Title page

Title: Ozone therapy for patients with COVID-19 pneumonia: a Quasi-Randomized Controlled Trial

Short running title: Ozonated blood as a therapy for COVID-19 pneumonia

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Conflict of interest statement. All the authors declare that there is no conflict of interest.

There is not any financial support for this study.

Clinical trial registration number: NCT04370223

Word Count: 3016

Abstract

Background: There is still no specific treatment strategies for COVID-19 other than supportive management. The potential biological benefits of ozonated autohemotherapy include reduced tissue hypoxia, decreased hypercoagulability, modulated immune function with inhibition of inflammatory mediators, improved phagocytic function, and impaired viral replication.

Objective: To determine the impact of the use of ozonated blood on time to clinical improvement in patients with severe COVID-19 pneumonia.

Design: A Quasi-Randomized Controlled Trial determined by admittance to the hospital based on bed availability.

Setting: Internal Medicine ward at Policlinica Ibiza Hospital, Spain.

Participants: Eighteen patients with COVID-19 infection (laboratory confirmed) severe pneumonia admitted to hospital between 20th March and 19th April 2020. The mean age of the cohort was 68 years-old and 72% (n=13) were male.

Intervention: Patients admitted to the hospital during the study period were pre-randomized to different beds based on bed availability. Depending on the bed the patient was admitted, the treatment was ozone autohemotherapy or standard treatment. Patients in the therapy arm received ozonated blood twice daily starting on the day of admission for a median of four days. Each treatment involved administration of 200 mL autologous whole blood enriched with 200 mL of oxygen-ozone mixture with a 40 μg/mL ozone concentration.

Main Outcomes: The primary outcome was time from hospital admission to clinical improvement, which was defined as either hospital discharge or a two-point improvement in clinical status measured on a six-point ordinal scale. Secondary outcomes were clinical improvement measured on the 7th, 14th and 28th day after admission, as well as time to a two-fold reduction in concentrations of C-reactive protein, ferritin, D-dimer and lactate dehydrogenase.

Results: Nine patients (50%) received ozonated autohemotherapy beginning on the day of admission. Ozonated autohemotherapy was associated with shorter time to clinical improvement (median [IQR]), 7 days [6-10] vs 28 days [8-31], p=0.04) and better outcomes at 14-days (88.8% vs 33.3%, p=0.01). In risk-adjusted analyses, ozonated autohemotherapy was associated with a shorter mean time to clinical improvement (-11.3 days, p=0.04, 95% CI -22.25 to -0.42).

Conclusion: Ozonated autohemotherapy was associated with a significantly shorter time to clinical improvement in this quasi-randomized controlled trial. Given the small sample size and study design, these results require evaluation in larger randomized controlled trials.

Introduction

The COVID-19 pandemic has led to more than 28.7 million cases and 920.847 deaths globally as of September 2020. [1] About 15% of infected adults develop severe pneumonia requiring supplemental oxygen, and an additional 5% progress to acute respiratory distress syndrome (ARDS) requiring mechanical ventilation often for several weeks. [2,3]

Supportive measures remain the cornerstone for treating COVID-19 in the absence of specific therapies. The potential biological benefits of ozonated autohemotherapy include reduced tissue hypoxia, decreased hypercoagulability, modulated immune function with inhibition of inflammatory mediators, improved phagocytic function, and impaired viral replication. [4-13] Ozone therapy is the administration of a mixture of gas of 97% oxygen and 3% ozone generated from a medical ozone generator. Ozone is a molecule which consists of three oxygen atoms all sharing the same electrons. Because there just are not enough electrons to go around, ozone is a relatively unstable molecule. This instability is why it is such a powerful biological stimulant. [14] Ozone therapy can be administered systemically by adding it to a sample of a patient's own blood sample and then reinfusing it, in what is termed ‘ozonated autohemotherapy’.

Ozone is a naturally occurring gas produced from oxygen atoms. Single oxygen atoms cannot endure alone without being regrouped into di-atomic oxygen molecules. In this recombination phase, some atoms will transform into loosely bound tri-atomic oxygen. This novel trioxygen molecule is called ozone which is found in the stratosphere where absorbs various ultraviolet radiation to protect us. Its molecular weight is of 48 g/mol with a solubility in water of 0.57 g/L at a temperature of 20 °C, (about ten-fold higher than oxygen). Consequently, the great solubility of ozone in water allows its immediate reaction with any soluble compounds and biomolecules present in biological fluids.

Ozone is generated by medical devices for medical purposes. Medical ozone is obtained from pure oxygen by passing it through a high voltage gradient (5-13 KV). This yields a gas mixture consisting of at least 95% oxygen and no more than 5% ozone. Thermodynamically is unstable and spontaneously reverts back into oxygen. Concentrations ranging from 10-70 μg/ml are commonly used for medical purposes. There are multiples routes for medical ozone administration. Inhalation route may be toxic to the pulmonary system and other organs. However, ozonated autohemotherapy has been shown to be safe in multiple randomized clinical trials, observational studies and meta-analyses. [15] The incidence of side effects of ozone therapy is very low (estimated at 0.0007%), and typically manifests itself as euphoria, nausea, headaches and fatigue. [16] In general, it is a very safe therapy when administered correctly, with the recommended dose. Complications like air embolism have been described but are caused by incorrect administration practices and by using non-certified equipment.

Several countries including Spain, Italy, Greece, Cuba, Russia, Portugal and Turkey have incorporated ozone therapy in medical practice for other indications. [17]

The pathogenesis of the virus is variable and not fully understood. It predominantly involves the lungs where diffuse alveolar damage with involvement of the microcirculation leads to marked hypoxia. [18,19] A dysregulation of the immune response is present and lymphocytopenia is a hallmark in the vast majority of these patients. [20] Innate immunity and coagulation pathways are intricately

linked. [21] COVID-19–associated macrophage activation, hyperferritinemia, cytokine storm, release of pathogen-associated molecular patterns and damage-associated molecular proteins can result in release of tissue factor and activation of coagulation factors that create a predisposition to hypercoagulability. [21]

Others have reported the use of ozonated autohemotherapy in patients with severe COVID-19 pneumonia, however, they had limitations. [22,23] The Scientific Society of Oxygen-Ozone Therapy (SIOOT) described a case series of 73 patients, of whom 32.8% were intubated and 67.1% were non-intubated. [24] Of all the intubated-patients, 62.5% were extubated after five sessions with ozone autohemotherapy. Of all the non-intubated patients, only 6% required intubation. Whereas, the group of patients with only usual care without ozone auothemotherapy, had only a 20% rate of recovery among the intubated patients. We, therefore, conducted a quasi-randomized controlled trial determined by admittance to the hospital based on bed availability to determine if ozonated autohemotherapy was associated with a shorter time to clinical improvement in patients with severe COVID-19 pneumonia.

Materials and methods

Study Design

This quasi-randomized controlled trial was performed at the Policlinica Ibiza Hospital in Spain. It was conducted in compliance with the Declaration of Helsinki and approved by a multidisciplinary human research ethics committee (HREC) at the institution. Each participant gave written informed consent for administration of any interventions, collection of relevant clinical data and ascertainment of outcomes. The study consisted of all adults (aged ≥18 years) who were admitted to the hospital with a diagnosis of severe COVID-19 pneumonia between 20th March to 19th April 2020. All included patients met the following criteria: confirmed COVID-19 infection (diagnosed by nasopharyngeal swab performed on admission); severe pneumonia with baseline chest X-ray

abnormalities; PaO_2/FiO_2 ratio <300 or oxygen saturation <94% on room air, and tachypnea with respiratory rate exceeding 30 per minute.

Pre-randomization

Patients admitted to the hospital during the study period were pre-randomized to different beds based on bed availability. Depending on the bed the patient was admitted, the treatment was ozone autohemotherapy or standard treatment.

Standard Clinical Care

Treatment for all COVID-19 pneumonia patients included supplemental oxygen therapy, hydroxychloroquine, lopinavir, ritonavir, corticosteroids, and antibiotics (including azithromycin) at the discretion of the individual patient's attending physician. Decisions on endotracheal intubation, mechanical ventilation and critical care unit admission were made following clinical standards and at the discretion of the patient's attending physician.

Ozonated Autohemotherapy Intervention

Ozonated autohemotherapy involved intravenous infusion of ozonated autologous whole blood. Initially, 200 mL of autologous whole blood was drawn from the patient's antecubital vein into a standard plastic disposable blood collection bag (certified $SANO_3$ bag) containing 35 mL of anticoagulant citrate dextrose solution (ACD-A). The blood was then enriched with 200 mL of gas mixture oxygen-ozone with an ozone concentration at 40 μg/mL obtained by *Ozonobaric P Sedecal*, an ozone generator with CE0120 certificate type IIb. The ozonized blood was then slowly re-infused into the same vein over approximately 10 minutes. [25]

Outcomes

The primary clinical outcome was time to clinical improvement during hospital admission.

Clinical evaluation

Clinical improvement was defined as a two-point reduction (relative to the patient's status on hospital admission) on a six-point ordinal scale, or discharge alive from the hospital, whichever came first. The six-point scale was as follows: death (6 points); extracorporeal membrane oxygenation or mechanical ventilation (5 points); noninvasive ventilation or high-flow oxygen therapy (4 points); oxygen therapy without need for high-flow oxygen or non-invasive ventilation (3 points); hospital admission without need for oxygen therapy (2 points); and discharged from hospital or reached discharge criteria (1 point). Discharge criteria were as evidence of clinical recovery (normalization of pyrexia, respiratory rate <24 per minute, oxygen saturation >94% on room air, and absence of cough) for at least 72 hours.

A six-point scale and definition of clinical improvement (i.e., two-point improvement in scale) has been used in prior research on intervention for relating to COVID-19 infection. [26] Personnel ascertaining outcomes were not blinded to whether patients received usual care versus ozonated autohemotherapy.

Secondary outcomes were clinical improvement as measured at the 7th, 14th and 28th days after hospital admission. Time to a two-fold decrease in concentrations of C-reactive protein, ferritin, D-dimer and lactate dehydrogenase were also daily measured. Patients also underwent repeat COVID-19 PCR testing at 5 and 15 days after hospital admission. Follow-up ceased at the point of hospital discharge, patient death, or 31 days following hospital admission, which ever came first.

Statistical Analysis

All analyses were performed using STATA version 13.0 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). Statistical significance was defined by a

2-sided P-value less than 0.05. The Shapiro-Wilk test was used to determine whether variables were normally distributed. Unadjusted differences between treatment and control arms were then calculated using the two-sample t-test (normally distributed continuous variables), Mann-Whitney U-test (continuous variables with evidence of non-normal distributions) and Fisher's exact test (categorical variables). Unadjusted times to clinical improvement were compared between the two study arms using Kaplan-Meier survival curves and the log-rank test. Patients were censored at the point of hospital discharge, death or 31 days following hospital admission, whichever came first. The adjusted association between ozonated autohemotherapy and mean time to clinical improvement was estimated using a multivariable linear regression model that adjusted for age, sex, and baseline quick SOFA score. These covariates were pre-specified on the basis of their clinical significance. Patients who had not achieved clinical improvement within the follow-up period were assigned a time value of 31 days. All patients admitted to the study site within a pragmatic one-month period were included in the study cohort.

Results

The cohort included 18 patients. The mean age was 68 years old (SD 15 years) and 72.2% (n=13) were male. The baseline characteristics of these patients are presented in Table 1. In total, 9 patients (50%) received ozonated autohemotherapy. The baseline characteristics of the two study arms were qualitatively similar, aside from age (mean age was higher in the usual care arm), weight (mean weight was higher in the usual care arm), and body mass index (mean value was higher in the supportive care only arm).

Table 1. Baseline characteristics

	Ozonated Autohemotherapy (n=9)	**Usual Clinical Care (n= 9)**	**p-value**
Age, mean (SD), years	64 (11)	71 (18)	0.35

Male sex, n (%)	7 (78%)	6 (67%)	1
Weight, mean (SD), kg	74 (17)	85 (23)	0.25
Height, mean (SD), cm	167 (10)	170 (7)	0.48
Body mass index, mean (SD), kg/m2	26.2 (4.5)	29.5 (7.1)	0.26
Hypertension, n (%)	4 (44%)	6 (67%)	0.34
Diabetes mellitus, n (%)	0 (0%)	2 (22%)	0.47
Chronic pulmonary disease, n (%)	2 (22%)	1 (11%)	1
Chronic cardiac disease, n (%)	1 (11%)	2 (22%)	1
Previous stroke, n (%)	0 (0%)	0 (0%)	1
Baseline hemoglobin, mean (SD), mg/dL	13 (2.1)	13 (3.0)	0.51
Baseline Quick SOFA score of 2 or 3, n (%)	1 (11%)	1 (11%)	1
Baseline WHO score, median [IQR]	3 [3-3]	3 [2-3]	0.60
Baseline Lactate Dehydrogenase, mean (SD), U/L	487 (168)	506 (123)	0.80
Baseline SpO2/FiO2 ratio, median [IQR]	350 [255-408]	339 [261-452]	0.96
Treatment			
Hydroxychloroquine, n (%)	4 (44%)	3 (33%)	0.23
Lopinavir/ritonavir, n (%)	1 (11 %)	2 (22%)	1
Corticosteroids, n (%)	2 (22%)	1 (11%)	1
Ceftriaxone, n (%)	1 (11%)	1 (11%)	1
Levofloxacin, n (%)	2 (22%)	2 (22%)	1
Azithromycin, n (%)	8 (89%)	7 (78%)	1
Therapeutic anticoagulation, n (%)	0 (0 %)	2 (22%)	1

Primary outcome: Time to clinical improvement

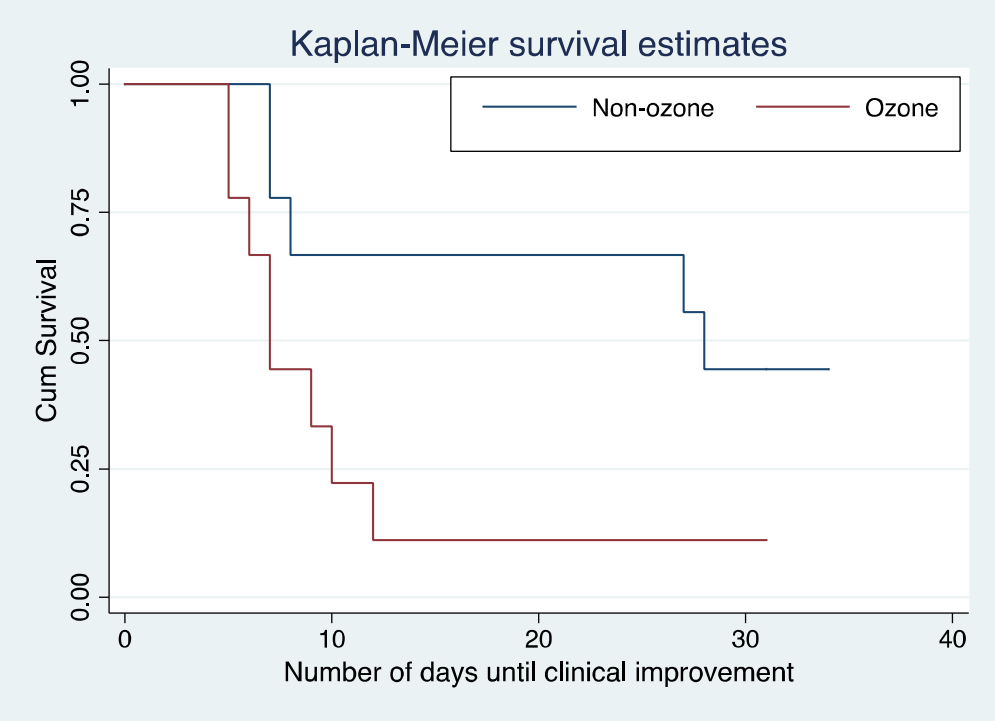
Ozonated autohemotherapy was associated with a significantly lower time to clinical improvement (median [IQR]), 7 days [6-10] vs 28 days [8-31], p=0.04) (Figure 1 and Table 2). In unadjusted linear regression analyses, the mean time to clinical improvement was 12.4 days shorter in the ozonated autohemotherapy arm (-12.4 days; p=0.01; 95% CI -22.49 to -2.39). In adjusted linear regression analyses, the mean time to clinical improvement in the ozonated autohemotherapy arm was 11.3 days shorter (-11.3 days, p=0.04, 95% CI -22.25 to -0.42). We conducted a post-hoc sensitivity analysis that adjusted for age, quick SOFA and weight – all of which were baseline characteristics with qualitative differences between study arms. The adjusted difference in time to clinical improvement (-11.6 days, p=0.05, 95% CI -23.3 to 0.41) was qualitatively similar in this sensitivity analysis. Unadjusted times to clinical improvement using Kaplan-Meier survival curves

and the log-rank test showed a significant difference between groups (Log Rank (Mantel-Cox) Chi-square 4,182. p=0,041) (Fig 1).

Table 2. Outcomes

	Ozonated Autohemotherapy (n=9)	**Usual Clinical Care (n= 9)**	**p-value**
Time to clinical improvement, median [IQR], days	7 [6-10]	28 [8-31]	0.04
Clinical improvement at day 7, n (%)	4 (44%)	2 (22%)	0.31
Clinical improvement at day 14, n (%)	8 (89%)	3 (33%)	0.01
Clinical improvement at day 28, n (%)	8 (89%)	5 (56%)	0.29
Time to Temperature <37C, median [IQR], days	1 [1-1.5]	4 [2-5]	0.10
Time to PCR COVID-19 negative, mean (SD), days	13.1 (5.7)	21.4 (7.4)	0.05
Time to a 2-fold decreased C-reactive protein, median [IQR], days	3.5 [3-28]	13 [8-25]	0.008
Time to a 2-fold decreased D-dimer, median [IQR], days	4 [1-10]	19.5 [10-28]	0.009
Time to a 2-fold decreased ferritin, median [IQR], days	8 [5-10]	15 [10-25]	0.016
Time to a 2-fold decreased Lactate Dehydrogenase, median [IQR], days	9 [7-9]	25 [12-26]	0.01
Ventilator-free days at day 28, median [IQR], days	28 [28-28]	28 [0-28]	0.14
Intubation required, n (%)	0 (0%)	2 (22.%)	0.47
ICU-length of stay, median [IQR], days	0 [0-0]	0 [0-0]	0.24
Hospital-length of stay, median [IQR], days	8 [7-12]	28 [8-31]	0.09
In-hospital mortality, n (%)	1 (11%)	2 (22%)	1
28-day hospital mortality, n (%)	1 (11%)	2 (22%)	1

Figure 1. Kaplan-Meier survival curves



Secondary Outcomes

Ozonated autohemotherapy was associated with clinical improvement at day 14 (88.8% vs 33.3%, p=0.01). Ozonated autohemotherapy was also associated with a shorter time to a two-fold decrease of C-reactive protein (3.5 days [3-28] vs 13 days [8-25], p=0.008), ferritin (8 days [5-10] vs 15 days [10-25], p=0.016), D-dimer (4 days [1-10] vs 19.5 days [10-28], p=0.009) and Lactate Dehydrogenase (9 days [7-9] vs 25 days [12-26], p=0.01). The mean time to negative PCR COVID-19 testing results was reduced [13.1 (SD 5.7) vs 21.4 (SD 7.4 days), p=0.05). There was no difference with respect to ventilator-free days at day 28 (median [IQR]), 28 days [28-28] vs 28 days

[0-28], p=0.14) or 28-days mortality (11.1% vs 22.2%; p=1). No adverse events were observed or unintended effects in both groups.

Discussion

In this quasi-randomized controlled trial of 18 patients with confirmed COVID-19 severe pneumonia, twice-daily ozonated autohemotherapy was associated with a significant reduction in the time to clinical improvement. This cohort study provides novel new data pointing to the potential role of ozonated autohemotherapy for treatment of severe COVID-19 pneumonia.

Our findings are consistent with recent reviews describing the potential biologically plausible benefits associated with ozonated autohemotherapy for COVID-19. [27-29]

There is a potential role for ozonated autohemotherapy for treatment of patients with severe COVID-19 pneumonia, with several biological plausible mechanisms of action. When human blood is exposed to a gas mixture of oxygen and ozone, oxygen equilibrates with the extracellular and intraerythrocytic water before becoming bound to hemoglobin until it is fully oxygenated. On the contrary, ozone, more soluble than oxygen, readily dissolves in water and reacts instantaneously with biomolecules, such as amino acids (particularly cysteine, tryptophan, methionine, phenylalanine, and tyrosine) and with lipids (particularly the unsaturated fatty acids contained in membrane phospholipids). The former can yield disulfides and methionine sulfoxide; the latter can yield hydrogen peroxide, aldehydes, and hydroxyhydroperoxides. The compounds generated during the reactions [reactive oxygen species (ROS) and lipid ozonation products (LOPs)] represent the "ozone messengers" and are responsible for its biological and therapeutic effects [30] so ozone can be considered as a pro-drug that produces biochemical messengers.

Ozone might improve blood circulation and oxygen delivery to ischemic tissue [4-7] as a result of the concerted effect of nitric oxide, [8] increase intra-erythrocytic 2,3-DPG level, [9] and increase of some prostacyclins such as PGI2. [10] These effects can help to decrease the hypercoagulation that has been observed in COVID-19 patients. [11] Another important role played by ozone in COVID-19 is its immunomodulatory effects. The inflammatory response is a hallmark of severe infection and cytokine modulation is key to avoid patient deterioration. Ozone is able to modulate and control cytokines releasing anti-inflammatory cytokines and reducing activity of pro-inflammatory such as IL-1, IL-6 and TNF-α counteracting the state of hyperinflammation seen in COVID patients, but in addition, ozone has potent anti-inflammatory properties through the modulation of the NLRP3 inflammasome which is recognized to play a crucial role in the initiation and continuance of inflammation in various diseases. [12] Ozone may also modulate the accumulation of neutrophils locally, the expression of IL-6, TNF-α, and albumin modified by ischemia in the kidneys, as well as increase local antioxidant capacity. [13]

Regarding to the specific potential action of the ozone against coronavirus, the effectiveness of ozone against pathogens is well known. The ozone appears to be the best agent available for sterilizing water [31], although the in-vivo virucidal activity of ozone in the dosage used in this present study is unknown. It has been suggested that ozone could act a signal molecule in the organism, being generated by human neutrophils and being necessary for antibody-catalyzed formation [32] which play a role in the natural humoral response to infection. [33] Ozone also is capable of inducing the release and modulation of IFN-γ, TNF-α and colony stimulating factors, [34, 35] and is also able to modulate and stimulate phagocytic function [36, 37] which may have a very positive effect in COVID-19 infection.

Finally, ozone may impair viral replication, as suggested in its effects on SARS and MERS. [38] Angiotensin-converting enzyme type 2 (ACE2) cell receptors has been identified as receptor for SARS-CoV-2 [39], which could be blocked with specific monoclonal antibodies but also through the control of the nuclear factor erythroid 2–related factor 2 (Nrf2) that regulates and blocks the activity

of this receptor. [40] Because ozone is able to cause a rapid Nrf2 activation, [41, 42] it seems very likely that this may be an important physiological mechanism to block endogenous COVID-19 reduplication by preventing contact with this receptor. Furthermore, spike proteins (S) is responsible for receptor binding and membrane fusion. [43] It contains a highly conserved transmembrane domain that consists of three parts: a N-terminal tryptophan-rich domain, a central domain, and a cysteine-rich C-terminal domain. Both, the cysteine-rich domain and tryptophan-rich domain, have been shown to be necessary for fusion. [43-45] Both cysteine and tryptophan, are sensitive to oxidation. It has been hypothesized that ozone metabolites could oxidize cysteine residues, making it difficult for the virus to enter the host cell and preventing viral replication. [46]

This proof of concept study points to the need for further research, such as a well-designed, well-powered multicenter randomized clinical trial. Limitations include the sample size of our cohort is small and single-centered. The 95% CIs for our adjusted estimates were wide, and do not exclude a 20–30% decrease in the coefficient for time (days) to clinical improvement. Outcome assessors were not blinded to the treatment arm assignment.

The strengths of this study include its pragmatic real-world COVID-19 population, use of objective primary clinical outcome and risk-adjustment using methods of regression modeling analyses.

In conclusion, ozonated autohemotherapy was associated with a significant shorter time to clinical improvement and shorter time to a two-fold decrease of C-reactive protein, ferritin, D-dimer and Lactate Dehydrogenase in severe COVID-19 pneumonia patients in this quasi-randomized controlled trial.

Acknowledgements

Dr. Vives affirm that has listed everyone who contributed significantly to the work.

Dr. Wijeysundera is supported in part by a Merit Award from the Department of Anesthesiology and Pain Medicine at the University of Toronto, and the Endowed Chair in Translational Anesthesiology Research at St. Michael's Hospital and University of Toronto.

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