy controls. Thus, differences between our study and others showing increased levels of autoantibodies in critically ill patients with COVID-19 [15, 16] could result from differences in experimental and analytical approaches. Indeed and

Figure 1: Levels of autoantibodies in control groups (Controls) and patients with severe COVID-19 (ICU COVID-19 positive), against common diagnostic markers of autoimmune disease.
The dotted line indicates cut-off for abnormal values. Values above the dotted lines are marked as cases. Groups: ICU-neg = ICU (sepsis) COVID-19 negative, Ward-Pos = ward COVID-19 positive, Healthy = healthy, uninfected controls consisting of two healthy control cohorts (see methods).

similar to our observations, when comparing between critically ill COVID-19 positive and critically ill COVID-19 negative patients, levels of anti-cardiolipin and other anti-phospholipid antibodies were similar between groups [18].

Furthermore, here we established cut-offs for identifying abnormally elevated levels of autoantibodies based on our regional uninfected healthy cohort (Ontario Negative). Following a similar approach, Lerma et al., found that when using manufacturer's suggested thresholds to classify a positive result, 25% (16/64) of patients who were hospitalized for COVID-19 had elevated levels of antibodies against anti-nuclear antigens such as SS-A (including both Ro52 and Ro60), SS-B, Sm, Sm/RNP, ribonucleoprotein (RNP), Scl-70/topoisomerase I, and Jo-1. However, when they used internally validated cut-offs based on regional healthy groups, the percentage of patients who had elevated autoantibodies against these antigens dropped to ~3% (2/64) [19]; notably the latter patients had a known history of systemic lupus erythematosus and autoantibodies, further suggesting that the observed autoreactivity was not induced as a result of COVID-19. Thus, our results, which are benchmarked against cut-offs based on regional healthy control cohorts, resemble the lower frequency range observed in the study by Lerma et al. [19]. Similarly, others showed that patients with severe COVID-19 did not present with elevated anti-phospholipid autoantibodies [20].

While this study shows that patients with severe COVID-19 do not have higher levels of autoantibodies against the autoantigens tested here, it is important to note some of its limitations. First, we do not have detailed clinical data for the healthy groups. While the individuals of the healthy groups were not infected with SARS-CoV-2 and had no known history of autoimmune disease at the time of sample collection, it remains unknown whether these individuals developed autoimmune disease later. Since the cut-off values were determined based on the regional healthy cohort (Ontario negative) in our study, we cannot exclude the possibility that our cut-off values may have been artifactually higher than normal if some of the healthy individuals had abnormally high autoantibodies linked with an unknown or subclinical autoimmune disease. Additionally, while we tested for 23 self-antigens, commonly used for diagnosing certain autoimmune diseases, we cannot exclude the possibility that other self-antigens could be better targets for autoimmune responses in COVID-19, during the time course selected here. Future studies using a discovery approach such as label-free mass spectrometry [21, 22], followed by labelled targeted approach similar to the technology used here, may be more useful for the identification and validation of novel autoantigens following infection by SARS-CoV-2. Lastly, here we looked at acute and post-acute time points of COVID-19, with a focus on pre-existing autoantibodies. However, such early observations do not allow us to know whether additional autoreactivity will develop at longer time points and how such changes will affect a patient's health in the long term. Thus, future studies should look at the development of autoantibodies at longer time points after COVID-19 diagnosis.

Notably, increased levels of autoantibodies against any self-antigen alone does not diagnose autoimmune disease. Rather, these tests are used in tandem with one's overall clinical presentation (symptoms, progression etc.) [23]. This is especially important when interpreting the results of our (and others) study, where it is evident that a small percentage of ICU-COVID-19 positive patients had elevated autoantibodies in some cases from the beginning of the study (e.g. ANCA/PR3, Jo-1, TPO, Scl 70) and in other cases, appearing during the course of the study (e.g. ACPA/CCP, Smith, Ro/SSA-52, TPO). These patients did not have any pre-existing autoimmune condition and it is unknown whether they developed autoimmunity later. However, we cannot diagnose autoimmune disease based on these elevated levels of autoantibodies alone. It is also important to note that some patients had elevated autoantibodies against multiple markers. For example, in one patient antibodies against ACPA/CCP and Smith increased within the first 10 days of ICU care, whereas another patient had elevated autoantibodies against ANCA/PR3, Jo-1, IA-2, ACPA/CCP and Smith throughout the study course; the latter patient had pre-existing diabetes –although the type of diabetes was not determined– and died after the study. Thus, while we cannot derive meaningful conclusions about the autoimmune status of severe COVID-19 patients based on the autoantibody levels alone, autoantibody profiling in a multiplex format could be useful for disease diagnosis and management, in a case-by-case scenario.

Taken together, we conclude that levels of autoantibodies against a preselected set of autoantigens that are used to diagnose common autoimmune diseases are not elevated significantly in patients with severe COVID-19 during ICU care (1–26 ICU days). Future studies including longer sampling time points are warranted to assess whether infection with SARS-CoV-2 results in *de novo* autoimmune responses or exaggeration of pre-existing autoimmune disease.

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Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013) and has been approved by the authors' Institutional Review Boards (Lawson Health Research Institute and Mount Sinai Hospital).

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