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Photo: *Courtesy of the Finlay Vaccine Institute, Havana.*

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Without Accessible Primary Care, We Are "Dangerously Unprepared" for the Next Pandemic

Polio, yellow fever, diphtheria, tetanus, pertussis and other deadly or debilitating diseases are routinely prevented and controlled with vaccines—when and where they are accessible.[1] Characterized as one of the most effective public health interventions ever available, WHO estimates that immunization saves four to five million lives every year.[2]

And yet. After another year working to contain the worst pandemic in living memory, evidence-based science and the life-saving vaccines it produces struggle to be heard above the din of bad (and fake) news. Anti-vaxxers and -maskers grab headlines; more transmissible variants travel the globe; vaccine inequity places everyone at higher risk; and the most economically vulnerable plumb even darker depths.

WHO set a global target of 40% COVID-19 vaccination coverage by the end of 2021 and 70% by mid-2022. The first target was not met. The second target will not be met. In the meantime, the hard, tragic reality is that many more people will become sick and some will die—needlessly.

In the United States, despite the availability of vaccines proven to prevent severe COVID-19, hospitalizations and deaths, just over half of youngsters aged 12–18 years are fully vaccinated.[3] It's a woeful statistic given that a recent study by the Centers for Disease Control and Prevention (CDC) found the Pfizer vaccine for those aged 12–17 prevented 94% of hospitalizations and 98% of ICU remittances.[4] The vaccine has been available since May 2021. According to the same study, nearly all the teenagers admitted to ICUs in 31 pediatric hospitals around the country with COVID-19 were unvaccinated; seven of them died. Each of these deaths might have been prevented.

Meanwhile, vaccine inequity slams the more vulnerable precisely when they are most vulnerable: by the end of December 2021, only 7% of people in low-income countries had received even a single dose of a COVID-19 vaccine, an unconscionable statistic.[5] Viruses like SARS-CoV-2 take swift advantage of the unvaccinated to spread and mutate into deadlier and/or more transmissible variants—exactly what happened with delta and is happening now with omicron. In the last week of December alone, infections increased by 50% and deaths by 11% in the Americas, according to PAHO,[6] while the USA registered a record 1.35 million new cases in a single day in January.[7]

Whether you are unwilling or unable to get a COVID-19 vaccine, the outcome is the same: more infections, more severe cases and more deaths.

It is no secret why vaccination rates are so uneven and infections soaring. Inadequate and inequitable vaccine strategies by and within individual countries have led to hoarding millions of doses, compounded by poorly-coordinated mechanisms for administering them. Meanwhile, distrust of health authorities and their messaging—too often muddled or poorly communicated—weakens adherence to preventive measures like masking and distancing. Indeed, the CDC has come under fire for issuing "confusing and flawed" guidelines that fail to communicate health policy clearly.[8] Misinformation, conspiracy theories and false news flourish in this environment, fueling vaccine hesitancy, mistrust of health authorities and anti-science in general. The world's health professionals are left to deal with the fallout—until they burn out, retire or are themselves infected, deepening the crisis.

Effectively tackling these problems is rife with hurdles. Moreover, necessary system-wide initiatives are easy targets for detractors. Such an overhaul is branded too costly. Too complicated. Too cumbersome. Competing interests too entrenched to bother. But what this argument conveniently ignores is the one strategy that has proven effective, both in terms of health outcomes and return on investment: primary health care.

According to a recent report by the US National Academies of Sciences: "high-quality primary care is the foundation of a high-functioning health care system and is critical for…enhancing patient experience, improving population health [and] reducing costs."[9] Nevertheless, family physicians and nurses, the foundation of primary care, were initially left out of the nation's COVID-19 vaccine strategy. Instead, drugstores were tasked with administering the majority of vaccines and diagnostic kits, while community primary care practices—where routine and childhood vaccines, along with preventive and other care is provided to patients who have an ongoing relationship with their doctors—were sidelined. This adversely affected rapid, equitable distribution of COVID-19 vaccines, especially in rural, African-American and Hispanic communities. The fact that it was very difficult to get vaccinated against COVID-19 at a doctor's office in the United States contributed to vaccine hesitancy by not involving the health professionals most trusted by their patients: primary care providers.[10]

In Cuba, equitable vaccination, delivered via a strong, coordinated primary health care system is a cornerstone of the country's population health strategy and is helping contain COVID-19. Family doctors, nurses and medical students went door-to-door early in the pandemic to educate families and screen for symptoms. Community-based polyclinics and their staffs were accredited to participate in clinical trials for Cuba's vaccine candidates. And thousands of family doctors and nurses received training and accreditation to vaccinate young and old in their catchment areas, making for a streamlined rollout.

As of this writing, nearly 90% of the Cuban population is fully vaccinated against COVID-19—including infants as young as two—with a triple-dose schedule using vaccines developed and produced on the island (see our exclusives this issue with Rinaldo Puga, Principal Investigator for the vaccines' pediatric trials and Verena Muzio, Director of Clinical Research at the Genetic Engineering and Biotechnology Center in Havana). The result is a 0.83% fatality rate (compared to 2.03% in the Americas and 1.69% worldwide), reinforcing public trust in science, vaccines and the professionals administering them.[11]

This trust was hard won over decades, built on a three-pronged strategy of community-based primary care, universal access and science. Despite COVID-19 and the resulting economic blow

...the key lesson from COVID-19 that must be implemented urgently is building accessible, equitable and sustainable primary healthcare systems that respond to science

dealt to sectors such as tourism, not to mention the brutal US sanctions that affect the health of every Cuban, the country's national childhood immunization program and annual polio vaccination campaign have continued during the pandemic, uninterrupted, once again relying on the primary healthcare subsystem.

Vaccines and related public health measures can contain the current pandemic, but what about long COVID, heart and lung damage, chronic kidney impairment and other possible lasting health effects for convalescents—to say nothing of the mental health toll and psychological distress many are experiencing? What about the next pandemic, which experts warn could be more lethal than COVID-19 and for which the world is "dangerously unprepared?" Rather than "fall back into the decades-long cycle of panic and neglect that will leave the world at grave risk for inevitable future public health threats,"[12] the key lesson from COVID-19 that must be implemented urgently is building accessible, equitable and sustainable primary healthcare systems that respond to science. For millions, it's already too late.

The Editors

NOTES & REFERENCES

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Influence of Inflammation on Assessing Iron-Deficiency Anemia in Cuban Preschool Children
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Pita-Rodríguez GM, Chávez-Chong C, Lambert-Lamazares B, Montero-Díaz M, Selgas-Lizano R, Basabe Tuero B, et al. influence of inflammation on assessing iron-deficiency anemia in Cuban preschool children. MEDICC Rev. 2021 Jul–Oct;23(3–4):37–45. Available at: https://mediccreview.org/influence-inflammation-assessing-iron-deficiency-anemia-cuban-children/

Page 39, right column, fourth paragraph, line 4, “Adjustments were applied only in the case of CRP >lnCRPref, AGP >lnAGPref, or both” should read “Adjustments were applied only in the case of CRP >CRPref, AGP >AGPref, or both”

Page 41, Table 4, column 6, headed by lnAGP, replace all “less than” signs (<) by minus signs (–).

Vaccines and Public Trust: Containing COVID-19 in Cuba

Verena Muzio-González PhD DSc

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Conner Gorry MA

As 2021 drew to a close, Cuba struggled to contain the highly transmissible omicron variant of SARS-CoV-2, braced for a new wave of infections and kept a close eye on other variants of concern popping up around the world—a common experience to countries everywhere as we head into the second year of the pandemic. In Cuba, however, there is one marked difference making all the difference: by early January, 87% of the population was fully vaccinated using a three-dose schedule of vaccines developed and produced on the island. [1] This massive vaccination campaign is complemented by a rapid booster rollout—also using Cuban vaccines—that began in December 2021 and was ongoing as we finalized this issue.

The island nation was able to achieve the third highest COVID-19 vaccination rate in the world[2] after decades of scientific investment, research, discovery and innovation; regulatory oversight and compliance; professional training; and increased production capacity. But a vaccine is only as effective as the health system charged with administering it—in a safe and timely manner, to as many people as possible. Here too, Cuba has decades of experience, including a national pediatric immunization program where 98% of children under 5 are immunized against 13 diseases,[3] an annual polio vaccination campaign (both launched in 1962 and uninterrupted since) and campaigns to contain epidemics such as H1N1.

When the first COVID-19 cases were detected on the island in March 2020, Cuba harnessed this vaccine experience, making a hard tack towards developing its own vaccines. Two of the main protagonists in the country's biotechnology development, the Finlay Vaccine Institute (IFV) and the Genetic Engineering and Biotechnology Center (CIGB), both with several groundbreaking preventive and therapeutic vaccines in their portfolios, led the search for a vaccine. Today, Cuba has three vaccines authorized for emergency use—Soberana 02 and Soberana Plus developed by IFV, and Abdala, developed by CIGB. Schedules with these vaccines have demonstrated more than 90% efficacy in clinical trials,[4] and after regulatory approval for emergency use, became the backbone of Cuban COVID-19 vaccination efforts. A fourth vaccine, Mambisa (CIGB), administered nasally, and a fifth, Soberana 01 (IFV) are still in clinical trials.

For this installment in ***MEDICC Review's*** series spotlighting leading women of Cuban science, we sat down with Dr Verena Muzio, Director of Clinical Research at CIGB. A pioneer of Cuba's biotechnology sector, she is an immunologist with a doctorate in biological sciences. Her professional trajectory began researching the genetically engineered hepatitis B surface antigen that led to the development of Cuba's recombinant hepatitis B vaccine in 1989. The same technological platform used in this vaccine was used to develop CIGB's Abdala vaccine against SARS-CoV-2—part of the reason Cuba was able to secure a vaccine so quickly. A phase 3 clinical trial determined a 92.28% efficacy rate for Abdala, with results to appear in forthcoming publications.

MEDICC Review: **The recombinant hepatitis B vaccine, Heberbiovac-HB, developed over 30 years ago by CIGB, foreshadowed the potential of Cuba's biotechnology sector. Can you tell us about that experience?**

Verena Muzio: Developing the hepatitis B vaccine was a huge challenge, involving a lengthy clinical development and production process which began in 1986 under the direction of Dr Luis Herrera, founder and former research director of CIGB [interviewed by *MEDICC Review*, April 2020, Vol 22, No 2, Eds]. This was our first foray into developing a recombinant vaccine—using genetic engineering technology to obtain the surface antigen used in the final product. It turned out to be a watershed event.

With any vaccine, you first need a safe, effective product. Just as important, however, is having an appropriate vaccine strategy for administering it to the population. Our strategy with Heberbiovac-HB was to vaccinate our entire population under the age of 20 against hepatitis B by the year 2000. After phase 1−3 trials demonstrated its safety and immunogenicity and following approval from our national

regulatory authority, Heberbiovac-HB was incorporated into our national pediatric immunization program in 1992; every child born since has been vaccinated against hepatitis B within 24 hours of birth using our vaccine. And since 2000, no Cuban child under 5 has contracted acute hepatitis B. I've always said we can develop the vaccines, but it's our health professionals who implement the strategy, who make it possible to protect our population. And this is true in any health system, not just ours.

MEDICC Review: **Heberbiovac-HB was not only the first recombinant vaccine developed wholly in Cuba, it was also the first to receive WHO prequalification, correct?**

Verena Muzio: That's right. In 2001, Heberbiovac-HB was the first Cuban vaccine and the first from Latin America to receive WHO prequalification. It's a rigorous evaluation process and every prequalified vaccine is submitted to periodic review—it's not a designation that's authorized once and then retains this status. In 2009, another CIGB product, Quimi-Hib, our *Haemophilus influenzae* type B (Hib) vaccines, also received WHO pre-qualification.

MEDICC Review: **Can you talk about how these early experiences informed the development of CIGB's COVID-19 vaccines, specifically Abdala, used to vaccinate the majority of the Cuban adult population?**

Verena Muzio: Our work on the COVID-19 vaccines benefited directly from the evidence and experience accrued developing, producing and administering the hepatitis B vaccine through all stages: research, development, pre-clinical, and clinical, as well as production and management processes and quality control. It also served as a fundamental learning experience for many of those working at CIGB today. In fact, many of us who developed Heberbiovac-HB are also working in one way or another on the COVID-19 vaccines—there's a continuum and link between the work we did then and the work we're doing now.

One link is related to the science: there were several strategies and projects under consideration simultaneously when we started looking for a COVID-19 vaccine in early 2020. In the end, what showed the most promise was a recombinant antigen vaccine—in this case the optimal antigen was the SARS-CoV-2 receptor-binding domain, RBD, protein—produced in the yeast *Pichia pastoris*. This is the same technology and production platform used in Heberbiovac-HB, providing an important advantage.

Since we decided to pursue a recombinant vaccine using the same surface antigen component used in our hepatitis B vaccine, we knew from the start that our clinical strategy would be based on administering three doses. At that time, other vaccine developers around the world using different technology platforms were emphasizing one-dose strategies, that a 'one and done' approach would be sufficient. But our experience with recombinant proteins told us that a three-dose strategy would be best. The immunization schedule could vary, but we knew it would take three doses.

Obviously, evidence accumulated during the laboratory, pre-clinical and clinical stages isn't sufficient to make a definitive determination about immunization schedules. Nevertheless, 30 years of clinical evidence applying our hepatitis B vaccine provided a road map. Within seven months of administering the first dose of Abdala in December 2020, we amassed the clinical efficacy data and met the other requirements needed to request emergency use authorization (EUA) from our national regulatory authority, the Center for Quality Control of Medicines, Equipment and Medical Devices (CECMED). This was granted in July 2021 and the vaccination of our adult population began.

MEDICC Review: **Given the urgency and severity of this global pandemic, some have questioned Cuba's decision to develop its own vaccines for COVID-19. Can you speak to this?**

Verena Muzio: We knew it would be very difficult for us to procure a vaccine from abroad—the prices would be out of reach, the demand would be enormous and we are Cuba, which is an issue for some—plus we knew we had the technological and scientific capabilities to make our own. And we did. Today we have five COVID-19 vaccines developed and produced in Cuba. Three of them have received EUA.

At the beginning some people asked: why so many vaccines? But our experience has shown that it's beneficial to have options. This increases production capabilities and provides alternatives for our immunization strategy—by combining vaccines, for example. I think this is another important element and we're exploring it further: we helped develop the vaccination strategy for our pediatric population together with IFV and CECMED, and are evaluating the feasibility of administering IFV's Soberana 02 with Abdala in a heterologous schedule for Cuban children, and making it available to other countries.

MEDICC Review: **Cuban and other vaccines helping control the pandemic were developed very quickly—in a year, as opposed to the typical five-to-ten years. In Cuba's case, what made this possible?**

Verena Muzio: First, our government and health authorities took early, coordinated action. In January 2020, Cuba convened an Innovation Committee of all research institutes, CECMED, manufacturers and other institutions to prioritize products and design strategies to control the pandemic. This Committee issued the first version of our National COVID-19 Prevention & Control Plan and we set out to forge solutions—developing a Cuban vaccine against COVID-19 paramount among them.

The urgency and severity of this health problem, with so many cases and deaths, not only in Cuba but around the world, presented a new challenge to develop a safe, effective vaccine in record time while adhering to rigorous scientific practices. Given these conditions, our regulatory authority, like others around the world, established mechanisms to shorten the vaccine timeline—faster revision times, seamless trials and more—to be able to address COVID-19 quickly.

Another factor is the close and fluid working relationship CIGB has maintained with CECMED—from pre-clinical to post-clinical processes—for all CIGB products introduced into our health system [see *MEDICC Review* interview with CECMED's Director Olga Lidia Jacobo-Casanueva, July-October 2021, Vol 23, No 3−4, —Eds.] This spans decades and predates the current pandemic of course, but has been advantageous given the urgency of the current health problem.

Last, both CIGB and IFV, developers of our COVID-19 vaccines, have manufactured vaccines for decades using the same technology platforms. There is public trust in our vaccines and immunization programs. Once a vaccine is approved for use and released to immunize our population, people know they are not being used as guinea pigs, they trust the vaccines and the science. This makes for a smooth vaccine rollout.

MEDICC Review: **Cuba's vaccines were developed quickly, but still have not received WHO emergency use listing or prequalification—a process CIGB knows intimately. Is this on the horizon?**

> There is public trust in our vaccines and immunization programs...This makes for a smooth vaccine rollout

Verena Muzio: The steps required of all vaccine manufacturers to obtain WHO prequalification are well established and begin with a formal request, which we submitted at the end of 2021. This request is for WHO emergency use listing (EUL), a designation used during global health emergencies; once we receive that, we can proceed to prequalification. Both EUL and prequalification typically take a long time. But just like national regulatory authorities have established mechanisms for shortening the timeline from research and development to EUA, WHO has also made their process more efficient, instituting rolling reviews that evaluate processes in stages, for example.

People say: since you have the clinical data and the vaccine demonstrates efficacy, what's holding things up? But WHO doesn't only evaluate vaccine clinical studies; they also inspect and evaluate production facilities, each step of lot production, quality control, warehousing, cold chain, adverse events, everything. And not only within the manufacturing institution but also the regulatory authority in their six basic functions—medicine registration, lot-by-lot vaccine release, inspections, clinical trial authorizations, laboratory access and post-marketing surveillance [CECMED is a WHO Level 4 National Regulatory Authority of Reference, —Eds.] It is very comprehensive and complex.

Throughout the pandemic, we also have maintained a close working relationship with PAHO about our progress, data and milestones concerning our COVID vaccines. In short, our formal request is with WHO now and we are preparing all the other materials required for the evaluation including the vaccine prequalification dossier, production and clinical data, etc.

MEDICC Review: **Is WHO prequalification necessary for Cuba to sell and/or donate your vaccines to other countries?**

Verena Muzio: WHO prequalification is not required for a country to authorize use of an imported vaccine—that is the role of each country's national regulatory authority. They conduct the established evaluations and analysis required to approve use of a vaccine for their population. We recognize the importance of WHO prequalification, it's always good to have and we are pursuing that, as well as exporting to other countries.

MEDICC Review: **Is Cuba exporting its vaccines now? What is the strategy for sales abroad?**

> WHO prequalification is not required for a country to authorize use of an imported vaccine—that is the role of each country's national regulatory authority

Verena Muzio: As you know, Cuba has a long history of global medical solidarity, not only in terms of health professionals but also regarding its pharmaceutical products, which have been authorized for use in dozens of countries. This is why we have a combination approach of vaccine donation and sales; between donations and sales, 17 million Abdala doses are now available overseas. This is in addition to the 40 million doses delivered to Cuba's Ministry of Public Health (MINSAP)—including the booster shots now being administered across the country.

Abdala has received EUA in various countries of the region including Venezuela, Nicaragua and Mexico, and elsewhere like Vietnam. This involves a rigorous evaluation process by each country's regulatory authority, reviewing the data, production facilities and more before the vaccines can be purchased or administered.

We are pursuing this process in various countries. Our approach is based on helping address health problems in these nations and what they need to do that, not to profit from illness.

MEDICC Review: **CIGB has had to scale up capacity during the pandemic. What role does the new CIGB Mariel Biotechnology Industrial Complex play?**

Verena Muzio: This mega-project within the Mariel Special Development Zone was inaugurated in November, 2021, but predates COVID-19, obviously. It was conceived several years ago with the attendant design, investment, construction, and equipment and technology acquisition, plus the training of professionals such a complex project implies. Given that CIGB produces pharmaceutical inputs for its own products and other scientific institutions and the number of products in our pipeline and portfolios is growing, especially oncological treatments, it became necessary to have another manufacturing facility to scale up capacity.

What's notable about the CIGB-Mariel project is that it brings together decades of biotech experience and knowledge under one roof, while integrating the latest and most advanced technology and equipment. It's a gold standard installation designed to produce what we need first in our health system and then, for export. It's fully financed by Cuba and will include everything: production, quality control, clinical trial design and analysis, etc.

It's important to note that this project is fully in line with our public health and biotechnology approach in that it is designed first and foremost to address health problems facing our population. No Cuban institution produces pharmaceuticals strictly for export—our commitment is to improve population health in Cuba, using our products in our health system. And the same applies to the facility at Mariel.

MEDICC Review: **CIGB has another vaccine candidate, Mambisa, administered intranasally. How is this research progressing?**

Verena Muzio: One of our early strategies was to develop an intranasal COVID-19 vaccine; CIGB already has a therapeutic hepatitis B vaccine in its portfolio, HeberNasvac, administered nasally [see *MEDICC Review*, January 2021, Vol 23, No 1, —Eds.] Mambisa is a new formulation combining the RBD protein used in Abdala with the recombinant antigen used in HeberNasvac—the latter contains properties that serve as an adjuvant in the nasal mucous membranes. Considering the properties of this vaccine, we knew it was not going to be our candidate for a massive vaccination campaign (for this we had Abdala), but we decided it could be developed as a next-generation vaccine and for different uses.

One of the challenges of a nasal vaccine is evaluating what delivery mechanism is most effective: drops or aerosol, if aerosol, which one. We also have to consider how practical and financially feasible it would be to scale up production. Nevertheless, a booster administered using a simple spray in the nose is a very attractive alternative—some people are afraid of needles and may avoid injections as a result. Offering Mambisa boosters to visitors to Cuba who request them could be another possibility with this easy-to-administer vaccine.

Right now, we're conducting phase 2 clinical trials in convalescent adults, and have just concluded a randomized multicenter phase 2 trial that evaluates Mambisa as a booster dose for the general population. We have preliminary data from this trial on how a Mambisa booster affects viral transmission. The main goal of COVID-19 vaccines is to protect people from developing severe forms of the disease. Nevertheless, cutting the transmission chain would be incredibly important—particularly with more transmissible variants like omicron—and we are looking at both possible effects in the case of Mambisa.

MEDICC Review: **Returning to Abdala, there are other studies being conducted with vulnerable populations including pregnant women. Can you elaborate?**

Verena Muzio: Pregnant women and other vulnerable populations are typically not included in the study universe of clinical trials for a new vaccine or therapeutic treatment due to their higher risk status. However, once you proceed to phase 3 trials, involving thousands of volunteers—48,000 people in the case of Abdala—some of them will become pregnant after being inoculated. Given this reality, we designed an evaluation strategy incorporating long-term follow-up of those volunteers who became pregnant after being vaccinated during clinical trials to analyze the safety data in this population.

Given the unfortunate reality that a number of pregnant women infected with COVID-19 have died in Cuba, the strategy to begin vaccinating this high-risk group was evaluated by MINSAP authorities, a panel of OBGYNs and directors of the National Maternal-Child Health Program (PAMI), CECMED and others. Once we had the pre-clinical and clinical data plus other information including adverse events from phase 3 trials, we applied for EUA from CECMED to being vaccinating pregnant women, which we received. On July 29, 2021, we began vaccinating this vulnerable population with Abdala. This helped reduce infections in this vulnerable group and we launched a post-authorization observational study in late 2021 to assess safety data in those pregnant women vaccinated during different trimesters of their pregnancies and the immunological status of their newborns.

This wasn't the first time we implemented a strategy to vaccinate pregnant women during a pandemic: in 2011, Cuba vaccinated pregnant women against H1N1 using an imported vaccine. The approval for use in our health system of this vaccine—like any imported pharmaceutical product—was issued by CECMED. Pregnant women were hit hard by this virus and the vaccine campaign yielded positive results.

MEDICC Review: **How about infants and children? Cuba is vaccinating its pediatric population, as well.**

Verena Muzio: Yes. We are vaccinating children as young as two, primarily with IFV's Soberana vaccines—results from the pediatric vaccination campaign are being prepared for publication. Additionally, we are getting ready to launch a series of clinical evaluations of children under two. Right now, this segment of the population is not vaccinated. Although we are studying the immunological protection passed from vaccinated mothers to their newborns, what happens to those babies when they approach their first birthday? It's possible that the vertical protection from the mother has diminished and these babies can be susceptible to infection—not necessarily severe forms, but they may become infected nonetheless. This study will begin by March 2022 and is based, again, on years of experience immunizing children under 2 with Heberbiovac HB, Quimi-Vio (a heptavalent pneumococcal conjugate vaccine against bacterial pneumonia and meningitis) and other pediatric vaccines produced in Cuba. This accumulated experience has informed clinical trial design and implementation for the current study.

MEDICC Review: **Developing these vaccines, under such stressful circumstances, is hard to grasp. What was the most difficult moment for you during this pandemic?**

Verena Muzio: The worst, I think, was when the virus spread across the country in the summer of 2021: we had this wave of infections and deaths and it was very hard. I personally didn't lose any family members but my brother and nephew were infected, in isolation centers and we were worried about their health. Some co-workers and their families were also infected, and I was concerned for them. The country was facing a shortage of medicines and at one point there was virtually no oxygen—it created a lot of pressure points at once. This, together with the urgency of our work, the intense pace and circumstances under which we were working to produce results—it was a lot.

MEDICC Review: **And the happiest moment for you?**

Verena Muzio: Definitely one of the happiest moments was in June 2021, when the team responsible for analyzing efficacy results for Abdala crunched the data from the first cutoff date. We couldn't believe how high the efficacy was! 'This can't be. Re-analyze the data, it's not possible' we said. We ran the data again, followed by data analysis from the second cutoff date, and then again when the phase 3 trial concluded and there it was: 92.28% efficacy. When we were sure and delivered these final results, there were tears of joy, it was very emotional.

In a broader sense, one of the most hopeful and positive results of working on these vaccines for me personally, was traveling across the country and working side-by-side with colleagues in Santiago de Cuba, Guantánamo and elsewhere during the clinical trials. I met so many good people. This country is full of hard working, professional people—statistical analysts, database entry clerks, regular folks, young people, it's not just us scientists—who have sacrificed and thrown themselves into the task of helping Cuba emerge from the pandemic. The potential feels limitless.

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Cuban COVID-19 Vaccines for Children: Rinaldo Puga MD MS

Principal Investigator, Pediatric Clinical Trials for Soberana 02 and Soberana Plus

Gail Reed MS

Cuba's decision in September 2021 to launch a massive vaccination campaign against COVID-19 for children as young as two years old turned heads around the world—of clinicians, immunologists, public health experts, governments and regulatory authorities alike. Since then—and just as pediatric COVID-19 hospitalizations reached record numbers globally—some two million Cuban children and adolescents have received the Cuban Soberana vaccines (1.7 million, or 81.3% of that population through December 16, 2021).[1]

Why did Cuban health authorities decide to vaccinate children? What clinical trials provided the evidence for such a course of action, especially for the youngest? And what have been the results thus far?

To answer these and other questions, ***MEDICC Review*** spoke with Dr Rinaldo Puga, principal investigator for the completed phase 1/2 clinical trials of the Finlay Vaccine Institute's Soberana 02 and Soberana Plus vaccines in pediatric ages. Dr Puga's 30 years as a practicing pediatrician have been accompanied by teaching and research, the latter earning him awards from the Cuban Academy of Sciences, among others. He is currently chief of pediatrics and chair of the Scientific Council at the Cira García Clinic in Havana, which granted him leave to lead the pediatric vaccine trials.

MEDICC Review: **Cuba is the first country to vaccinate children as young as two years old for COVID-19. As a pediatrician, what circumstances led to this decision and does it make sense to you?**

Rinaldo Puga: At the start of the pandemic, we had relatively few pediatric cases: 1308 patients in 2020. But later, as happened in the rest of the world and particularly as new variants appeared, household transmission accelerated, accounting for a notable increase of COVID-19 in younger ages. Thus, over 2020–2021, pediatric cases totaled 176,708, with 11,692 of these in infants under one year old. We have had a total of 18 deaths among children and adolescents as a result of COVID-19, associated with other conditions that worsened their prognosis, representing a survival rate of 99.9% in the pediatric age group. So right now, we have over 176,000 convalescent youngsters, who can also be vaccinated.

Of course, the main goals of vaccinating youngsters are to prevent a greater proportion of severe forms of the disease and resulting deaths from COVID-19 by protecting them, and at the same time to achieve added protection for other vulnerable people in their social circles, such as family members. Thus, we can reduce incidence of infection in the community and decrease transmission. In turn, this permits reopening schools under much safer conditions for children and teens.

MEDICC Review**: You've been the principal investigator for the pediatric clinical trials of Soberana 02 and Soberana Plus, both vaccines approved for emergency use in children and adolescents by Cuba's regulatory authority. All Cuban COVID-19 vaccines and vaccine candidates have been described as relying on the same 'classic biotechnology platforms' already in use for other Cuban vaccines. Is this the case?**

Rinaldo Puga: Yes. The other Cuban vaccines and vaccine candidates that have shared the same platforms are those for *Haemophilus influenzae* type b (in the case of Soberana 02), for meningococcal disease serogroups B and C (Soberana 01) and hepatitis B (used in developing the Abdala COVID-19 vaccine).

Cuba's was the first synthetic conjugate polysaccharide vaccine for *Haemophilus influenzae* type b and was introduced in the National Immunization Program in 2004. More than 40 million doses have been administered since then. The Cuban meningitis vaccine has also been applied domestically and in other countries since the late 1980s, totaling over 30 million doses.

Soberana 01 is the recombinant SARS-CoV-2 receptor binding domain (RBD) dimer added to *Neisseria meningitidis,* external membrane vesicles, potentiating innate immunity. Soberana 02 antigen is the recombinant SARS-CoV-2 RBD covalently linked

to tetanus toxoid as carrier protein, potentiating T-cell response and immunological memory. Soberana Plus is also an RBD dimer. All have aluminum hydroxide as adjuvant. Soberana Plus has proved very effective as a booster in persons vaccinated with other Soberanas and in COVID-19 convalescents with a natural immunity against the virus. These vaccines and the other Cuban vaccine, Abdala, are based on protein subunits, the antigen being a recombinant copy of the viral protein; due to the absence of viral and genetic material, they are very safe. Each of them can be used as a booster for persons vaccinated with RNA- and viral-vector vaccines, among others. In short, these vaccines are highly safe and efficacious.

Other protein subunit vaccines in development include:
- NVX CoV 2373 (Novavax, USA)
- SF-UZ Vac 2001 (Zifivax, Anhui Zhifei, China)
- Sanofi-GSK COVID 19 (France-UK)
- SCB 2019 (Clover Bioph, GSK Dynavax, China-UK-USA)
- COVAX 19 (Australia-Iran)

MEDICC Review: **What can you tell us about the clinical trials for Soberana 02 and Soberana Plus in pediatric age groups?**

Rinaldo Puga: We carried out trials in two pediatric groups: the first trials included children and adolescents who had never been infected with the virus, and a later group comprised those who were convalescent.

The first study involving healthy children and adolescents (phase 1/2) used a heterologous vaccine schedule (the same used in adults): two doses of Soberana 02, 28 days apart, combined with one dose of Soberana Plus on day 56. This was an open-label, adaptive, multicenter and sequential trial. After the safety results were confirmed for 12–18 year-olds, we proceeded with the 3–11 age group. It was an open trial, since we didn't use a placebo. The study included the Juan Manuel Márquez Pediatric University Hospital and the 5 de Septiembre and Carlos Juan Finlay community-based polyclinics in Havana's Playa and Marianao municipalities, respectively. We initiated the trial on June 14, 2021 (See table. —Eds.).

For the phase 1/2 clinical trial in convalescent youngsters, one dose of Soberana Plus was used. This was an open, adaptive study and began in Havana on October 5, 2021, with participants at least 8 weeks post-COVID. A total of 520 children and teens were involved, initially in Havana (at the Juan Manuel Márquez Pediatric University Hospital) and later also at the Paquito González Cueto Pediatric University Hospital in Cienfuegos province. Phase 1 included 40 participants: 20 aged 12 to 18 years, and 20 aged 2 to 11. Phase 2 included an additional 480 participants in the same age ranges.

A phase 3 trial was not organized, given the negative ethical implications of using a placebo for this age group. In order to grant emergency use authorization for children and adolescents, the regulatory authority considered the excellent results obtained in adults with the same heterologous schedule, as well as the phase 1/2 results in the pediatric population.

MEDICC Review: **Did the pediatric trials differ from those in adults?**

Rinaldo Puga: The rigor required for the clinical research, study ethics compliance and approval levels are virtually the same for trials in the adult and pediatric populations. And the formulations and vaccine schedules were also the same in this case (a heterologous schedule of two doses of Soberana 02 followed by one dose of Soberana Plus).

But there were differences regarding consent: parents gave written informed consent, but adolescents were also asked to approve their own enrollment in the study. There were also differences regarding the selection criteria, post-vaccination observation time (a full one hour) and active follow-up through periodic checkups.

MEDICC Review: **Did you have difficulties recruiting children and teens for the clinical trials? Were parents resistant or hesitant? We have seen that in other countries.**

Rinaldo Puga: Our experience in Cuba has been just the opposite: we really didn't experience resistance from parents and in fact, the list of volunteers seemed endless. This isn't surprising, since we're talking about a country with over 98% coverage for its vaccines, and of course this applies mainly to childhood vaccinations. So the response to our call for volunteers exceeded our expectations. The recruitment process took place several weeks before the studies began, mainly in the polyclinic health areas I mentioned. The trials were explained, and for children under 12 years old, parents provided consent; older participants recruited provided their own approval as well.

MEDICC Review**: What were the most important results?**

Rinaldo Puga: The results were quite favorable, since more than 90% of the youngsters showed seroconversion after two doses, and 100% after the third dose. Induction of neutralizing antibodies against circulating viral variants was also demonstrated (except omicron, still being studied). There were no serious adverse events related to vaccination.

The Soberana vaccines have proven to be very safe, with quite low reactogenicity, reflected in the fact that adverse events have been local, mild and mainly as expected, not serious or severe. As a result, there has been no need to suspend trials in any of their phases for any age group. These vaccines have also demonstrated very good immunogenicity, evaluated through anti-RBD IgG antibodies, as well as inhibition of RBD–ACE2 receptor interaction, and viral and molecular neutralization tests.

MEDICC Review: **What kinds of adverse events presented?**

Rinaldo Puga: Almost all were those you would expect, and fundamentally local. The most frequent was pain at the injection site, and others expected included local swelling or heat, induration, itching in the injection area and so on. Systemic events with low frequency included general discomfort, fever and headache.

MEDICC Review: **Are heterologous vaccination schedules beneficial then? That is, applying two different vaccines instead of one?**

Rinaldo Puga: Yes, that's been the experience. In our case, the two initial Soberana 02 doses are equivalent, in the best sense of the term, to training a person's immune system to act as if they had already had the disease, and then reinforcing that training

with one dose of Soberana Plus. So, while the two-dose results in children are excellent, since we are facing a mutating virus we opted for a third heterologous dose. This sort of approach is being used a lot now worldwide in booster strategies, and it's an approach we have consolidated with the Soberana vaccines.

MEDICC Review: **You were also principal investigator for studies completed recently of Soberana Plus in COVID-19 convalescent youngsters.**

Rinaldo Puga: Yes, the results after one dose of Soberana Plus have been very good, even better than expected, since at first, we thought convalescent children and adolescents who had been asymptomatic or had mild symptoms might need more than one dose, that is, a full three-dose schedule. But results showed that the anti-RBD IgG titers, percent inhibition of RBD–ACE2 interaction, and the molecular and viral neutralization titers increased significantly compared with the levels after natural infection. Even children who were asymptomatic throughout their illness responded quite favorably to a single dose of Soberana Plus (See table. —Eds.).

MEDICC Review: **The Soberanas aren't the only Cuban COVID-19 vaccines. Abdala has also received emergency use authorization. Are these vaccines similar? Why did vaccinations among children and adolescents begin first with the Soberanas?**

Rinaldo Puga: The Soberanas and Abdala are all protein subunit vaccines, built on different tried-and-true biotech platforms used extensively in Cuba and elsewhere for decades, as I mentioned.

We already had conditions in place for a heterologous pediatric study involving the Soberanas based on earlier results in adults, which meant we could begin the clinical trials with those vaccines first and thus these were the first to obtain emergency use authorization for children and adolescents. So, in September 2021, when Cuba decided to vaccinate this age group, we used the Soberanas, while at the same time Abdala was being used to continue vaccinating the country's adult population.

Later, pediatric trials for Abdala were carried out in Camagüey province, duly registered and approved by the regulatory authority. So now, both the Soberanas and Abdala have received emergency use authorization for the pediatric population.

MEDICC Review: **As principal investigator for the Soberana pediatric clinical trials, how was the rigor of these studies ensured? What processes were in place?**

Rinaldo Puga: From the early stages, a research ethics committee delved deeply into the trial design to review and suggest modifications. Cuba's regulatory authority, the Center for State Control of Medicines, Equipment and Medical Devices (CECMED) received an exhaustive presentation, and periodically supervised the clinical trial. Of course, the studies were listed in the Cuban Registry of Clinical Trials (https://rpcec.sld.cu), and accompanied, reviewed and corrected throughout the process by specialists at the National Clinical Trials Coordinating Center (CENCEC). An external committee on data management carried out their analysis, in our case composed of specialists from the Pedro Kourí Tropical Medicine Institute and the Molecular Immunology Center, among others.

Also participating in the supervision of the entire process were the national Maternal–Child Health Program, the National Pediatrics Expert Group, the Cuban Pediatrics Society, and both the National and Provincial Immunization Groups.

The trials also received extraordinary assistance from the San Miguel del Padrón Pediatric Teaching Hospital in Havana, and the San Alejandro isolation center that acted as an extension of the Juan Manuel Márquez Pediatric University Hospital…and from others such as the CIMEQ Hospital, the Immunoassay Center and the Civil Defense laboratories in San José de las Lajas municipality.

Mobilization of resources was key to ensure the trials' quality: vaccine refrigeration and transportation, ambulances at all the community vaccination sites in case of a serious adverse event, guaranteeing safe, non-toxic meals for all participants (provided by Cuba Catering) …a whole range of services.

MEDICC Review: **What were the main challenges during preparation, the trials themselves and data analysis?**

Rinaldo Puga: The main challenge was to create the necessary infrastructure and optimal conditions to ensure the correct sequence of activities for everyone involved: workshops for healthcare staff on good clinical practices, certification of vaccination sites and of the nursing staff applying the vaccines, seminars and instruction workshops for parents, and so forth. Plus, the detailed attention to ensure correct data processing, including sampling, digitalization and statistical analysis of results.

MEDICC Review: **You also serve as chief of pediatrics at the Cira García Clinic, where international patients are seen. Have children from other countries received the Cuban COVID-19 vaccines?**

Rinaldo Puga: Yes, children of permanent residents, or whose parents are here in Cuba with various foreign companies or international agencies, children of diplomats and some who have traveled from their home countries through Cuban Medical Services expressly for the vaccines. In the case of such visitors, Abdala is used since its schedule is shorter. For those living in Cuba and for convalescent children, we are using the Soberanas. As you know, all three vaccines have emergency use authorization for the pediatric population from CECMED.

MEDICC Review: **Globally, a growing consensus is emerging about the need to vaccinate the pediatric population against COVID-19. Will Cuba be sharing its results in this age group with WHO to apply for emergency use listing for use of these vaccines in children and adolescents?**

Rinaldo Puga: Yes, results of the pediatric Soberana clinical trials will be included in the dossier provided to WHO for emergency use listing. Not all COVID-19 vaccines have such a formidable safety record in pediatrics as these protein subunit vaccines, and thus our clinical trial results are quite important, coupled with those evaluating the impact of vaccination in Cuba's pediatric population.

We are not the first country to apply COVID-19 vaccines in children, but we are the first to carry out a massive, nationwide vaccination campaign for our children and teens. And we are the first and still

COMPLETED CLINICAL TRIALS FOR SOBERANA COVID-19 VACCINES IN THE PEDIATRIC POPULATION (as of January, 2022)

	CLINICAL STUDY DESCRIPTION	SELECTION CRITERIA, CLINICAL SITES	VACCINATION SCHEDULE & DOSES	GENERAL CONCLUSIONS	EMERGENCY USE AUTHORIZATION
SOBERANA PEDIATRIA VACCINES: FINLAY-FR- 2 (SOBERANA 02) and FINLAY-FR-1A (SOBERANA PLUS) REGISTRATION IN PRIMARY REGISTRY: June 10, 2021 ISSUING AUTHORITY-SPONSOR: Finlay Vaccine Institute (IFV) PRINCIPAL INVESTIGATOR: Rinaldo Puga MD MS	**Study type:** Interventional **Purpose:** Prevention **Study design:** Single group **Phase:** 1/2 **Target sample size:** 350 Phase 1/2 study, sequential during phase 1, open-label, adaptive and multicenter to evaluate the safety, reactogenicity and immunogenicity of a heterologous two-dose schedule of SARS-CoV-2 prophylactic vaccine FINLAY-FR- 2 and one dose of FINLAY-FR-1A, in Cuban children and adolescents.	**Population type:** Children/Adolescents **Sex:** M/F **Minimum age:** 3 years **Maximum age:** 18 years **Participant type:** Healthy volunteers **Clinical sites:** Juan Manuel Marquez Márquez Pediatric University Hospital, Havana 5 de Septiembre Polyclinic, Playa Municipality, Havana Carlos J. Finlay Polyclinic, Marianao Municipality, Havana	FINLAY-FR-2: 25 µg of RBD-TT, by intramuscular route, 0.5 mL, in scheme 0–28 days. FINLAY-FR-1A: 50 µg of d-RBD + Aluminum hydroxide gel, by intramuscular route, 0.5 mL as dose booster 56 days	The FINLAY-FR-2 vaccine is well tolerated following 2-dose schedule in children and adolescents (3–18 years). There were no serious or severe adverse events following immunization. The safety profile is similar to that for young adult subgroup (19–29 years) in phase 1/2 study. Immune response is >50% from day 14 post-second dose in phase 1/2 for 3–18 year-olds, and >90% of anti-RBD IgG seroconversion post-second dose. All immunological variables had similar results as those in young adults (19–29 yrs) in phase 2 trial, and superior to those in the 19–80 years population, and significantly superior to those in the Cuban convalescent pediatric COVID-19 panel. As of September 6, 2021, results for the third dose (FINLAY-FR-1A) became available for the adolescent subgroup (12–18 years). After this dose, seroconversion was 100%, with a significant increase in IgG titers, and molecular virus neutralization.	September 3, 2021 Note: Children 2–3 years of age were included in the authorization based on results of the study and the clinical-epidemiological arguments endorsed by the National Pediatrics Expert Group.
SOBERANA PLUS PEDIATRIA VACCINE: FINLAY-FR-1A (SOBERANA PLUS) REGISTRATION IN PRIMARY REGISTRY: September 28, 2021 ISSUING AUTHORITY-SPONSOR: Finlay Vaccine Institute (IFV) PRINCIPAL INVESTIGATOR: Rinaldo Puga MD MS	**Study type:** Interventional **Purpose:** Prevention **Study design:** Single group **Phase**: 1/2 **Target sample size:** 520 Phase 1/2, open-label, adaptive study to evaluate the safety, reactogenicity and immunogenicity of the prophylactic vaccine candidate FINLAY-FR-1A against SARS-CoV-2 in COVID-19 pediatric-age convalescents.	**Population type:** Children/Adolescents **Sex:** M/F **Minimum age:** 2 years **Maximum age:** 18 years **Participant type:** Healthy volunteers (recovered from COVID-19) **Clinical sites:** Juan Manuel Márquez Pediatric University, Havana Paquito González Cueto Pediatric University Hospital, Cienfuegos	One dose of the prophylactic vaccine candidate FINLAY-FR-1A a minimum of two months after medical discharge. **Dosage:** 50 µg of RBD + adjuvant, 0.5 mL by intramuscular route.	The study demonstrated that a single dose of the vaccine is safe, and provided indications of immunological benefits against possible risk of reinfection by SARS-CoV-2.	December 7, 2021 Emergency use authorization for COVID-19 convalescent pediatric patients (from 2 years and 1 day through 18 years), after at least 2 months of hospital or home-hospital release.

Sources: CENCEC, CECMED, Finlay Vaccine Institute blog (https://www.finlay.edu.cu/blog/resumen-de-los-resultados-de-ensayo-clinico-soberana-pediatria/)

today only country to have vaccinated the pediatric population as young as 2 years old, and through age 18. We have also vaccinated convalescent children and adolescents in the same age range.

MEDICC Review: **In 2021, COVID-19 took its toll in every aspect of Cuban life, and in particular in the Maternal–Child Health Program and infant mortality rates. How many deaths have occurred in pediatric ages from this disease? What are your hopes for the future, and in particular for under-five mortality?**

Rinaldo Puga: Certainly the pandemic has created a more complex situation in the country, for the population as a whole and for the health system itself. Since the first pediatric case was diagnosed on March 21, 2020, as I mentioned, 18 children and adolescents have died from COVID-19 in Cuba, not all of them infants, of course, for an overall survival rate of 99.9%.

Nonetheless, the increase in infant mortality from 4.9 per 1000 live births in 2019 to 7.6 in 2021 has multiple causes, only some of which may be directly or indirectly related to COVID-19. Low birth weight and prematurity have heavily influenced this change, according to initial assessments by National Maternal–Child Health Program experts.

With respect to under-five mortality, our hope is the same as for all Cuban children and adolescents: that the introduction of our vaccines will continue to improve the indicators by reducing progression to severe forms of the disease or death.

We can't be 100% optimistic, since we know that vaccines alone cannot solve the problem. On the contrary: if we don't comply with classic public health measures such as physical distancing, handwashing and sanitizers, as well as masking, then the situation could become very tense.

MEDICC Review: **Other reflections on your experience? Future studies?**

Rinaldo Puga: I'm very grateful for the generous support I've received throughout from my director at the Cira García Clinic and my colleagues, in order to dedicate myself full-time to the Soberana pediatric studies, and am grateful to the Finlay Vaccine Institute for the confidence they placed in me.

We're generating further research to evaluate the effectiveness of the vaccines in the pediatric population, measuring the B- and T-cell responses, duration of the immune response over time, the overall impact of the vaccination campaign, the need (or not) to modify vaccine schedules, given the appearance of more highly transmissible variants of concern such as omicron, to prevent severe forms of the disease and deaths. In fact, we're obtaining quite interesting results concerning omicron's behavior related to our vaccines that we look forward to sharing publicly.

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In Haiti, Cubans Among First Responders, Again: Luis Orlando Oliveros-Serrano MD

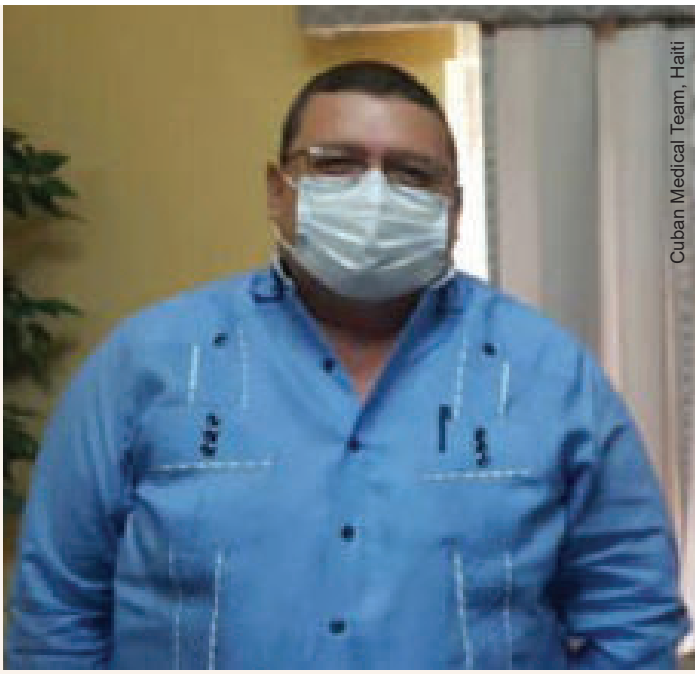
Coordinator, Cuban Medical Team in Haiti

Conner Gorry MA

Soaring summer temperatures, systematic urban and political violence, unreliable infrastructure—power outages, water shortages, sporadic transportation and interruption of other basic services—plus the illness, death and economic straits wrought by COVID-19, are what Haitians awake to every day. On the morning of August 14, 2021, they also woke to the earth in the throes of violent, lethal convulsions caused by a 7.2-magnitude earthquake, along the same fault line responsible for the devastating 2010 disaster and stronger still. As if this weren't enough, Tropical Storm Grace was bearing down on the nation, about to dump biblical amounts of rain on the heels of Tropical Storm Fred.

When the Haitian President was assassinated on July 7, Haiti still had not received a single dose of any COVID-19 vaccine—indeed, it was the last country in the Americas to receive vaccines. Later that month, 500,000 doses arrived in the country, donated by the United States via COVAX, the WHO-led initiative to assure at least some vaccines reached low- and middle-income countries. In Haiti, getting those vaccines into the arms of the population is beset by cold chain, distribution and bureaucratic problems, and compounded by widespread vaccine hesitancy; when the earthquake struck, only 14,074 of those doses had been administered.[1,2]

Suddenly there was a new, more urgent tragedy, the earthquake leaving thousands of dead, injured and displaced—perhaps hundreds of thousands once the real tally emerges. As in the 2010 quake, the doctors, nurses and technicians comprising Cuba's medical team in Haiti—a commitment Cuba has maintained with its Caribbean neighbor since 1998—were among the first responders. The 2010 relief effort included an additional 1500 health professionals and specialists from Cuba's Henry Reeve Emergency Medical Contingent.



Cuban Medical Team, Haiti

Just 24 hours after the August 14th quake, ***MEDICC Review*** spoke by phone with Dr Luis Orlando Oliveros-Serrano in Port-au-Prince, where he coordinates Cuba's medical team in Haiti. His disaster response experience had already taken him to Haiti twice before and to Pakistan, Bolivia and beyond.

MEDICC Review: **Can you tell us what the situation is like on the ground and if all the members of the Cuban team are accounted for?**

Luis Orlando Oliveros: We have 253 professionals working here—the majority women—distributed throughout the country. Everyone is okay. The situation is very tense, especially in the southern region most affected by the earthquake [the epicenter was 5 miles from the town of Petit Trou in the Nippes department, 80 miles west of the capital, Eds.]. This was a very strong earthquake, causing the kind of widespread destruction—loss of human life, collapsed buildings, difficulty in accessing health services—that our team has seen with other natural disasters. It is a very difficult situation, made more so by the spread of COVID-19.

All of our health professionals staffing hospitals in the towns of Aquín, L'Asile and Jérémie, closest to the epicenter, are attending the injured. Patients are being treated in triage areas set up outside, on hospital grounds.

MEDICC Review: **Are you working with other organizations? Who is coordinating the relief effort?**

> It is a very difficult situation, made more so by the spread of COVID-19

Luis Orlando Oliveros: Our medical team works with the Haitian Ministry of Public Health—all the services we provide and the actions we take are coordinated with them. We aren't an autonomous entity making decisions independently; we aren't free electrons roaming about. Rather, we work with the Ministry in an integrated way to help address health problems in the country—as we've been doing on a continual basis for 22, going on 23 years. In that respect, this post-earthquake scenario is no different.

We are not in this alone. A command center has been set up in the capital and we always do our best to collaborate with any other organization or entity to forge optimal solutions.

***MEDICC Review*: A number of Haitian doctors staffing the public health system received their degrees from Cuba's Latin American Medical School (ELAM). Are you working with them as well?**

Luis Orlando Oliveros: Our medical team works with many of the Haitian ELAM graduates. They direct health centers and services where our professionals are posted and this requires close collaboration. We work shoulder-to-shoulder with them. We maintain a very good relationship and communication that we continually strengthen through regular meetings about new developments and health initiatives. Additionally, many of the Haitian graduates are pursuing their medical residencies with the Cuban team here.

***MEDICC Review:* After the 2010 earthquake, Cuba's Henry Reeve Emergency Medical Contingent was dispatched to Haiti for more than six months. Are there any veterans of that relief effort on the ground now?**

Luis Orlando Oliveros: I was here in 2010, coordinating logistics for the Henry Reeve Contingent after the earthquake. Other members of the team currently serving in Haiti also have post-earthquake and epidemic experience.

***MEDICC Review:* The destruction wrought by an earthquake of this magnitude is staggering—unimaginable in the middle of a global pandemic. Can you describe the COVID-19 situation? Are there any special measures you're taking?**

Luis Orlando Oliveros: Of course, we take all the standard precautions—wearing masks, distancing, frequent hand washing—to prevent infection because we can't afford to get sick: we need to stay healthy to continue providing free health services. Additionally, every member of our team is vaccinated against SARS-CoV-2, giving them a level of protection that allows them to continue working within the extraordinarily complex pandemic scenario that we're seeing around the world.

For over a year, we have been treating patients with COVID-19 in a hospital here in Port-au-Prince equipped for that purpose. In fact, there are Cuban health professionals working in public health services in all ten departments across the country, who have amassed 15 months' experience managing cases and contributing to the fight against the pandemic. It helps that we have excellent rapport and communication with the population we serve. We live and work in Haitian communities and live under the same pandemic parameters as the patients we see.

Right now, we are focused on mitigating the quake's damage to the people we serve as quickly as possible, above all in the southern part of the country, which was the hardest hit and where there is the most injury and suffering.

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Asymptomatic SARS-CoV-2 Infection in Havana, Cuba, March–June 2020: Epidemiological Implications

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ABSTRACT

INTRODUCTION The percentage of asymptomatic COVID-19 cases worldwide is estimated at 18–50%; 53% in Cuba specifically, and 58% in Havana, the Cuban capital and the 2020 epicenter of the country's COVID-19 epidemic. These figures, however, do not represent the transmission capacity or behavior of asymptomatic cases. Understanding asymptomatic transmission's contribution to SARS-CoV-2 spread is of great importance to disease control and prevention.

OBJECTIVE Identify the epidemiological implications of asymptomatic SARS-CoV-2 infection in Havana, Cuba, during the first wave of the epidemic in 2020.

METHODS We carried out a cross-sectional study of all confirmed COVID-19 cases diagnosed in Havana, Cuba, from March 16 through June 30, 2020. The information was obtained through review of the standardized form for investigation of suspected and confirmed cases. Examined variables included age, sex, occupation, case type and source of infection. Cases were divided into asymptomatic and symptomatic groups, and transmission was characterized through the creation of a contact matrix. Analysis was carried out in Epidat and R.

RESULTS We studied 1287 confirmed cases, of which 57.7% (743) were asymptomatic, and 42.3% (544) were symptomatic. Symptomatic presentation was the most common for both imported and introduced cases, while asymptomatic presentation was more common in autochthonic cases and infections from an undetermined source. Asymptomatic infection was more common in groups aged <20 and 20–59 years, while symptomatic infection was more common in those aged >60 years. In the contact matrix, 34.6% of cases (445/1287) were not tied to other cases, and 65.4% (842/1287) were infectious–infected dyads, with symptomatic–symptomatic being the most common combination. The majority of primary cases (78.5%; 1002/1276) did not generate secondary cases, and 85.6% (658/743) of asymptomatic cases did not lead to other cases (although one asymptomatic superspreader led to 90 cases in a single event). However, 63.2% (344/544) of symptomatic primary cases generated secondary cases, and 11 symptomatic superspreaders spawned 100 secondary cases in different events.

CONCLUSIONS Asymptomatic SARS-CoV-2 infection was the most common form of COVID-19 in Havana during the study period, but its capacity for contagion was lower than that of symptomatic individuals. Superspreader events under specific conditions played an important role in sustaining the epidemic.

KEYWORDS COVID-19, SARS-CoV-2, pandemics, asymptomatic infection, Cuba

INTRODUCTION

Research on COVID-19, the disease caused by the SARS-CoV-2 virus, is evolving, addressing questions like different transmission routes, the infectious dose (the amount of virus required for transmission), the characteristics of those most susceptible to infection, situations that facilitate contagion events, the proportion of individuals who remain asymptomatic throughout the course of infection, the specific factors that drive asymptomatic and pre-symptomatic transmission, as well as the proportion of infections transmitted by asymptomatic and pre-symptomatic individuals.[1]

The overall percentage of COVID-19 cases that are pre-symptomatic (infected individuals who are currently asymptomatic but will present with symptoms during a later stage of infection) or asymptomatic (individuals who will never present symptoms) is estimated at 18%–50% worldwide, although this figure has been much higher in certain contexts.[1–3] The data available in Cuba for the first three months of the epidemic (whose first case was officially reported on March 11, 2020) place the proportion at about 53% of all confirmed cases nationwide,[4] and about 58% of all cases diagnosed in Havana.[5]

IMPORTANCE Identification of asymptomatic SARS-CoV-2 infection and its role in COVID-19 spread and transmission contributed to the implementation of effective disease control measures in Havana, Cuba during the pandemic's first wave in 2020.

The aforementioned data, however, represent the total proportion of asymptomatic or pre-symptomatic COVID-19 cases, and not the role of asymptomatic patients in SARS-CoV-2 transmission. According to a study by the University of Padua and the Imperial College of London, asymptomatic COVID-19 patients are about as infectious as symptomatic patients,[6] but there are still no reliable estimates of the contagiousness of asymptomatic individuals as compared with symptomatic individuals.

Due to its clinical importance, an understanding of the magnitude of the role asymptomatic transmission plays in the spread of SARS-CoV-2 is much needed. Additionally, it cannot be assumed that a lack of symptoms means there is no harm being done

to the asymptomatic individual.[7] A thorough understanding of asymptomatic transmission is also important from an epidemiological point of view, as the diagnosis, confirmation and subsequent isolation of symptomatic cases (as established in Cuban national action protocols for case and contact management)[8]—if not accompanied by similar measures for asymptomatic cases—may have a limited effect in reducing overall community transmission.

The basic reproduction number R_0 (the average number of secondary infections caused by a primary case in an epidemiologically naïve population) for SARS-CoV-2 person-to-person transmission was estimated at two to three cases, for the original wild-type virus circulating when this study was done. Fewer secondary cases have been reported in some countries than would have been expected with this R_0, suggesting that not all primary cases cause secondary transmission.[9,10] Some literature suggests that 10%–20% of infected individuals are responsible for 80%–90% of all transmitted cases.[10,11]

All of this suggests the need for epidemiological studies estimating not only R_0 for asymptomatic SARS-CoV-2–positive individuals (estimated as 1%; 95% CI: 0%−2%), but also other important parameters such as the k dispersion parameter, a measure of person-to-person transmission variation that is especially important in calculating superspreader events in which a single person infects tens or hundreds of others.[11,12]

Given the importance of this information for pandemic control, this article explores asymptomatic and presymptomatic transmission in the Havana pandemic epicenter during the first half of 2020, although the authors recognize that these epidemiological parameters have changed both in Cuba and worldwide with the appearance of new SARS-CoV-2 variants.

METHODS

Study design and participants We carried out a descriptive cross-sectional study that included all confirmed SARS-CoV-2–positive cases in Havana, Cuba, from March 16 (following the declaration of the first cases in Cuba and the beginning of the Cuban epidemic) through June 30, 2020 (the end of the first wave of the Cuban epidemic).

Study variables The following sociodemographic variables were collected: age (≤20 years, 21–59 years, ≥60 years); sex (male or female); and occupation (health worker or other).

We also included the following clinical variables:

- Asymptomatic case: a case manifesting no signs or symptoms of the disease at diagnosis.
- Symptomatic case: a case manifesting signs and symptoms compatible with any of the five clinical forms recognized by WHO and included in the COVID-19 Cuban protocol (uncomplicated or mildly symptomatic disease, uncomplicated lower respiratory tract infection or mild pneumonia, acute respiratory distress syndrome, sepsis, or septic shock syndrome).[8]
- Confirmed case: any patient who tested positive for SARS-CoV-2, with or without symptoms.

The epidemiological variables were:[8]

- Case type: index case defined as the one that introduces an infection into a group or population; primary case, defined as the first case identified in an outbreak or event, capable of generating other cases, the recognition of which is generally retrospective; or secondary case, defined as any case infected by a primary or index case.
- Number of secondary cases: secondary cases generated by primary cases; any primary case that generated ≥6 secondary cases was considered to be a superspreader.
- Conditions facilitating contagion: places facilitating close contact (≤1 meter between persons), such as those with high concentrations of people, high social mobility, enclosed places with limited ventilation, and detention centers.
- Source of infection: imported if the known source of infection occurred outside Cuba; introduced, if infection occurred through contact with travelers from abroad; autochthonous, when infection was acquired locally via contact with confirmed cases, or cases of unknown infectious sources, if no relationship to other cases was identified.
- Country of origin: any country as a probable source of infection pertaining to cases included in the sample.

Procedures, data collection and management Sociodemographic, clinical, and epidemiological data were obtained through a document review of primary sources (the standard form specifically designed for investigation of suspected and confirmed COVID-19 cases, results of which are housed in Havana province's database of confirmed cases at the Provincial Hygiene, Epidemiology and Microbiology Center, the quality and reliability of which was refined by, and compatible with, Cuba's national database of confirmed cases, housed in the Ministry of Public Health, MINSAP).

We selected confirmed COVID-19 cases in Havana from the country's database and established two study groups: symptomatic and asymptomatic. We recorded the symptom onset date for symptomatic cases, and the confirmation date for each patient, regardless of whether they were symptomatic or not.

We constructed a contact matrix or transmission tree for all cases to characterize both the groups and their transmission routes. To do this, we identified isolated cases, or primary–secondary contact pairs (infective–infected) in which both cases were symptomatic, both were asymptomatic, or only one patient was symptomatic. Contact matrices (epidemiological networks) were constructed with the index, primary and secondary cases forming part of either outbreaks (defined as ≤9 secondary cases) or events (≥10 generated cases); a few of which included superspreader cases.

We performed a percentage analysis of asymptomatic and symptomatic cases, according to age, sex, occupation and infection source, and bivariate analyses through calculation of prevalence ratios with 95% confidence intervals. The dispersion threshold was calculated using the 99th percentile of the Poisson distribution ($\lambda = R_0$) where $Pr(Z \leq Z(99)|Z \sim Poisson(R_0) = 0.01$; where R_0 is the baseline reproductive number and Z is the number of secondary cases derived from an infected person in a susceptible population.[12] Given that the value of R_0 for COVID-19 (wild-type) has been estimated at 2–3,[7,9,10] the superspreader threshold was established at Z = 6 secondary cases. From this value, all subjects who generated six or more secondary cases were considered superspreaders.

For transmission chains, the effective reproduction number (R) was estimated from the negative binomial distribution's

mean adjusted to the distribution of observed secondary cases.[12] This was completed for all pairs within the matrix clusters and for purposes of comparison, was additionally completed for all chains started by symptomatic and asymptomatic cases.

Statistical analysis Data analysis was performed in EPIDAT 3.1 and R, version 3.4.0 (SERGAS, Spain, 2017) with 95% confidence intervals (CI).

Ethical considerations This study was approved by the Specialized Scientific Commission for Epidemiology and the Pedro Kourí Tropical Medicine Institute's Ethics Committee, as well as Havana's Provincial Hygiene, Epidemiology and Microbiology Center. Patient anonymity was guaranteed; informed consent was unnecessary, as only information excluding patient identity from the Ministry of Public Health's database was utilized.

RESULTS

As of June 30, 2020 (statistical week 27), 7438 suspected COVID-19 cases had been studied in Havana's molecular biology laboratories, of which 1287 (17.3%) were confirmed positive by RT-PCR (real-time polymerase chain reaction). Of this total, 743 (57.7%) were asymptomatic, while 544 (42.3%) had various clinical symptoms (Figure 1).

Symptomatic cases were the most common during the beginning of the epidemic, from the confirmation of the first case on March 16 (statistical week 12), until statistical week 16 (April 12–18.) After this period, asymptomatic cases were the most common. This pattern was maintained until the end of the study period (Figure 2).

Figure 1: Confirmed SARS-CoV-2 case distribution, according to presentation and infection source. Havana, Cuba, March–June, 2020

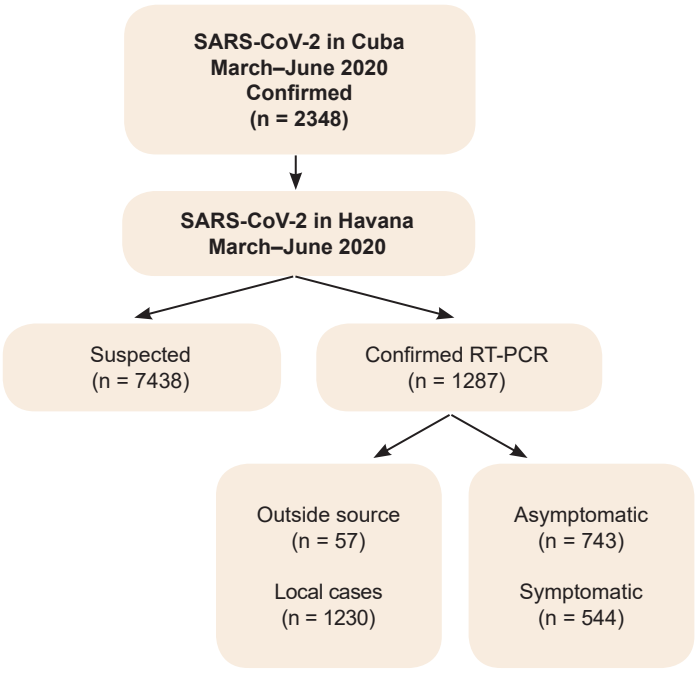
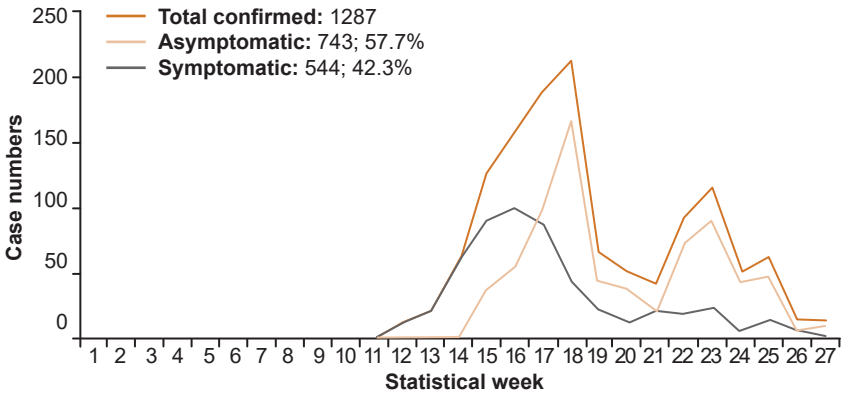


Figure 2: Distribution of asymptomatic and symptomatic SARS-CoV-2 cases by statistical week. Havana, Cuba, March–June, 2020



Autochthonous infection was predominant at 89.0% of all cases (1145/1287). In imported and introduced cases, symptomatic infection was the most prevalent, at 91.2% (52/57) and 97.1% (66/68), respectively, while autochthonous cases (63.4%; 726/1145) or cases without a specified infection source (58.8%; 10/17) were most commonly asymptomatic (Table 1).

Among the 15 countries that contributed imported cases, Spain was the most represented (22.8%; 13/57), followed in order of frequency by the USA (15.8%; 9/57), Mexico (12.3%; 7/57), France and Panama (both of which were 10.5%; 6/57of cases). Between one and three cases were identified for each of the other countries listed.

Confidence intervals for the prevalence ratios (PR) for sex and occupation in symptomatic cases contained the value 1, which is not the case for individuals ≥60 years of age, which have a remarkably higher likelihood (2.13) of being symptomatic than individuals aged ≤20 years. Asymptomatic infection was most common in individuals <60, while adults ≥60 were more likely to be symptomatic (Table 2).

The contact matrix allowed us to identify 445 isolated cases (34.6% of the total) and 842 interrelated cases (65.4%), for a total of 644 infector–infected dyads, among which symptomatic–symptomatic pairings were the most common (144/644; 22.4%).

During the study period, 177 transmission chains or clusters began in Havana, 49 of which had an asymptomatic individual as the first case, while 128 were initiated by a symptomatic case (Figure 3). Clusters with asymptomatic primary cases generated a smaller number of secondary cases (193), of which 80.3% were

Table 1: Asymptomatic and symptomatic case distribution, according to infection source

Infection source	Asymptomatic		Symptomatic		Total	%
	n	%	n	%		
Imported cases	5	8.8	52	91.2	57	4.4
Introduced cases	2	2.9	66	97.1	68	5.3
Autochthonic cases	726	63.4	419	36.6	1145	89.0
Undetermined	10	58.8	7	41.2	17	1.3
Total	743	57.7	544	42.3	1287	100.0

Table 2: Asymptomatic and symptomatic case distribution, according to age, sex and occupation; prevalence ratios and 95% confidence intervals

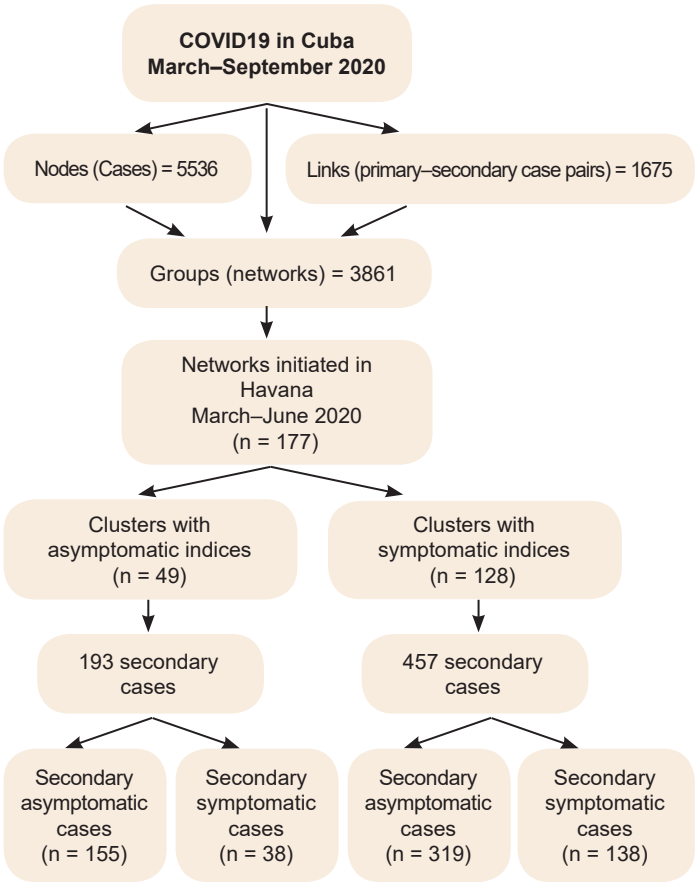
Factors	Asymptomatic		Symptomatic		Total	%	PR	CI	
	n	%	n	%				IL	SL
Age groups									
≤20 years	100	73.5	36	26.5	136	10.57	-	-	-
20–59 years	523	59.8	352	40.2	875	68.14	1.52	1.13	2.03
≥60 years	120	43.5	156	56.5	276	21.29	2.13	1.57	2.86
Sex									
Male	370	57.1	278	42.9	648	50.75	0.97	0.76	1.18
Female	373	58.4	266	41.6	639	49.65			
Occupation									
Health worker	134	55.1	109	44.9	243	18.88	1.08	0.77	1.45
Other	609	58.3	435	41.7	1044	81.12			

PR: prevalence ratio, CI: confidence interval; IL: inferior limit; SL: superior limit

asymptomatic (155/193). Clusters generated by symptomatic individuals resulted in 457 secondary cases, of which 69.8% were also asymptomatic (319/457).

Among asymptomatic cases, a single individual was identified as a superspreader who generated 90 secondary cases in a single event (closed internment institution). Among symptomatic cases, 11 primary cases acted as superspreaders, leading to 100 secondary cases, related to either high social mobility, close contact between individuals in closed spaces with limited ventilation, or places with high concentrations of people (Table 3).

Figure 3: General contact matrix of confirmed SARS-CoV-2 cases. Havana, Cuba, March–June, 2020



We obtained a global value of the effective reproductive number (R = 0.51; 95% CI: 0.43–0.60), which was lower in the asymptomatic group (R = 0.34; 95% CI: 0.25–0.44) compared to the symptomatic group (R = 0.77; 95% CI: 0.65–0.91).

DISCUSSION

Claims that asymptomatic and pre-symptomatic transmission represent the main source of the pandemic's spread[13,14] are debatable, particularly given the emergence of new virus variants and new strategies to address them. What is not in doubt is the high proportion of asymptomatic individuals that are carriers of the virus in different contexts,[15–17] so research is essential to determine the true role of asymptomatic carriers in SARS-CoV-2 transmission.

The upward linear trend in asymptomatic case notification in Havana during the epidemic's first wave was similar to that reported by other national[4,5,18] and international studies;[6,19,20] but differed from reports from researchers in Korea[21] and China.[22]

By the time this study was done, Cuba's national strategy, contained in the country's national action protocols for all phases and stages of the pandemic,[8] included extensive research at the primary care level: testing everyone who had either direct or indirect contact with a confirmed case; isolation of all suspected or confirmed cases in hospitals, regardless of the presence or absence of symptoms; quarantine of contacts of confirmed cases in isolation centers; epidemiological surveillance of travelers; and other control measures in accordance with WHO guidelines.[23] Most of these protocols, with the exception of strict isolation measures, are still in effect.

A shift in transmission from a predominance of symptomatic to asymptomatic cases during the first wave was likely due to

Table 3: Asymptomatic and symptomatic primary case distribution, according to secondary case generation

Number of secondary cases	Asymptomatic		Symptomatic		Total
	n	%	n	%	
0	658	0.868	344	0.664	1002
1	62	0.082	88	0.170	150
2	20	0.026	34	0.066	54
3	10	0.013	24	0.043	34
4	3	0.004	12	0.026	15
5	4	0.005	5	0.010	9
6	0	0.000	5	0.010	5
7	0	0.000	1	0.002	1
8	0	0.000	1	0.002	1
9	0	0.000	1	0.002	1
10	0	0.000	1	0.002	1
11	0	0.000	1	0.002	1
12	0	0.000	1	0.002	1
24	0	0.000	1	0.002	1
90	1	0.001	0	0.000	1

increased diagnostic capacity and contact tracing, in which more asymptomatic and pre-symptomatic cases were counted that would have gone undetected at the very beginning of the pandemic. At the same time, there was wide community transmission and defined diagnostic testing criteria, in an asymptomatic population that was mostly young and healthy and associated with more benign clinical progression.

This situation continued until the end of 2020, when borders closed due to the pandemic began to open, and cases in foreign visitors and Cubans visiting from abroad introduced new SARS-CoV-2 variants, which are associated with more severe forms of COVID-19 that are more likely to be symptomatic,[24] a situation that has continued through 2021.

The first Cuban travel restrictions were implemented on March 21, 2020, and borders were definitively closed on April 7, 2020.[25] Most cases were either imported or introduced during this period. From then on, outbreaks in closed communities or institutions, where confirmed cases were not linked to travelers from affected areas, suggest that autochthonous transmission was a fundamental aspect of the pandemic, confirmed by this and other national studies.[4,5,18,26,27]

The observation that imported and introduced cases mainly presented as symptomatic before the borders were closed could be related to the fact that Cuba's epidemiological surveillance system during the very beginning of the pandemic defined a 'suspected case' of COVID-19 as an individual who presented with symptoms compatible with COVID-19 who came from countries or territories with documented transmission. After studies began of all contacts, identification and diagnosis of asymptomatic cases increased, which led to a change in the definition of 'suspected case.'

SARS-CoV-2 can affect people of all ages. In this instance, both globally and within groups, infection was more common in middle-aged adults, similar to results found in additional studies in Cuba,[5,18] and other countries,[2,28] with the exception of those carried out in Santiago de Cuba[26] and Camagüey[27] provinces, in which older adults were more susceptible to infection. These results could be due to the fact that middle-aged adults comprise the group with the highest levels of social interaction, as they continue to work and carry on family activities, while children remained at home after schools were closed and older adults self-isolated, among other posible causes.[2]

In our study, asymptomatic infection was most common in individuals <60 years of age, while older adults were more likely to manifest the disease symptomatically. This result is consistent with other literature on the same period of the pandemic, in which children were generally less susceptible to infection and manifested milder symptoms than adults, suggesting however that they posed a high risk of transmitting the disease through asymptomatic presentation. In adults, advanced age is directly correlated with greater risk of infection, as well as more severe clinical presentations, and death.[29–33]

In some studies, the highest rates of infection were reported among men,[5,33,34] while others report the opposite.[26] In this study, both sexes showed the same probability of becoming infected and presenting with symptomatic (or asymptomatic) forms.

According to official information published in the Cuban media,[35,36] health workers (during the study period) were not among the risk groups with the highest SARS-CoV-2 infection rates, nor did theirs compare with such rates in other countries. Public health programming within the country led to increased staff awareness and compliance with biosafety protocols. The proportion of cases with occupations outside the healthcare sector was higher in this study; however, the overall proportion of symptomatic and asymptomatic cases was similar in both groups.

The degree to which SARS-CoV-2–positive individuals spread the virus depends to a great extent on environmental and seasonal conditions, and social behavior.[37] COVID-19 transmission dynamics are characterized by the fact that much of disease's spread is due to a small group of infected individuals.[38]

The epidemiological networks and transmission chains of each group in this study led to the identification of a lower R_0 among asymptomatic cases, with a lower percentage of transmission chains initiated by asymptomatic individuals, which translates directly into generation of a smaller number of secondary cases; a finding in direct contrast with one Chinese study,[11] and similar to that found by Ruiz Nápoles in Cuba's Holguín province and another study performed in China during the beginning of the epidemic.[39,40]

We also found that superspreader events occurred more often among symptomatic patients, except for one asymptomatic super-spreader case that occurred in a closed internment institution.

The results of this study affirm that it is not possible to know in advance which persons have the ability to transmit the infection, so from an epidemiological point of view it is advisable to promote and respect measures to avoid specific socio-environmental conditions that favor transmission: not frequenting crowded public places or closed spaces with poor ventilation, maintaining physical distancing, masking, and frequent hand washing in all circumstances.

In agreement with Nishiura,[41] estimating the percentage of asymptomatic cases and their role in the spread of the epidemic has substantially expanded knowledge of the disease, and has improved understanding of SARS-CoV-2 transmission.

New SARS-CoV-2 variants of concern Molecular surveillance carried out by the Pedro Kourí Tropical Medicine Institute in Havana has shown that COVID-19 transmission patterns in Cuba during the beginning of the epidemic were similar to those in Wuhan, China. During almost all of 2020, the mutation in position 614 (D614G) was the most common, coinciding with the period in which asymptomatic or pre-symptomatic infection was the most predominant form of infection. After borders re-opened in November 2020, the alpha and beta variants of concern (VOCs) were identified and later isolated in all Cuban provinces. VOC beta was associated with significant increases in case numbers and with case severity in certain regions, including Havana and Jagüey Grande, Matanzas province. This situation worsened after the introduction of VOC delta, first identified in a traveler at the end of April 2021 and detected in local transmission cases by May. Within a short period of time, this variant spread throughout most provinces, especially Havana, Matanzas, Ciego de Ávila, Cienfuegos, Holguín and Guantánamo.[42,43] Delta's rapid

extension—associated with characteristics that facilitate greater transmissibility—led to its predominance over other variants, including beta, also the case in the Americas.[44]

The change caused by the new variants in the proportion of symptomatic and asymptomatic cases at the time of diagnosis is remarkable. In 2020, asymptomatic patients exceeded 70% of all patients during certain periods, while by the end of August 2021, asymptomatic cases represented only 5% of daily confirmed cases. [42] The increase in the proportion of symptomatic cases, together with a shortening incubation period, an increase in viral load and lengthened transmissibility periods suggest that the contribution of symptomatic cases could continue to be an important factor in community transmission of SARS-CoV-2 compared with asymptomatic transmission, as was observed in 2020.

Study limitations As we used information from the Cuban database of confirmed cases, pre-symptomatic cases may have been included in the asymptomatic case group, as there was no clinical follow-up as to whether these cases became symptomatic. It was also not possible to calculate disease attack rates, as it would have been necessary to know the exposed population in each epidemiological network.

CONCLUSIONS

Asymptomatic SARS-CoV-2 infection was the most common form of COVID-19 in Havana during the study period, and such individuals were less likely to spread the infection than those who were symptomatic. Superspreader events, most likely under specific conditions, played an important role in sustaining the epidemic.

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Cerebral Hemodynamic Reserve Abnormalities Detected Via Transcranial Doppler Ultrasound in Recovered COVID-19 Patients

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ABSTRACT

INTRODUCTION SARS-CoV-2 infection can produce endothelial injury and microvascular damage, one cause of the multiorgan failure associated with COVID-19. Cerebrovascular endothelial damage increases the risk of stroke in COVID-19 patients, which makes prompt diagnosis important. Endothelial dysfunction can be evaluated by using transcranial Doppler ultrasound to study cerebral hemodynamic reserve, but there are few of these studies in patients with COVID-19, and the technique is not included in COVID-19 action and follow-up guidelines nationally or internationally.

OBJECTIVE Estimate baseline cerebral hemodynamic patterns, cerebral hemodynamic reserve, and breath-holding index in recovered COVID-19 patients.

METHOD We conducted an exploratory study in 51 people; 27 men and 24 women 20–78 years of age, divided into two groups. One group comprised 25 recovered COVID-19 patients, following clinical and epidemiological discharge, who suffered differing degrees of disease severity, and who had no neurological symptoms or disease at the time they were incorporated into the study. The second group comprised 26 people who had not been diagnosed with COVID-19 and who tested negative by RT-PCR at the time of study enrollment. Recovered patients were further divided into two groups: those who had been asymptomatic or had mild disease, and those who had severe or critical disease. We performed transcranial Doppler ultrasounds to obtain baseline and post-apnea tests of cerebral hemodynamic patterns to evaluate cerebral hemodynamic reserve and breath-holding indices. We characterized the recovered patient group and the control group through simple descriptive statistics (means and standard deviations).

RESULTS There were no measurable differences in baseline cerebral hemodynamics between the groups. However, cerebral hemodynamic reserve and breath-holding index were lower in those who had COVID-19 than among control participants (19.9% vs. 36.8% and 0.7 vs. 1.2 respectively). These variables were similar for patients who had asymptomatic or mild disease (19.9% vs.19.8%) and for those who had severe or critical disease (0.7 vs. 0.7).

CONCLUSIONS Patients recovered from SARS-CoV-2 infection showed decreased cerebral hemodynamic reserve and breath-holding index regardless of the disease's clinical severity or presence of neurological symptoms. These abnormalities may be associated with endothelial damage caused by COVID-19. It would be useful to include transcranial Doppler ultrasound in evaluation and follow-up protocols for patients with COVID-19.

KEYWORDS: SARS-CoV-2; COVID-19; breath holding; ultrasonography, Doppler, transcranial; endothelium, vascular; cerebrovascular circulation; Cuba

INTRODUCTION

SARS-CoV-2 infects humans by binding its spike protein to angiotensin-converting enzyme 2 (ACE2) receptors expressed in lung, heart, kidney and intestinal cells, and in the vascular tree's endothelial cells. Direct or immune-mediated viral infection results in endothelial and microvascular dysfunction, one cause of multiple organ involvement in COVID-19.[1–2]

SARS-CoV-2 spreads via systemic circulation or by passing through the cribriform plate of the ethmoid bone,[3] which can affect brain tissue and result in neurological signs and symptoms.[4,5]

IMPORTANCE Cerebral hemodynamic reserve, an expression of endothelial involvement, is altered in recovered COVID-19 patients. This alteration, which increases stroke risk, can be detected via transcranial Doppler ultrasound.

Varga described multifocal microvascular lesions in the brain and olfactory bulbs in patients who died of COVID-19, but did not find direct viral infection of the brain tissue, so infection or inflammation of the endothelium itself seems to be important.[1]

The cerebral microcirculatory damage that causes endotheliitis in SARS-CoV-2 infection can be diagnosed at a patient's bedside by evaluating cerebral hemodynamic reserve (CHR) through transcranial Doppler ultrasound. However, the technique is not included in international or domestic action guidelines for addressing acute SARS-CoV-2 infection or recovery, and research on the matter is limited.

Transcranial Doppler ultrasound approximates cerebral hemodynamic study by evaluating flow velocities circle of Willis arteries.[6] The technique has proved a useful diagnostic tool for patients with microvascular disease of different etiologies, in which the cerebral hemodynamic reserve may be abnormal even though the cerebral hemodynamic pattern is within normal ranges. CHR is defined as the ability of arterioles and capillaries to dilate in response to increased neuronal activity or to a metabolic

or vasodilator stimulus,[7] which can include CO_2 inhalation, acetazolamide administration, and the breath-holding test (BHT). BHT consists of a period of voluntary apnea that causes cerebral vasodilation by secondary increase of CO_2 levels.[8,9] The result represents the percentage change of cerebral blood flow velocity from baseline to maximum values. The inclusion of apnea time with BHT more closely approximates CHR, defined as breath-holding index (BHI).[8,10] The decrease in CHR has been linked to greater probability of stroke.[8,11,12]

Considering that abnormalities in cerebral microcirculation can be diagnosed with transcranial Doppler ultrasound, we propose estimating baseline cerebral hemodynamic pattern, CHR and BHI in recovered COVID-19 patients.

METHODS

General study design and sample screening The study universe comprised 51 people, 27 men and 24 women aged 20–78 years, divided into two groups. The first group included 25 participants recovered from COVID-19, who had had different degrees of disease severity and no neurological symptoms or disease at the time of study enrollment. The second group included 26 healthy people with no history of COVID-19, and with negative SARS-CoV-2 real-time polymerase chain reaction (RT-PCR) tests at study enrollment; participants in this group were recruited from the medical and nursing staff of Havana's Medical Surgical Research Center (CIMEQ).

We enrolled the group of recovered COVID-19 patients from those who came in for follow-up visits, from Februrary through May 2021, two weeks through one month following hospital visits. Patients were admitted to a medical center during their illness with different degrees of COVID-19 clinical severity, as defined by WHO,[13] and were treated according to the Cuban protocol.[14] Participating patients were stratified into two groups: one included asymptomatic patients and those who had mild forms of the disease, and the other included those who had severe forms of the disease.

Of the patients who had recovered from COVID-19, 14 had asymptomatic infections (56%, 14/25), 7 had mild forms of the disease (28%, 7/25), 2 had severe, and 2 had critical forms (16%, 4/25). Of the patients, 3 had experienced headache (12%, 3/25), 2 a decreased sense of smell (8%, 2/25), and 1 loss of taste (4%, 1/25). All were asymptomatic at the time the study was conducted.

The ages, distribution by sex, and comorbidities of the recovered patients and control group are shown in Table 1.

Data acquisition and collection Transcranial Doppler ultrasound (Doppler-Box X1, DWL, Germany) was performed to determine baseline cerebral hemodynamic patterns and estimate cerebral hemodynamic reserve through a BHT following completion of a baseline exam. All studies were conducted by the same evaluator, who has more than 10 years'experience in the technique.

Table 1: Study group demographics and medical history

Variable	Recovered Patients	Control Participants
N	25	26
Age, mean (SD)	38.6 (14.7)	39.6 (11.8)
Female	7	17
Male	18	9
Hypertension	8	6
Diabetes mellitus	1	1
Bronchial asthma	3	1

Baseline recordings and BHTs were performed while the person was lying in a supine position. Vessels were examined using a 2 MHz pulsed-wave Doppler transducer (DWL, Germany). Sample volume of the studied vessel, and gain and selected power were kept constant during the recording. We studied the right and left middle cerebral arteries (MCA) through both temporal windows, at depths of 45–55 mm. The values acquired in the MCAs were used once a bilateral symmetrical pattern was confirmed. MCA blood flow velocities were considered asymmetrical if the difference was greater than 20%. Arterial flow (velocity and spectral waveform) remained constant for at least 30 seconds before recording study variable values.

We evaluated peak systolic velocity (PSV), end diastolic velocity (EDV) and mean velocity (MV) in the baseline hemodynamic pattern.

Once a baseline pattern reading was established, participants were instructed to stop breathing for 30 seconds after taking a normal breath (to avoid a Valsalva maneuver). In the next 5–10 seconds, we obtained the maximum values of the same hemodynamic variables used in the baseline studies. If the participant could not hold his or her breath for 30 seconds, the shortened breath-holding time was recorded.

CHR evaluated by BHT was defined as the percentage increase of post-apnea rMCAMV, and was calculated as the increased percentage of rMCAMV in apnea over baseline rMCAMV. There are no national studies to provide reference values, so we used a control group. Normal reported values differ in the international literature because individual pCO_2 varies based on the condition of the person being evaluated.[15]

BHI was calculated by dividing rMCAMV's increased percentage, averaged out in the time after breath-holding, by seconds of apnea according to the following formula:

BHI = [(apnea rMCAMV – baseline rMCAMV) / (baseline rMCAMV)] x 100] / 30 seconds (or seconds of apnea achieved).

Normal BHI value was established at 1.2 (SD 0.6).[10]

Statistical analysis We estimated the arithmetic means and standard deviations (SD) of quantitative variables, and calculated percentages for the qualitative variables.

Ethics We obtained written informed consent from participants. We maintained patient confidentiality, following the Declaration of Helsinki guidelines on research involving human subjects. Ethics protocols were approved by the scientific and ethical advisory board at the Medical–Surgical Research Center (CIMEQ) in Havana, Cuba.

RESULTS

Baseline hemodynamic variable means were similar between recovered COVID-19 patients and the control group. The post-apnea hemodynamic pattern variables were lower in the recovered patient

Table 2: Baseline and post-apnea cerebral hemodynamic pattern, cerebral hemodynamic reserve and breath-holding index, by study group

Variable	Recovered Patients Mean (SD)	Asymptomatic/Mild Mean (SD)	Severe/Critical Mean (SD)	Control Participants Mean (SD)
Baseline systolic velocity (cm/s)	78.1 (19.5)	78.0 (18.8)	78.5 (26.1)	84.6 (18.6)
Baseline diastolic velocity (cm/s)	39.4 (11,3)	40.1(11.8)	36.0 (8.3)	43.4 (11.4)
Mean baseline velocity (cm/s)	52.3 (13.6)	52.7 (13.8)	50.2 (14.2)	57.2 (13.2)
Post-apnea systolic velocity (cm/s)	90.7 (23.5)	91.0 (23.4)	89.3 (27.4)	112.9 (27.1)
Post-apnea diastolic velocity (cm/s)	48.7 (14.5)	49.5 (15.0)	44.9 (12.2)	60.4 (14.5)
Mean post-apnea velocity (cm/s)	62.7 (17.1)	63.3(17.6)	59.7 (16.3)	77.9 (18.5)
Cerebral hemodynamic reserve (%)	19.9 (13.2)	19.9 (11.9)	19.8 (21.1)	36.8 (14.7)
Breath-holding index	0.7 (0.4)	0.7 (0.4)	0.7 (0.7)	1.2 (0.5)

group, with lower systolic, diastolic and mean velocities. Mean CHR and mean BHI were lower in recovered patients (Table 2).

Baseline cerebral hemodynamic pattern, post-apnea hemodynamic pattern variables, CHR and BHI were similar in all recovered patients, degree of disease severity notwithstanding (Table 2).

DISCUSSION

The endothelium corresponds with cerebral hemodynamic vasoreactivity since the endothelium produces constricting and relaxing factors, based on its integrity. Cerebral hemodynamic reserve may be a marker of endothelial function in cerebral arteries.[16,17]

Hypertensive patients <60 years of age, with no neurological signs or symptoms, with mild white matter conditions diagnosed by MRI, and in whom CHR was decreased, had a progressively higher risk of symptomatic stroke and lacunar infarct chronic vascular lesions than hypertensive patients of the same age with normal CHR.[18]

The endothelium has been called 'the Achilles' heel' of patients with COVID-19.[19] Cytokines and proinflammatory mediators shift endothelial functions from homeostasis to defense,[20] and microvascular lesions found in the brain and olfactory bulbs in patients who died from COVID-19 show that the virus infects brain vessel endothelia and can cause abnormalities in vasoreactivity.[1]

Sonkaya[21] evaluated cerebral hemodynamics in 20 patients hospitalized with COVID-19 who had neurological symptoms (headache, seizures, stroke, alterations in consciousness, ageusia, anosmia) and compared them with control participants. Patients had higher mean MCA velocity and lower CHR—evaluated by BHI with transcranial Doppler ultrasound—than control participants. These results are consistent with ours, the difference being that participants in our study had recovered and did not have neurological symptoms.

Marcic[22] studied cerebral hemodynamics through color-coded transcranial duplex ultrasound and estimated BHI in 25 patients who had recovered from mild COVID-19 (according to WHO classification), who were not hospitalized, and who came in for a neurology visit due to neurological symptoms 28–50 days after a negative SARS-CoV-2 RT-PCR. Patients had lower mean MCA velocities and lower mean BHI values (related to CHR abnormalities), compared with a healthy control group, which is also consistent with our results.

Unlike other studies, ours included patients from the entire range of COVID-19's clinical forms, several days after hospital discharge, who had no neurological or cardiorespiratory symptoms during CHR and BHI recordings. Therefore, the decrease in these variables indicates that vascular abnormalities remained after the acute phase of the disease had passed, that they continued to exist even though the patients had no neurological manifestations, and that these endothelial abnormalities occurred even with minor damage caused by COVID-19.

As of this writing, there has been no report that the decline in CHR and BHI in asymptomatic recovered patients or patients who had recovered from mild forms of the disease is similar to the rates in those with severe or critical forms of the disease. This is why the findings reported in the previous paragraph are important and constitute one of the main contributions of this research.

It is interesting to note the difference in baseline hemodynamic patterns reported in other research with different study populations.[21,22] In our research, the baseline hemodynamic pattern was normal, and the abnormality was found by evaluating CHR. This finding is consistent with cerebral microvasculature damage that exists in different diseases, including COVID-19.[1,23]

CHR decline is an expression of the endothelial damage that characterizes SARS-CoV-2 infection and is a warning sign of stroke risk, [8,11,12] even if patients were absent any neurological signs and symptoms during the disease's initial clinical presentation or during their recovery period, regardless of COVID-19's clinical severity.

Convenience sampling did not allow for an equal distribution of patients by sex. Due to the epidemiological situation related to COVID-19, we did not invite healthy individuals who would have to come to the hospital center, possibly putting themselves at risk, to participate in the study. Regarding sex and its influence on CHR, some authors report that CHR is lower in women in response to hypercapnia.[24,25]

Additional studies are needed to confirm these results, but the limited research we do have suggests transcranial Doppler ultrasound should be included for non-invasive neurological monitoring in COVID-19 action protocols and patient studies.

CONCLUSION

Patients who have recovered from COVID-19 have CHR and BHI impairment, regardless of initial infection severity and absence of neurological symptoms. This impairment can be attributed to the endothelial damage caused by the viral infection. We recommend including transcranial Doppler ultrasound in treatment protocols for recovering COVID-19 patients to alert providers to patients with elevated risk of stroke.

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Thrombotic Microangiopathy in Patients Recovering from COVID-19

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ABSTRACT

INTRODUCTION During the pandemic caused by the SARS-CoV-2 virus, some patients who develop severe forms of COVID-19 present thrombotic microangiopathy in the course of the disease's clinical progression.

METHODS Data came from direct patient observation and clinical records. We performed a kidney biopsy and used optical microscopy and immunofluorescence techniques.

RESULTS We present the case of a 78-year-old male patient, mestizo, overweight with a history of high blood pressure, ischemic cardiopathy and chronic obstructive pulmonary disease who was first admitted to the hospital due to respiratory symptoms and diarrhea related to COVID-19, from which he recovered. He was subsequently readmitted with symptoms of acute renal dysfunction accompanied by mild anemia and thrombocytopenia; at the same time, he resulted negative for COVID-19 via a real-time polymerase chain reaction test. A kidney biopsy revealed thrombi in glomerular capillaries, acute tubular necrosis, thickening of extraglomerular blood vessel walls, and C3 deposits in the glomerular tufts.

CONCLUSIONS We describe a case of thrombotic microangiopathy with kidney biopsy in a patient recovering from COVID-19. Acute renal dysfunction is a form of thrombotic microangiopathy that has been observed in patients recovering from COVID-19.

KEYWORDS COVID-19, thrombotic microangiopathy, kidney, biopsy, Cuba

INTRODUCTION

COVID-19 presents an ongoing challenge to global public health.[1] Many patients with severe forms of COVID-19 present coagulation abnormalities, such as disseminated intravascular coagulation and thrombotic microangiopathy (TMA), with thrombi in several organs and tissues, marked endothelial damage and high mortality.[2]

Clinically, TMA is characterized by hemolytic anemia, thrombocytopenia and acute renal dysfunction. TMA development in COVID-19 has several potential causes, and its identification and prompt treatment can affect a patient's clinical progress.[3] However, reports of TMA in patients recovering from COVID-19 are not common and its diagnosis can be difficult, since clinical signs may not be pronounced.[4] The objective of this study is to present and describe a case of TMA with acute renal damage in a patient recovering from COVID-19.

METHODS

All data were collected from direct patient observation and from the patient's clinical records. Two kidney biopsy samples were taken with an automatic 16-gauge core biopsy gun. Optical microscopy (OM) was used to analyze one of the samples with the following tests: hematoxylin-eosin test, Masson's trichrome, Jones methenamine silver and periodic acid Schiff (PAS). The other was analyzed by immunofluorescence (IF) microscopy using IgA, IgG, IgM (immunoglobulins A, G, and M, respectively), C3, C1q (complement component 1q), fibrinogen, and kappa and lambda antisera.

Ethical considerations The patient provided written informed consent for his case to be presented without revealing his identity. The study was approved by the Ethics Committee and the Scientific Council of the Dr Abelardo Buch López Nephrology Institute in Havana, Cuba.

IMPORTANCE We describe Cuba's first case of thrombotic microangiopathy in a patient recovering from COVID-19.

RESULTS

The study describes a 78-year-old male patient, mestizo, overweight (BMI of 29.3 kg/m^2), ex-smoker with a history of hypertension, ischemic cardiopathy and chronic obstructive pulmonary disease treated with diltiazem, enalapril and hydrochlorothiazide. He sought emergency care three days following symptom onset of productive cough, fever, weakness, diarrhea and polypnea. In the physical exam, the patient presented mild crepitations in both lung bases and a heart rate of 115 beats/min. The patient also presented hypoxemia (SO_2 of 78% without supplemental oxygen). The chest X-ray showed patches of diffuse inflammatory lesions. A real-time reverse polymerase chain reaction (RT-PCR) test for COVID-19 was conducted, which was positive. The patient was admitted to an intermediate care unit and treated with oxygen, chloroquine, α2b interferon, Kaletra (lopinavir and ritonavir), methylprednisolone and ceftriaxone.

Laboratory test results conducted upon admission are presented in Table 1. The patient recovered from the respiratory dysfunction and diarrhea without the need for mechanical ventilation. After being hospitalized for 21 days, he tested negative for COVID-19 (by RT-PCR) and was discharged (without anticoagulant therapy and with basic treatment, as previously described).

Two weeks after discharge, the patient presented asthenia, anorexia, diarrhea and reduced urine output, along with elevated levels of creatinine (746.1 µmol/L). He was readmitted (with a negative RT-PCR) and required hemodialysis (six sessions). The remaining exams are presented in Table 1. Renal ecography showed a slight rise in ecogenicity in both kidneys, and conserved perfusion. Given the acute renal dysfunction of unknown origin with

Table 1. Laboratory test results

Test (normal values)	During first hospitalization	During second hospitalization
Hemoglobin (132−166 g/L)*	156	90
Leukocytes (4.0−11.0 x 10^9/L)	7.5	9.1
Granulocytes (55%−70%)	89.5	78.8
Lymphocytes (1.5−3.5 x 10^9/L)	7.5	18.2
Monocytes (0.2−0.8 x 10^9/L)	3	3
Platelets (150−400 x 10^9/L)	181	121
Peripheral lamina		Absence of schistocytes or fragmented red cells
Coombs test		Negative
Creatinine (65.4−119.3 µmol/L)*	107.8	746.1
Uric acid (202−416 µmol/L)*	439	659
Urea (2.1−8.5 mmol/L)		20.86
Cholesterol (<5.18 mmol/L)	3.41	5.61
Triglycerides (<1.7 mmol/L)	3.39	3.19
Total proteins (60−83 g/L)		48.6
Albumin (38−64 g/L)		23.21
Total alkaline phosphatase (25−290 U/L)		96
ALT (≤49 U/L)		10
AST (≤49 U/L)		16
LDH (200−400 U/L)		467
Gamma-glutamyl-transpeptidase (10−45 U/L)		45
Total bilirubin (≤1.2 mg/dL)		1.2
Indirect bilirubin (≤0.94 mg/dL)		0.82
Direct bilirubin (≤0.3 mg/dL)		0.38
Blood glucose (4.20−6.11 mmol/L) (RV:)		3.49
Creatine phosphokinase [CPK] (24−195 U/L)		42.5
VDRL (Serology for syphilis)		Negative
Coagulogram		PT:14.4 sec (control: 12.6 sec) aPTT:27.3 sec (control 27.1 sec) Retracting clot Bleeding time: 1 min Coagulation time: 8 min
C3 (0.9−1.8 g/L)		1.3
C4 (0.1−0.4 g/L)		0.3
Nuclear antibodies		Negative
2-hour Addis count (elements/min)		Red blood cells: 55,000 Leukocytes: 21,000 Casts: 0
Proteinuria 24 hours (g/day)		0.57
Protein/creatinine ratio (g/g)		0.76
Stool culture		Negative
Fresh fecal sample		Negative
Hepatitis C antibodies		Negative
HIV antibodies		Negative
Hepatitis B surface antigen		Negative

*Normal range for adult males
ALT: Alanine aminotransferase; aPTT: Activated partial thromboplastin time; AST: Aspartate aminotransferase; LDH: Lactate dehydrogenase; PT: Prothrombin time

hematuria, mild anemia (no schistocytes) and thrombocytopenia, a kidney biopsy was performed.

In the kidney biopsy sample analyzed by OM, 11 glomeruli were found (two were obsolescent, one had an area of sclerosis, and several others had dilated glomerular capillaries and intracapillary thrombi) (Figures 1A, 1B, 1C and 1D). The tubular epithelium was simplified with loss of brush border and sloughing of cells toward the tubular lumen (Figure 1A). The extraglomerular blood vessels had marked thickening of the walls, vacuolization and intimal myxoid degeneration (Figures 1E and 1F). In the IF technique, diffuse deposits of C3 were observed, as well as traces of IgG, and lambda and fibrin light chain depositions in glomerular capillaries lumens (Figure 1C). The anatomical–pathological conclusion was TMA with acute tubular necrosis.

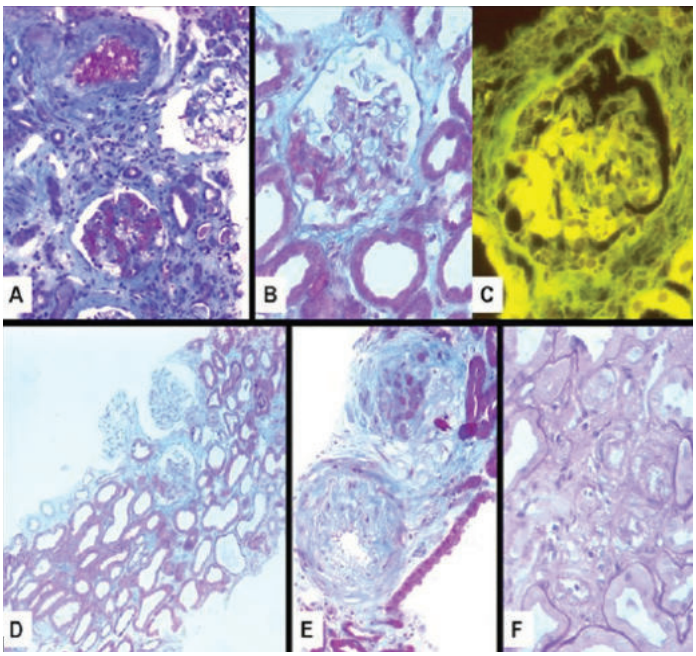
In the differential diagnosis to determine the origin of acute renal failure associated with COVID-19, multiple potential causes should be considered, such as collapsing glomerulopathy, interstitial nephritis, TMA, and acute tubular necrosis (ATN).[5] ATN in this case was most likely associated with renal hypoperfusion secondary to diarrheal episodes in an older patient with extremely damaged renal vasculature. The patient showed no signs of rhabdomyolysis (normal CPK), which has been described as a cause of ATN in COVID-19.[5] TMA was undoubtedly the most notable finding of the kidney biopsy; the literature has reported cases of COVID-19–associated TMA identified by kidney biopsy, one of which was in a recovering patient.[4,6]

While the TMA's clinical profile was atypical (with no evidence of schistocytes in the peripheral lamina, mild thrombocytopenia, and only moderately elevated LDH—lactate dehydrogenase—levels) the biopsy findings left no doubt. Other reports are incomplete when it comes to recording clinical manifestations of COVID-19–associated TMA.[7]

TMA's origin is particularly important for preventing damage in these patients. Possible origins include antiphospholipid syndrome, thrombotic thrombocytopenic purpura (TTP) (due to deficiencies in disintegrin and metalloprotease with thrombospondin type 1 motif 13 [ADAMTS-13]), typical and

atypical hemolytic-uremic syndrome (HUS) and multisystem inflammatory syndrome (MIS).[2]

Figure 1: A. Thrombi in glomerular capillaries and small arteries. Area of interstitial fibrosis and tubular atrophy. Masson's trichrome X200. **B.** Glomeruli with intracapillary thrombi. Masson's trichrome X400. **C.** Thrombi in glomerular capillaries. IF technique X400. **D.** Area of acute diffuse tubular necrosis. **E.** Extraglomerular blood vessels with severe thickening of the wall. Masson's trichrome. X200. **F.** Twisted arterioles with marked thickening of the wall and partial obliteration of the vascular lumen. PAS X400.



Antiphospholipid syndrome has been described in patients with COVID-19. However, it is unlikely that this was the trigger in this case, for several reasons. There were no signs of macrothrombosis; serology was negative for syphilis (cardiolipin antibodies), which, while not a measure of phospholipid antibodies, tends to be positive in patients with no history or signs of autoimmune disease; and nuclear antibodies were negative.[8]

This syndrome cannot be completely ruled out as an initiating cause, however, since phospholipid antibodies were not directly measured (in any case, not an ideal test since these antibodies can fluctuate upward during the course of an infection or exposure to drugs).[9] Antibody presence 12 weeks following symptom onset must be identified to confirm this diagnosis.[10]

Thrombotic thrombocytopenic purpura secondary to ADAMST-13 deficiency is another potential diagnosis in cases of patients recovering from SARS-CoV-2 infection. A lack of resources made measuring ADAMTS-13 levels infeasible, so it cannot be ruled out. In the present case, however, the PLASMIC score[11] (at <5) suggests no notable enzyme deficiency.

Another plausible diagnosis in this case is typical HUS with diarrhea, as the patient presented with diarrhea at symptom onset and again when readmitted. HUS is more common in children and tends to affect other systems. For this patient, no other organs were affected, stool samples were negative, and the diarrhea did not contain blood.[12]

Another potential diagnosis previously identified in patients with COVID-19 is atypical HUS with activation of an alternative complementary pathway,[2] generally caused by mutations in gene coding for complementary proteins such as C3 and the H, B, and I factors; or by antibodies against complementary regulatory factors.[2,6] However, atypical HUS has been described as secondary to infections that directly cause endothelial damage or that deregulate the alternative complementary pathway with heavy deposits of C3 in the kidney biopsy, as occurred with this patient.[13] Atypical HUS is a probable diagnosis, despite the patient's normal C3 levels. It is important to keep in mind that fewer than half of atypical HUS cases have low C3 levels.[12,14]

MIS (multi-syndrome inflammatory syndrome)—a Kawasaki-like disease—has been described in children recovering from COVID-19, and one report describes an adult with COVID-19–associated TMA. While that case shares some similarities with ours, absence of any extra-renal abnormalities suggests that MIS is an unlikely diagnosis. [4,15]

Despite the shortage of diagnostic resources needed to determine a more accurate differential diagnosis, all indications point to basic vascular damage due to age, adverse lipid profile and excess weight, which worsened due to the vascular cytopathic effect of SARS-CoV-2 infection, as observed in the kidney biopsy. During convalescence, the pro-thrombotic condition caused by COVID-19 triggered TMA following secondary atypical HUS.

CONCLUSIONS

We describe TMA in a patient recovering from COVID-19, demonstrating that TMA should be considered a possible cause of renal dysfunction in patients with COVID-19 and in those recovering from the disease.

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Effects of Physical Exercise on Burnout Syndrome in University Students

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ABSTRACT

INTRODUCTION Burnout syndrome has a negative impact on university students' health worldwide. Global prevalence of each dimension of the syndrome is estimated at 55.4% for emotional exhaustion, 31.6% for cynicism and 30.9% for academic inefficacy.

OBJECTIVE Evaluate the efficacy of physical exercise in reducing burnout levels in university students.

METHODS We carried out an investigation in students from the Technical University of Ambato, Ecuador. Students were in different career tracks, randomly selected, and were assigned to three different groups with pre-test and post-test measurements: two intervention groups (aerobic and strength exercise) and one control group (no exercise). The evaluation instrument was the Maslach Burnout Inventory-Student Survey, whose dimensions are exhaustion, cynicism and academic inefficacy. We also evaluated heart rate variability.

RESULTS The aerobic exercise group reduced cynicism by 21.1% (d = 0.252), inefficacy 13.1% (d = 0.397) and exhaustion by 31.0% (d = 0.532). The strength exercise group reduced cynicism by 27.4% (d = 0.315), inefficacy by 21.7% (d = 0.704) and exhaustion by 19.6% (d = 0.299). In the control group, exhaustion and inefficacy increased by 10.1% (d = 0.128) and 4.4% (d = 0.129) respectively; instead, cynicism was reduced by 7.3% (d = 0.062).The aerobic exercise group had the greatest increase in heart rate variability (at 16.8%), followed by the strength group (16.6%) and the control group (5.2%).

CONCLUSIONS Physical exercise (both aerobic and strength) was effective in reducing burnout levels in university students.

KEYWORDS Exercise; burnout, psychological; burnout, professional; mental health; Ecuador

INTRODUCTION

Burnout syndrome (BS) is a health issue with great—and often underacknowledged—societal repercussions. In students, BS is defined as a persistently negative emotional response related to being a student, which consists of feelings of tiredness, not being able to perform study-related tasks (exhaustion); a cynical or detached attitude regarding the meaning and utility of studies undertaken (cynicism); and a feeling of academic incompetence (inefficacy). BS is measured with the Maslach Burnout Inventory-Student Survey (MBI-SS), which incorporates these dimensions.[1]

BS has broadened as a field of study, as research is now carried out on university students,[1] since they face pressures and overloads related to academic work, like workers with their specific work.[2] Additionally, they are exposed to many demands concerning their educational context: learning, academic performance, work overload, time pressure and paucity, lack of self-management opportunities, competition among peers, perceived irrelevance, poor teaching and poor relationships, among others, and in the academic environment, students are considered among the most vulnerable to BS.[1–4]

Global prevalence of BS dimensions in students is estimated at 55.4% for emotional exhaustion, 31.6% for cynicism and 30.9% for academic inefficacy.[1,2]

IMPORTANCE
This is the first study in Latin America and among the first worldwide demonstrating the benefits of physical exercise in reducing burnout levels in university students.

The practice of regular physical exercise has beneficial effects for overall physical,[3,4] mental[4,5] and socio-affective[5] health. It directly supports mental health, as the endorphins released during physical exercise act on the brain and produce sensations of well-being and immediate relaxation;[6] in addition, they inhibit the neurotransmitters that transmit pain, resulting in analgesia and mild sedation. We also observed reductions in stress and emotional fatigue, which in turn can lead to better sleep quality and improved well-being, and decreased BS and fatigue.[6] Regular physical exercise could be an effective intervention to reduce burnout in students.

In the absence of an effective physical exercise program, students may suffer from mental health problems with their studies, because the risk of mental health issues increases if academic studies are accompanied by certain predisposing factors.[6]

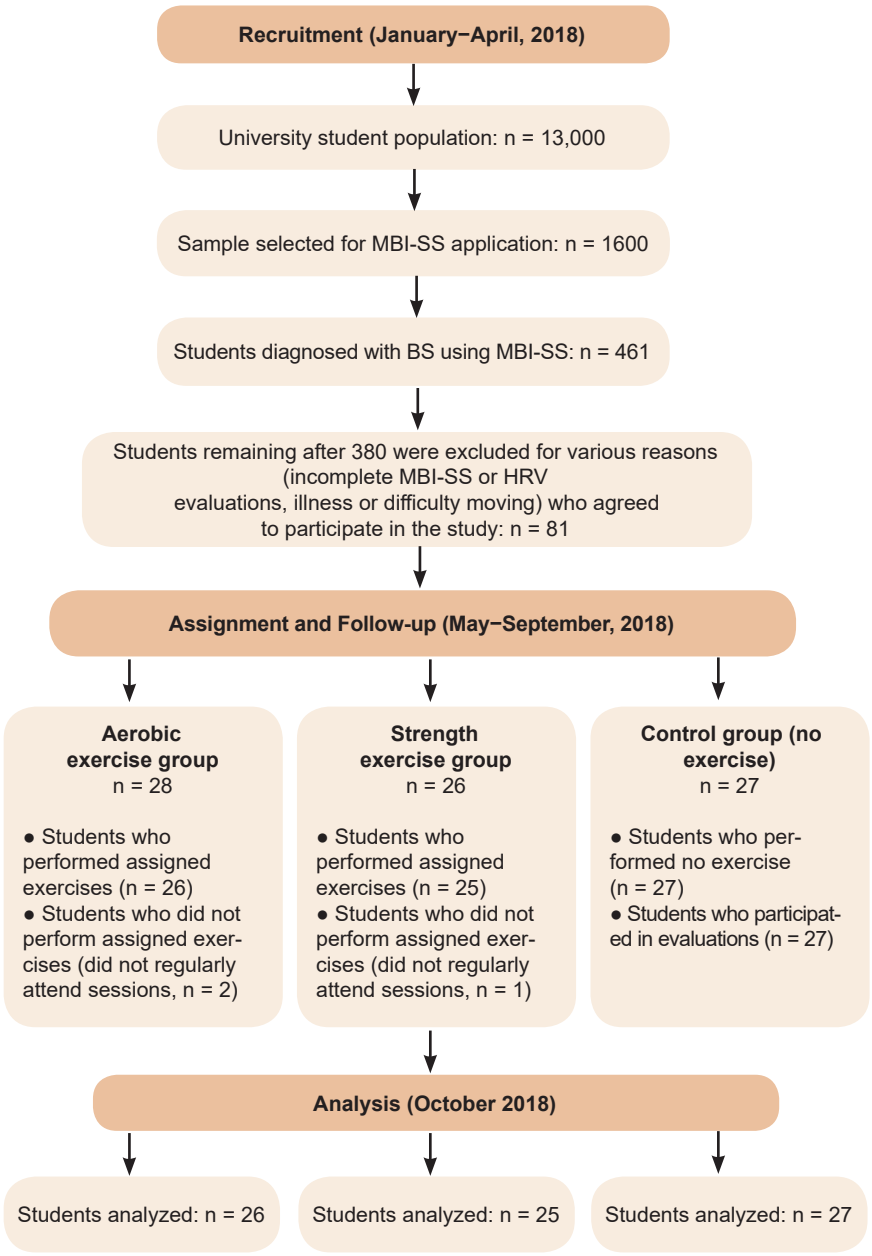
Programs designed to prevent or treat BS are essential in improving student health, and yet to date, few studies have been conducted on the usefulness of physical exercise in combatting BS in university students,[5] the objective of our research.

METHODS

Study and participants We carried out an investigation from January through October, 2018 in students from the Technical University of Ambato, Ecuador, which at the time of our study enrolled 13,000 students. A sample of 1600 undergraduate students from all departments, career tracks and years of study was selected via stratified random sampling. MBI-SS was administered to students by a psychologist qualified to do so.[7,8] Among the students selected, 461 had moderate or high levels of BS; 380 students were excluded for various reasons (incom-

plete MBI-SS and heart rate variability evaluations, illness, movement difficulties) leaving a total sample of 81 students. These students were randomly divided into three similarly composed groups: two intervention groups (aerobic and strength-training exercises) and a control group (no assigned exercise). The mean age of the control group was 23.13 years (SD = 3.77), that of the aerobic exercise group was 22.74 (SD = 3.05), and that of the strength-training group was 22.97 (SD = 3.31) (Figure 1).

Figure 1: Study protocol flowchart



BS: Burnout syndrome; HRV: Heart rate variability; MBI-SS: Maslach Burnout Inventory-Students Survey[8]

Study variables

Burnout syndrome in students was evaluated using the MBI-SS,[1,8] structured to evaluate three dimensions: the feeling of not being able to give more of oneself, both physically and psychically (exhaustion), devaluation and loss of interest in studying (cynicism) and doubts about one's ability to carry out efficacious academic work (inefficacy). All items in each of these subscales were scored on a 7-point frequency scale, ranging from 0 (never) to 6 (always). Of the items, 5 evaluate exhaustion, 4 cynicism, and 6 academic inefficacy, for a total of 15 items. The scale has adequate levels of global internal consistency (α = 0.82) and validity as measured by the Kayser, Meyer & Olkin test (KMO = 0.87).[8]

The scale was evaluated according to the following:[7,8]

- Exhaustion: No BS: <1.2; Low: 1.3–2.0; Moderate: 2.1–2.8; High: ≥2.9
- Cynicism: No BS: <0.5; Low: 0.6–1.24; Moderate: 1.25–2.25; High: ≥2.26
- Inefficacy: No BS: <2.83; Low: 2.83–3.83; Moderate: 3.84–5.16; High: ≥5.17

Heart rate variability (HRV) is a good marker of overall health status and can quickly aid in the diagnosis of states of stress, burnout, fatigue, overtraining, exhaustion, or anxiety in the general population. It is an excellent cardiovascular biomarker for prevention and early detection of BS.[9] To measure HRV, a transmitter band and the Android application Elite HRV were used, by which the mean (RR), standard deviation (SDNN) and square root of the mean value of the sum of the squares of the difference of all RR intervals (RMSSD) are generated between R waves with each heartbeat.

Physical exercise Our definition and procedures regarding physical exercise were taken from the American College of Sports Medicine's (ACSM) latest prescribed exercise guidelines.[10]

Procedures The 81 study participants were randomly assigned to one of the three groups, as previously described: two intervention groups (one assigned aerobic physical exercise and one assigned resistance/strength-training exercise) and a control group, to which no treatment was applied (Table 1). In the two exercise groups, physical exercise was governed by the latest ACSM prescribed exercise guidelines[10] at three weekly sessions of one hour on alternating days for 16 weeks, always from 5:00 PM to 6:00 PM.[9] The MBI-SS was applied at the beginning of the study, and HRV was measured in all participants. The two instruments (MBI-SS and HRV) were reapplied to the 3 groups at week 17, and comparisons were made between groups.

The aerobic exercise group was assigned jogging, walking or stationary bicycling for 30–50 minutes, divided into an initial warm-up/stretch, the planned aerobic exercise, and a recovery period.[10] The strength/resistance training group was assigned push-ups, abdominal exercises, high bar and leg squats, in sessions of 30–50 minutes, divided into warm-up and stretching, planned strength exercises, and a recovery period.[10] No exercises were assigned to the control group.

Data analysis All data were analyzed with SPSS version 25.0 (IBM, USA). Descriptive statistics (mean X and standard deviation SD) were calculated and assumption of distribution normality was verified using the Shapiro–Wilks test. Comparisons within groups between pre- and post-tests were made using the Wilcoxon test for Likert-like scale instruments and physiological tests (HRV). We calculated the effect size of the biserial point correlation (bpc) to quantify the magnitude of changes, and converted the results into Cohen's d, a measure of the effect size as a standardized mean difference, reporting how many standard deviations exist between the two groups compared—experimental group and control group, or the same group before and after the intervention.

The reference points used were: trivial (d <0.2), small (d = 0.2–<0.5), moderate (d = 0.5–<0.8), large (d = 0.8–1.2) and very large (d >1.2). We set the assumed level of statistical significance at p <0.05.

The aerobic exercise group included 28 students: all performed the assigned exercises, although only 26 were included in the analysis, as 2 students did not regularly exercise at least 3 days per week. The strength-training group included 26 students, all of whom performed the exercises, but only 25 were included in the analysis, as 1 student did not perform the exercises regularly. The control group comprised 27 students who performed no exercises, and all were included in the analysis (Figure 1, Table 1).[11]

Ethical considerations Students received explanations regarding the nature and aims of the research and informed consent was obtained from each participant. The study was approved by the ethics committee of the National Health Directorate and the Technical University of Ambato, both in Ecuador, under the code: CEISHSOLCAQ.OBS.19.129.

RESULTS

In the aerobic exercise group, exhaustion showed the greatest change, with a 31% percent reduction, followed by reduced cynicism (21.1%) and academic inefficacy (13.1%) (Table 1). However, in the strength-training group, the greatest change was obtained in cynicism, with a 27.4% reduction, followed by inefficacy (a 21.7% reduction) and exhaustion (a 19.6% reduction). In the control group, the mean exhaustion level increased by 10.1%. Inefficacy also increased (4.4%) (Table 1).

The BS dimension showing the greatest reduction over time in the aerobic exercise group was exhaustion, with a moderate effect size (d = 0.532), but not cynicism or inefficacy, which exhibited small change effects (d = 0.252 and 0.397, respectively). However, in the strength-training group, the dimension with the greatest reduction over time was inefficacy, with a moderate effect size (d = 0.704); here the change in exhaustion levels was small (d = 0.299) as the change in cynicism (d = 0.315). The effect size on exhaustion, cynicism and inefficacy in the control group were all considered trivial (d = 0.128, 0.062 and 0.129, respectively).

Reduction in HRV is related to mental health problems, overtraining or poor physical condition. In the aerobic exercise group, SDNN had the largest percent change, at 33.0%, while RR and RMSSD had lower percent changes of 16.8% and 19.7%, respectively.

There was a very large change in effect size in the mean RR in the aerobic and strength groups (d = 1.281 and 1.328 respectively), unlike SDNN, which was large (d = 0.943 and 0.833), and RMSSD, which was small (0.425 and 0.318) in both groups. However, in the control group the effect sizes were small (0.449, 0.457) and trivial (0.120) (Table 1).

DISCUSSION

The study results show that physical exercise reduced BS intensity in participating students, although not all dimensions of BS were reduced considerably. Some of our results coincide with those of other authors: de Vries evaluated the effectiveness of an exercise intervention in reducing BS in patients working in various professions, using a set exercise routine lasting six weeks.[12] The intervention groups reported lower BS than the control group, as was the case in this study.

Table 1: Pre-test and post-test comparison between exercise and control groups

		Exercise group Aerobic (n = 28; M / F: 11/17)*				Exercise group Strength (n = 26; M / F: 10/16)**				Control group (n = 27; M / F: 12/15)			
Variable		Pre-Test X (SD)	Post-Test X (SD)	Percent Change	Effect size (d)	Pre-Test X (SD)	Post-Test X (SD)	Percent Change	Effect size (d)	Pre-Test X (SD)	Post-Test X (SD)	Percent Change	Effect size (d)
Burnout syndrome	Exhaustion	2.00 (1.24)	1.38 (0.99)	–31.0	0.532	1.80 (1.19)	1.45 (1.11)	–19.6	0.299	1.68 (1.27)	1.85 (1.31)	10.1	0.128
	Cynicism	1.18 (1.03)	0.93 (0.97)	–21.1	0.252	1.32 (1.12)	0.96 (1.22)	–27.4	0.315	1.24 (1.22)	1.15 (1.69)	–7.3	0.062
	Inefficacy	4.27 (1.38)	3.71 (1.49)	–13.1	0.397	4.24 (1.37)	3.32 (1.29)	–21.7	0.704	4.27 (1.34)	4.46 (1.65)	4.4	0.129
Heart rate variability	RR (ms)	865.69 (102.08)	1011.37 (128.32)	16.8	1.281	859.92 (100.76)	1002.77 (117.92)	16.6	1.328	862.53 (101.24)	907.72 (104.05)	5.2	0.449
	SDNN	71.32 (20.11)	94.87 (29.86)	33.0	0.943	70.34 (20.18)	89.92 (27.24)	27.8	0.833	70.91 (21.24)	81.13 (24.27)	14.4	0.457
	RMSSD	68.71 (31.10)	82.24 (33.78)	19.7	0.425	69.75 (31.76)	79.81 (32.73)	14.4	0.318	70.01 (30.97)	73.69 (31.39)	5.3	0.120

* The analysis included only 26 participants who performed the exercises regularly.
** The analysis included only 25 participants who performed the exercises regularly.
M / F: Male/Female; RMSSD: Square root of the mean values of the sum of squares of the differences of all RR intervals; RR: interval mean; SDNN: RR Standard deviation; X (SD): Mean (Standard deviation).

Eskilsson applied a 12-week aerobic training program of moderate intensity for BS patients.[13] A total of 88 patients diagnosed with BS (average age: 45) were randomly divided into two groups; an intervention group assigned aerobic exercise and a control group with no assignments. The final evaluation showed a decrease in BS levels in the intervention group, with a much smaller decrease in the control group, consistent with our results.

Our study used both strength and aerobic exercise to combat BS, and both types of exercise were found to be effective. It is well known that physical exercise improves blood circulation and, consequently, oxygenation, and stimulates the brain to produce chemical mediators like serotonin, a neurotransmitter that aids in preventing depression. Increased heart rate during exercise strengthens the heart and aids in circulation.[12,13]

One of the possible strengths of this study is the methodology. Exercises assigned to each group followed the latest ACSM recommendations.[10] Another strength is the relationship it draws between mental health and physical exercise, and contributes to the recognition of physical exercise as a therapeutic resource, which has good acceptance among patients and providers, is low-cost, non-pharmacological, and may reduce other risk factors affecting overall health, lifestyle, and well-being.

Regarding the study's limitations, we recognize the infeasibility of maintaining the exercise program for longer than initially planned, which would have allowed us to know whether or not there was a change in the intervention's effectiveness over a longer period. Also, tests related to the immune system and inflammation—important biomarkers for BS effects in human physiology and biochemistry—could not be applied. Furthermore, the true extent of burnout (in both frequency and intensity) was not assessed. It would be useful to scale up the intervention involving physical exercise as a strategy to combat BS in students. This study could provide the initial results necessary to support development of intervention programs using exercise to help combat burnout syndrome in university students.

CONCLUSIONS

Our results show that physical exercise (aerobic or strength) reduced burnout syndrome levels in university students.

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Percutaneous Coronary Intervention Versus Myocardial Revascularization Surgery in Multivessel Coronary Artery Disease: Four-Year Followup

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ABSTRACT

INTRODUCTION In Cuba, 29,939 deaths from ischemic heart disease were recorded in 2020. Myocardial revascularization surgery and percutaneous coronary intervention are well-established methods of treating patients with multivessel coronary artery disease. These methods can reduce overall deaths, but choosing the optimal strategy for treating left main coronary ischemia is a source of debate among specialists.

OBJECTIVE Estimate survival and major cardiac and cerebrovascular events in patients treated with percutaneous coronary intervention versus myocardial revascularization surgery and their relationships with pre-existing patients' clinical and angiographic characteristics.

METHODS We conducted a retrospective cohort study in 41 patients; 35 men and 6 women aged 40–85 years who had been diagnosed with multivessel coronary artery disease and treated with percutaneous coronary intervention (n = 17) or myocardial revascularization surgery (n = 24) at the Medical–Surgical Research Center in Havana, Cuba, in 2016. The main variable under consideration was the occurrence of major adverse cardiovascular events over a four-year period following these interventions. We collected clinical and angiographic characteristics, and used the Kaplan–Meier test to calculate survival curves. Survival probabilities were compared using the log-rank test. A value of p <0.05 was considered statistically significant. The Cox proportional hazards model was used to estimate the hazard ratio, with 95% confidence intervals used for both procedures.

RESULTS There were a total of 20 major adverse cardiovascular events, 75% (15/20) of which occurred in patients who underwent percutaneous coronary intervention and 5% in patients who had myocardial revascularization surgery. The probability of survival was 70.6% in surgery and 37.5% in interventionism; p = 0.043; hazard ratio 1.58 (95% confidence interval 0.987–2.530), p = 0.047. The need to repeat a revascularization procedure was the only major cardiovascular event that showed significant differences between methods (log-rank p = 0.015), and was more frequent in percutaneous intervention.

CONCLUSIONS Myocardial revascularization surgery offers a better chance of survival than percutaneous coronary intervention. Major adverse cardiovascular events are more frequent in patients with coronary interventionism, due to the need to repeat revascularization.

KEYWORDS Coronary disease, myocardial revascularization, coronary artery bypass, angioplasty, Cuba

INTRODUCTION

In Cuba, 29,939 people died in 2020 from heart disease, at a rate of 267.3 per 100,000 population. Of these, 18,572 died from ischemic-type diseases.[1] Among ischemic heart diseases, multivessel coronary artery disease is a heterogenous group, due to its anatomical and functional complexity. This requires a similarly complex approach to treatment that focuses on each patient's individual characteristics when choosing the best available therapeutic strategy.

Myocardial revascularization surgery (MRS) or percutaneous coronary intervention (PCI) are common methods used in treating these patients, but the ideal method for multivessel coronary artery disease or ischemia of the left main coronary artery (LMCA) is controversial among interventional cardiologists and cardiovascular surgeons. Although clinical practice guidelines lean toward MRS, decisions on treatment options changed for many patients after PCI was introduced.[2]

IMPORTANCE
This paper provides information on survival rates and complications following percutaneous coronary intervention and myocardial revascularization surgery over four years comparing these procedures. This may help determine the optimal revascularization strategy for patients with multivessel coronary artery disease.

The Cuban cardiocenter network practices both revascularization methods, but few reports are available on long-term survival for the two procedures. We set out to estimate survival and major adverse cardiac and vascular events (MACE) in patients treated with PCI or MRS, and the relationships these events have with patients' clinical and angiographic characteristics prior to both procedures.

METHODS

Study type and sampling We carried out a cohort study in 41 patients; 35 men and 6 women aged 40–85 years, who were diagnosed with either three-vessel coronary artery disease or LMCA, treated with percutaneous coronary intervention (n = 17) or myocardial revascularization surgery (n = 24) at the Medical–Surgical Research Center (CIMEQ) in Havana, Cuba, in 2016. MRS was

performed in most of the more complex lesions. Patients were followed up for 48 months after PCI or MRS.

Inclusion criteria
- Patients undergoing revascularization for the first time with three-vessel arterial disease or LMCA
- Patients with stenoses ≥50.0% in vessels 1.5 mm in diameter

Exclusion criteria
- Patients who underwent previous interventions (PCI or MRS)
- Patients with ST-segment elevation acute myocardial infarction (MI)
- Patients with MRS concomitant with other types of cardiac or vascular surgery

PCI patients had either conventional or paclitaxel-eluting stents (a drug limiting growth of scar tissue after stent placement).

The primary focus was MACE, defined as: death from any vascular cause, cerebrovascular events, MI, or the need for repeat revascularization. MI was defined by the fourth universal definition[3] and cerebrovascular disease was defined as a focal neurological deficit lasting >72 hours. Demographic, clinical and angiographic variables were examined. The anatomical complexity of coronary artery disease (CAD) was graded by SYNTAX scoring.[4]

Data were obtained from medical records, MRS operation reports, PCI reports and followup records.

Statistical analysis MACE-free survival curves were constructed using the Kaplan-Meier method. Survival probabilities were compared using the log-rank test. A value of $p < 0.05$ was considered statistically significant. Cox proportional hazards modeling was used to estimate hazard ratios (HR) using 95% confidence intervals (CI), comparing PCI and MRS.

Ethics This research was approved by CIMEQ's research ethics committee and followed the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from patients before inclusion in the study. Identifying information was kept confidential.

RESULTS

Participants' mean age was 62.2 years; 15% of patients were older than 75. Heart failure prior to revascularization was the only clinical variable that demonstrated a clear association with the chosen procedure. The angiographic variables showing notable differences between groups were total occlusions, bifurcation/trifurcation, severe calcification and tortuosity. The SYNTAX scores were higher in patients with coronary artery bypass grafting (MRS) (Table 1).

Most complex injuries (64.7%) included LMCA plus two or three additional vessels (Table 1).

Median followup was 30 months (with an interquartile range of 4–48 months). At the end of the 4-year study period, there were 20 MACE; 75% (15/20) in PCI patients and 25% (5/20) in MRS patients. The probability of survival at the end of the followup period was 70.6% in MRS patients and 37.5% in PCI patients.

In the survival analysis, HR = 1.58 (95% CI: 0.987–2.530) (Figure 1).

Table 1: Baseline clinical and angiographic patient characteristics

Characteristic	MRS group (n = 17)	PCI group (n = 24)	Total n = 41
Age (median) (SD)	60.0 (8.2)	64.7 (11.5)	62.2 (10.2)
Male sex n (%)	16 (94.1)	19 (79.2)	35 (85.4)
Female sex n (%)	1 (5.9)	5 (20.8)	6 (14.6)
Risk factors n (%)			
Diabetes mellitus	4 (23.5)	4 (16.6)	8 (19.5)
Hypertension	14 (82.3)	18 (75.0)	32 (78.0)
Smoker	15 (88.2)	16 (66.6)	31 (75.6)
Dyslipidemia	8 (47.0)	6 (25.0)	14 (34.1)
Medical record n (%)			
Previous MI	6 (35.2)	12 (50.0)	18 (43.9)
cardiac insufficiency	6 (35.2)	1 (4.1)	7 (17.0)
Clinical presentation n (%)			
Unstable angina	4 (23.5)	12 (50.0)	16 (39.0)
Stable angina	11 (64.7)	10 (41.6)	21 (51.2)
Asymptomatic with positive test	2 (11.7)	2 (8.3)	4 (9.8)
SYNTAX median (SD)	33.6 (9.02)	21.7 (8.0)	26.6 (10.2)
Median number of lesions (SD)	3.5 (1.2)	4.4 (1.7)	3.8 (1.5)
Diffuse disease n (%)	14 (82.3)	19 (79.1)	33 (80.4)
Coronary disease extension n (%)			
Only 3 vessels	5 (29.4)	14 (58.3)	19 (46.3)
LMCA	0 (0.0)	2 (8.3)	2 (4.9)
LMCA + 1 vessel	1 (5.9)	0 (0.0)	2 (4.9)
LMCA + 2 vessels	6 (35.3)	4 (16.7)	10 (24.4)
LMCA + 3 vessels	5 (29.4)	4 (16.7)	8 (19.5)
Total occlusion (n)*	15	13	28
Bifurcation/ Trifurcation (n)*	20	13	33
Thrombi (n)*	0	2	2
Severe calcification (n)*	13	5	18
Ostial aortic injury (n)*	6	8	14
Length >20 mm (n)*	25	36	61
Tortuosity (n)*	14	2	16

*Referring to total lesions; LMCA: left main coronary artery; MI: myocardial infarction; MRS: myocardial revascularization surgery; PCI: percutaneous cardiac intervention; SD: standard deviation
Note: SYNTAX scores were calculated from data analyzed in the catheterization laboratory

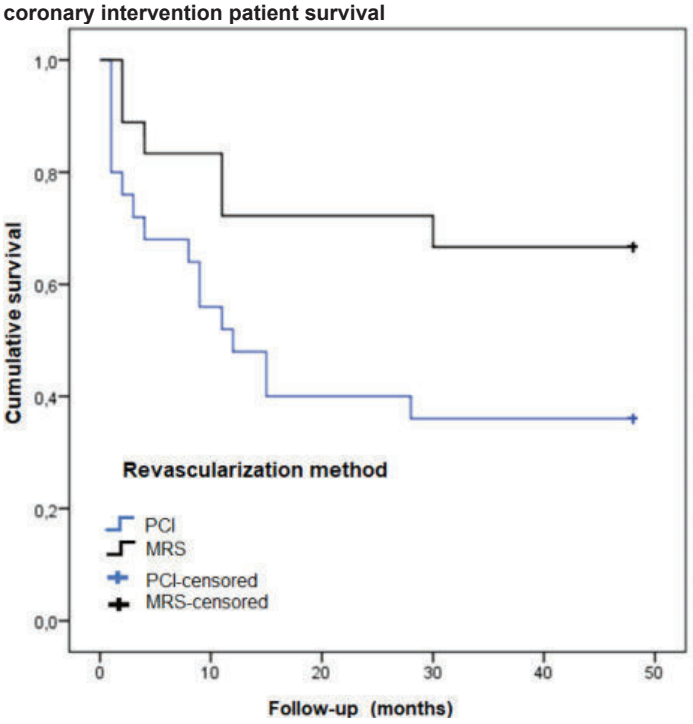
In PCI patients, MACE included the need for repeat revascularization (60%; 9/15), death from any cause (26.7%; 4/15) and MI (13.3%; 2/15). In MRS, MACE included death from any cause (40%; 2/5), cerebrovascular disease (40%; 2/5), and the need for repeat revascularization (20%; 1/5).

DISCUSSION

Although there are protocols and resources guiding these procedures in high-income countries, there have been no studies in Cuba evaluating long-term results of the two revascularization methods. Accordingly, this study—although limited to Cuba—could prove useful to low- and middle-income countries where PCI has been more accessible.

Since the publication of the SYNTAX study in 2009, attempts have been made to relate pre-existing clinical and angiographic

Figure 1: Myocardial revascularization surgery and percutaneous coronary intervention patient survival



MRS: Myocardial revascularization surgery
PCI: Percutaneous coronary intervention

variables to revascularization methods.[5] In the present study, we found an association with age and heart failure. Left ventricular ejection fraction deterioration has been reported as a good independent predictor of death and other major adverse events after revascularization, and particularly surgery. It is common to include this variable in predictive models.[6] In a meta-analysis comparing revascularization methods, Hlatky found age and diabetes mellitus to be associated with the choice of revascularization method.[7]

In several studies, angiographic variables showed stronger associations with the revascularization methods than clinical variables.[5,7] Among the angiographic variables associated with revascularization, lesion lengths >20 mm were an exception, which may be due to the fact that this variable is imprecise and exhibits high interobserver variability, as reported by Mohr.[8] These variables seem to have a greater influence on patients treated with PCI, likely related to greater anatomical complexity.

A SYNTAX study with a 5-year followup showed a higher MACE incidence in the PCI group than the MRS group (37.5% vs. 24.2%, p <0.001). Death/acute stroke/MI were higher in the PCI group (22% vs. 15.0% in MRS, p <0.001), with significant differences in all causes of death and in occurrence of angina during followup. The need for repeat revascularization was higher among PCI patients.[9]

The SYNTAX study was the first to evaluate survival after PCI with drug-eluting stents, as compared with MRS. After a 10-year followup period, proportions of all-cause deaths between PCI and MRS were similar. Subgroup analyses showed MRS had significantly fewer all-cause deaths in patients with three-vessel artery disease, but not in patients with LMCA disease.[10]

Occurrence of MACE was less frequent after MRS, mainly due to the need for repeat revascularization in PCI patients, and there were no differences in the categories of MI, death from any cause, or cerebrovascular disease. The difference in the need for repeat revascularization could be due to the type of stent used in PCI, but we did not include this variable in our analysis. A randomized prospective longitudinal study of 318 patients—159 assigned conventional stents and 159 assigned paclitaxel-eluting stents—with a followup of 3 years, concluded that although patients who received paclitaxel-eluting stents had a greater probability of survival, "they evolved similarly in terms of MI incidence, death from any cause, and stent thrombosis."[11]

The only Cuban study that compares the two strategies in 178 patients (87 PCI and 91 MRS), after a 2-year followup, found higher rates of cardiac mortality and MI in MRS and greater need to repeat revascularization in PCI, and concluded that there were no differences in survival between the two strategies.[12] However, the researchers did not perform a log-rank test for this variable, and the periods of followup and intervention for the two strategies were not simultaneous and this is a potential source of bias.

The main limitation of this study is its quasi-experimental design and the fact that it is based on a retrospective cohort. The typical random assignment of clinical trials is not feasible in this type of study. Other limitations include the lack of stratification by cause of death (as this information was not available), which also limited more rigorous control of confounding variables. We also did not compare the use of conventional stents and drug-eluting stents in PCI patients.

CONCLUSIONS

Patients with multivessel coronary artery disease or ischemia of the left main coronary artery have a better chance of survival if they undergo myocardial revascularization surgery than if they undergo percutaneous coronary intervention. The number of major adverse cardio-cerebrovascular events is greater in patients with coronary interventionism, relating to the need for repeat revascularization.

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Wound Chronicity, Impaired Immunity and Infection in Diabetic Patients

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ABSTRACT

BACKGROUND Diabetic foot ulcers are a common diabetic complication leading to alarming figures of amputation, disability, and early mortality. The diabetic glucooxidative environment impairs the healing response, promoting the onset of a 'wound chronicity phenotype'. In 50% of ulcers, these non-healing wounds act as an open door for developing infections, a process facilitated by diabetic patients' dysimmunity. Infection can elicit biofilm formation that worsens wound prognosis. How this microorganism community is able to take advantage of underlying diabetic conditions and thrive both within the wound and the diabetic host is an expanding research field.

OBJECTIVES 1) Offer an overview of the major cellular and molecular derangements of the diabetic healing process versus physiological cascades in a non-diabetic host. 2) Describe the main immunopathological aspects of diabetics' immune response and explore how these contribute to wound infection susceptibility. 3) Conceptualize infection and biofilim in diabetic foot ulcers and analyze their dynamic interactions with wound bed cells and matrices, and their systemic effects at the organism level. 4) Offer an integrative conceptual framework of wound–dysimmunity–infection–organism damage.

EVIDENCE AQUISITION We retrieved 683 articles indexed in Medline/PubMed, SciELO, Bioline International and Google Scholar. 280 articles were selected for discussion under four major subheadings: 1) normal healing processes, 2) impaired healing processes in the diabetic population, 3) diabetic dysimmunity and 4) diabetic foot infection and its interaction with the host.

DEVELOPMENT The diabetic healing response is heterogeneous, torpid and asynchronous, leading to wound chronicity. The accumulation of senescent cells and a protracted inflammatory profile with a pro-catabolic balance hinder the proliferative response and delay re-epithelialization. Diabetes reduces the immune system's abilities to orchestrate an appropriate antimicrobial response and offers ideal conditions for microbiota establishment and biofilm formation. Biofilm–microbial entrenchment hinders antimicrobial therapy effectiveness, amplifies the host's pre-existing immunodepression, arrests the wound's proliferative phase, increases localized catabolism, prolongs pathogenic inflammation and perpetuates wound chronicity. In such circumstances the infected wound may act as a proinflammatory and pro-oxidant organ superimposed onto the host, which eventually intensifies peripheral insulin resistance and disrupts homeostasis.

CONCLUSIONS The number of lower-limb amputations remains high worldwide despite continued research efforts on diabetic foot ulcers. Identifying and manipulating the molecular drivers underlying diabetic wound healing failure, and dysimmunity-driven susceptibility to infection will offer more effective therapeutic tools for the diabetic population.

KEYWORDS Diabetic foot, amputation, infections, biofilms, microbiota

INTRODUCTION

Diabetes mellitus (DM) is characterized by the onset and progression of a constellation of multi-organ complications resulting from multifactorial interactions—including biochemical derangements and epigenetic factors—which ultimately translates to irreversible tissue changes as a response to glucooxidative processes.[1] Of all diabetic complications, the development of diabetic foot ulcers (DFUs) is among the most common and debilitating.[2,3] Classic concepts define DFU as deep tissue damage of the lower limb, frequently preconditioned by, and associated with, neuropathy or peripheral arterial disease.[4] It is recognized as a major and growing public health problem, a scientific challenge and a socioeconomic burden;[5] and remains the main causal factor of lower extremity amputations, disability and early mortality.[6] Armstrong introduced the 'cancer analogy' concept to highlight the fact that five-year mortality rates associated with foot ulceration and amputation surpass those registered for common cancers.[7–10]

IMPORTANCE This article contrasts wound healing processes in healthy individuals and diabetics, establishing and conceptualizing the reciprocal links between diabetic dysimmunity, susceptibility to infection, diabetic foot ulcer chronicity and insulin resistance amplification.

Diabetic glucooxidative stress impairs the healing response and disrupts the flow of overlapping healing phases, ultimately promoting the onset of a 'wound chronicity phenotype'.[11–13] Aside from healing impairment, a common occurrence in diabetic patients is ulcer recurrence after primary closure.[6] These non-healing wounds are a major predisposing factor or entry point for wound infection[11] and accordingly, more than 50% of DFUs become infected.[14] Infection acts as a primary deterrent to physiological healing responses[15] and a risk factor for lower-limb amputation,[16–18] especially when deep tissues and bones are compromised.[19,20] Although diabetics are particularly vulnerable to bacterial infections,[21–23] DFUs have a complex and highly organized polymicrobial community that frequently contributes to undesirable outcomes in DFU-affected individuals.[22] This microbiota–biofilm comprises symbiotic bacteria, yeast and fungal loads and can silently spread, amplify the underlying healing deficit, increase antibiotic resistance, disrupt host metabolism and further dampen immune response.[24–27]

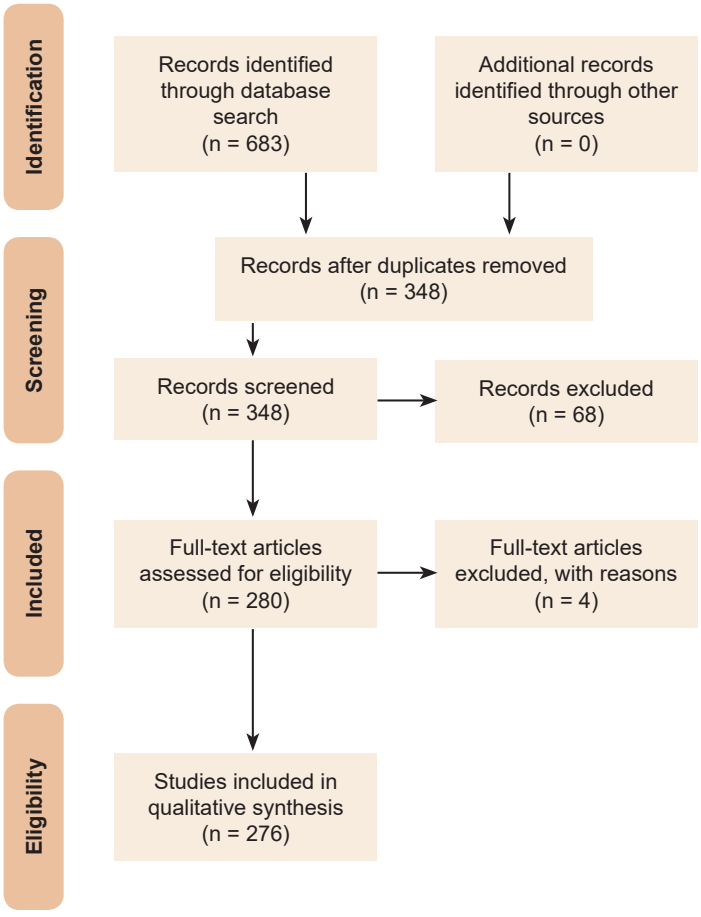
Globally speaking, DM and infection increasingly go together.[28,29] Diabetic individuals are prone to peripheral-tissue infections; given dysregulations in primary surveillance, recognition, activation and neutralization mechanisms within the innate immunity repertoire.[21,30,31] Furthermore, diabetic individuals exhibit antigen presentation failure, contraction of T-cell–mediated immune function[32] and a particular predisposition to bacterial adhesion to epithelial linings.[33,34]

DFU is a unique battlefield where host–microorganism interactions shape ulcer progression. Consequently, numerous studies have addressed the role of biofilm on DFU and its impact within the ulcer bed and the host itself.[35–38] We reviewed this critical issue, given its etiopathogenic relevance to basic aspects of DFU pathology: 1) Why are diabetic persons more susceptible to wound infections? 2) How is DFU biofilm organized? 3) How does a microorganism's pathogenic potential and concentration impact DFU outcomes? and 4) How does biofilm impair the healing response?

EVIDENCE ACQUISITION

We retrieved articles indexed in Medline/PubMed, SciELO, Bioline International, and Google Scholar using the following keywords/phrases: DFU, limb AND amputation, DFU AND infection, DFU AND biofilm, immune system AND diabetic patient, microorganism AND immune system. A total of 683 articles were retrieved and exported a reference manager. Duplicate articles were removed (Figure 1). Our final selection included 280 research and review articles. Titles, objectives and abstracts were carefully screened and reviewed. The search was limited to the English language without date restrictions. All compiled information was structured under four principal headings: 1) a general overview of the normal healing response in a healthy organism; 2) an overview of the cellular and molecular foundations of the impaired healing process in the diabetic population; 3) diabetic dysimmunity; and 4) conceptual definition and pathogenic implications of diabetic foot infection (DFI) in its interaction with the host.

Figure 1. Literature review process



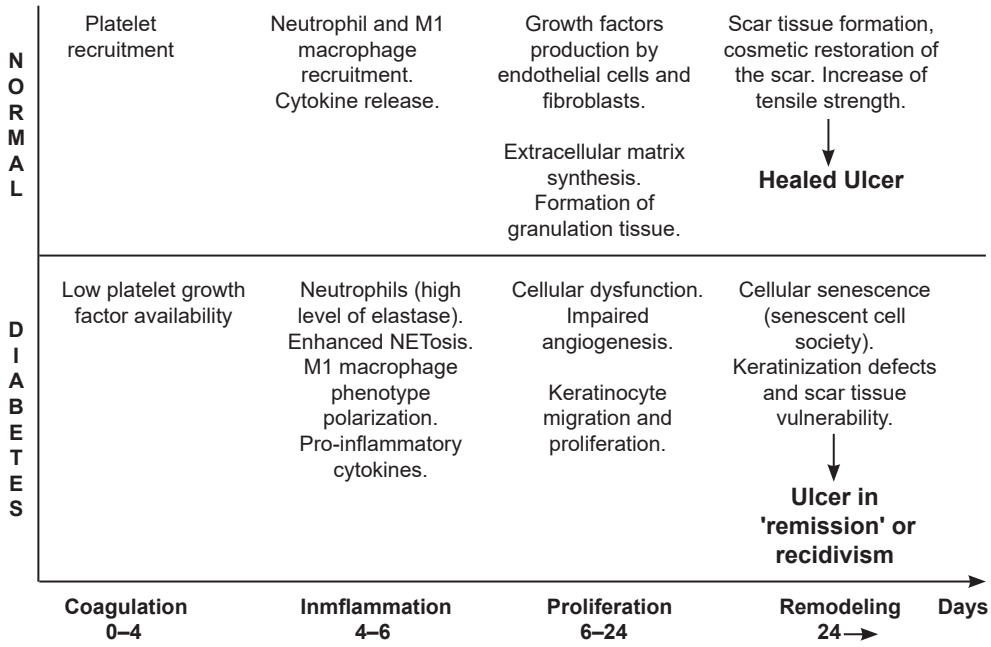
DEVELOPMENT

Brief overview of normal healing response Wound healing is a dynamic and complex process that ultimately results in restoration of anatomic integrity with analogous function.[39–41] Of note, however, skin wound healing represents an evolutionary advantage for organism survival, given its role in restoring barrier function, as well as preventing internal tissue damage and infection dissemination.[42] This evolutionary advantage involves a complex and intricate, but finely regulated, crosstalk between cells and soluble mediators.[43] A normal healing process is made up by four overlapping phases: 1) coagulative, 2) inflammatory, 3) proliferative, and 4) remodeling (Figure 2). Each phase takes place during a temporary window involving a certain cell population, a specific set of cytokines and a particular chemical composition within the extracellular matrix (ECM).[44,45] The coagulation process, aside from ensuring hemostasis, has two other relevant functions: 1) the fibrin clot and fibrinogen byproducts act as a scaffold and chemoattractant for the recruitment and anchorage of inflammatory cells, fibroblasts and other mesenchymal-derived cells that will participate in tissue granulation formation; and 2) platelet degranulation promotes primary growth factors. Platelets represent the first group of resident cells with fibroangiogenic soluble messengers, including platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).[46,47]

Of particular relevance for healing trajectory and for the ultimate scar phenotype are the infiltrating inflammatory cells and their biochemical signalers, as they exist in interaction with granulation tissue-resident cells as fibroblasts, myofibroblasts and endothelial cells.[44] Since cutaneous injury is linked to the release of 'danger and pathogen signals', innate immune receptors become activated and eventually trigger an inflammatory phase.[48]

The influx of polymorphonuclear neutrophils (PMNs) is ensured mainly by a complex cascade of vasoactive signalers and chemoattractants, so that these cells invade and are anchored within the wound matrix, initiating the wound's acute phase that may last up to four days.[49] PMNs pattern recognition receptors are activated by local damage-associated molecular patterns (DAMP) released during cell injury and necrosis, and by bacterial pathogens' associated molecular pattern (PAMP). Upon activation, these cells release pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and IL-6, which amplify the inflammatory response and pave the way for macrophage infiltration and activation.[50] Activated PMNs 'clean' the wound bed of tissue debris via an armamentarium of degradative and antimicrobial proteases (cathepsins, defensins, lactoferrin and lysosomes) stored in cytoplasmic granules. This sanitizing process is largely dependent on the formation of neutrophil extracellular traps (NETs), web-like structures that capture and eliminate exogenous bacteria, fungi and viruses.[51] This process of NET release is termed NETosis and has broad implications for different forms of inflammation.[52] Recent studies show that circulating PMNs from diabetic subjects are biased toward excessive release of NETs,[53] and that uncontrolled NETosis impairs the healing process in diabetic mice and humans.[54] These cells also generate reactive oxygen species (ROS) that help eliminate invading pathogens.[55] Altogether, the role of PMNs in wound healing is undoubtedly important, but an extension of their local residence time and their functional profile may lead to wound chronicity (Figure 2).

Figure 2. Normal vs. diabetic healing



Disruption in phase progression, delaying normal progress.
NETosis: neutrophil extracellular traps release

A second generation of inflammatory infiltrating cells comes from local resident macrophages that are differentiated from infiltrating monocytes, which are activated via DAMP, PAMP and a cytokine surge.[56] Anti-macrophage depletion studies have revealed the physiological significance of these cells for the late stage of the inflammatory phase and for initiation of the proliferative one.[41,57,58] This late stage of the inflammatory phase is mostly characterized by the M1 subset of macrophages, which themselves are characterized by phagocytic and pro-inflammatory activities. Later, macrophages polarize to an M2 subpopulation with anti-inflammatory activity by expressing interleukin-receptor antagonists and a collection of fibroangiogenic growth factors that enhance fibroblast proliferation, extracellular matrix synthesis, angiogenesis,[59,60] ultimately reducing inflammation.[47] Macrophage subclass shift from M1 to M2 subsets is a meaningful event, given its role in turning off inflammation, clearing the wound bed of apoptotic PMNs (efferocytosis), ensuring proliferative phase progression, and preventing autoreactivity to released self-antigens.[15,57,61] Conclusively, inflammation is a time-restricted, finely controlled sequential event, whose expansion is paradigmatically associated with a torpid healing phenotype, or to wound chronification and senescence of granulation tissue productive cells.[16,17,62]

Granulation tissue is subsequently organized and populated by a broad spectrum of extracellular matrices, secreting cells that are in active and dynamic engagement with the substrate, and progressively modulating the structure and composition of the wound's extracellular matrix.[63] Although granulation tissue is a temporary organ, it is important as a 'welding material' filling wound gaps, preventing environmental threats, and providing support for cell adhesion, migration, growth and differentiation during wound repair.[63,64]

The proliferative phase also embraces three important processes: angiogenesis, wound contraction and re-epithelialization. Angiogenesis is an exciting biological process actively regulated by pro-angiogenic growth factors, chemokines, integrin receptors, bone marrow-derived progenitor cells, and transcriptional and post-transcriptional epigenetic regulators; it restores blood inflow and outflow, and therefore oxygen delivery and CO_2 extraction. Its role in a normal healing trajectory is essential.[65] In response to pro-angiogenic signals like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), PDGF-B, TGF-β, and angiopoietins, endothelial cells initiate angiogenesis by sprouting, proliferation and migration.[66] At the same time, locally secreted antiangiogenic factors are able to counterbalance and limit excessive angiogenesis.[67] The lack of an appropriate angiogenic response is a representative hallmark of DFUs, and identification of molecular forces underlying diabetic microangiopathy has been extensively examined.[68,69]

Wound contraction and epithelial resurfacing are two integrated mechanisms that ensure complete wound closure in most mammalian species. Contraction, a physiological and necessary event in wound closure, appears to be mediated by myofibroblasts, specialized cells responsible for force generation, that are differentiated from migrating fibroblasts during granulation tissue formation.[70] Although current theories posit that alpha smooth muscle actin-expressing myofibroblasts contribute to wound contraction,[71] others attest that contraction progresses through fibroblast-derived traction forces via thick collagen fiber secretion.[72] Irrespective of the contraction-responsible cell and the molecular drivers behind them, limited contraction is associated with torpid healing in diabetic wounds.[12]

Instrumental for complete and successful wound closure is re-epithelialization. This event demands integration of leading-edge keratinocyte proliferation, migration and differentiation in order to re-establish epidermal integrity.[73] This is perhaps one of the most complex and unexplored processes in wound healing.[74–76] Simplistically described, keratinocytes at the wound edge and epithelial cells from hair follicles in the vicinity migrate and proliferate. Signals that promote keratinocyte proliferation include heparin-binding EGF-like growth factor (HB-EGF), EGF, transforming growth factor alpha (TGF-α), and FGF secreted from platelets, macrophages and dermal fibroblasts.[77] For migration to progress, there is a reduction of desmosomes and hemidesmosome connection, cytoskeleton reorganization, morphological reprograming and changes in the pattern of keratin expression.[78] In general, there is a loss of physical tension at points of cell attachment to the basal lamina.

Re-epithelialization progresses when the basement membrane is reconstituted by upper dermal fibroblasts and keratinocytes in a cooperative effort.[79] In this context, the interaction between integrin receptors and the neomatrix determines the speed of

migrating keratinocytes, a process that in turn is regulated by growth factor gradients.[80] Also important are plasmin and other plasmin-related degradative enzymes that degrade fibrin and other matrix glycoproteins, facilitating keratinocyte migration. Other collagenases and gelatinases are expressed by migrating keratinocytes.[77] Once keratinocytes attach to the basement membrane, they initiate a process of upward migration and differentiation to create a mature stratified, squamous epithelium that covers the wound.[81] Failed or delayed re-epithelialization is an obvious sign of wound stagnancy.[82] Compelling evidence documents the deleterious role of hyperglycemia and other downstream biochemical signalers on fibroblast and keratinocyte proliferation and migration.[83–85]

The ultimate phase—remodeling—begins approximately 14–21 days post-injury and may continue for years.[86–88] Its main function is the formation of normal epithelia and maturation of scar tissue;[89] excessive matrix is eliminated by a set of metalloproteinases, while new type-1 collagen fibers are produced and horizontally aligned in a more organized and esthetic manner. Inflammatory infiltration has ceased and different cell populations gradually enter into apoptosis.[90] The initial granulation tissue progressively collapses and is replaced by a new wound chemical milieu, so that the scar ECM architecture increasingly approaches original tissue morphology. The complexity involved in wound tissue remodeling has led us to hypothesize the existence of structural, positional and organizational memory in late wound cells, enforced by topographic 'home address signals'. At this point, wound tensile strength is restored, and antiangiogenic mediators are locally released to turn off angiogenic sprouting and ensure excessive vessel regression. In parallel, other anatomical structures including the epidermis, nerves and myofibers are synchronously remodeled forming a functional unit.[78,86,91]

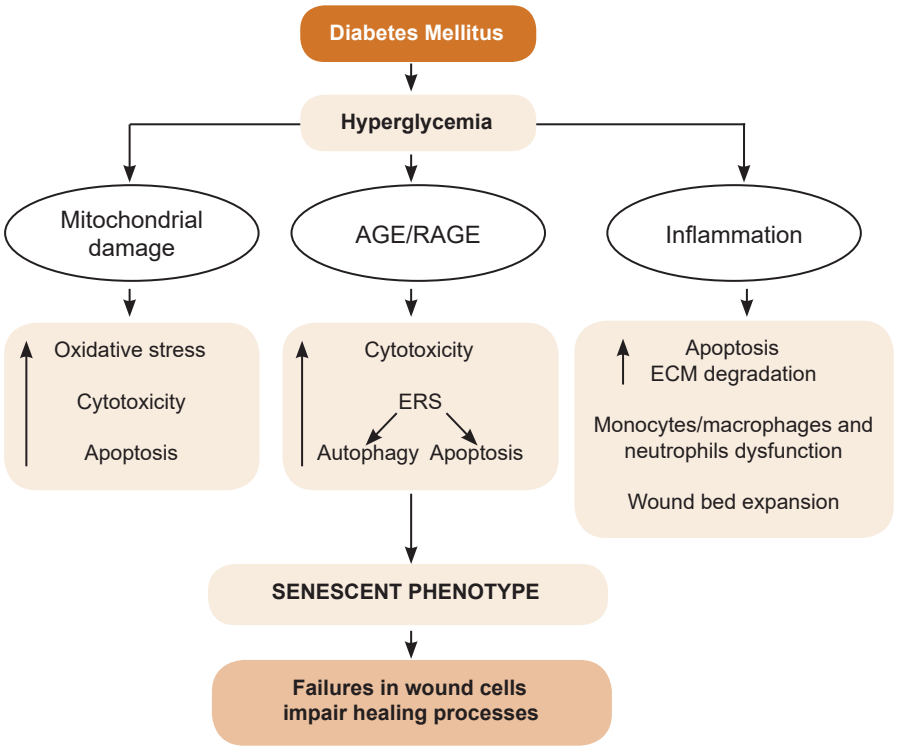
The diabetic foot ulcer: overview of the molecular bases of the torpid healing process Although it is generally accepted that time-to-heal determines a wound's clinical classification as acute or chronic, the conceptual definition of wound chronicity has remained controversial.[92] Nevertheless, it is generally accepted that a wound is considered chronic when it fails to proceed in "an orderly and timely reparative process that results in sustained restoration of anatomic and functional integrity".[92] DFU is archetypical of chronic wounds.[93–95] It is generally accepted that a primary hallmark of diabetic wounds is their persistent arrest in an unproductive inflammatory phase, associated with impaired formation and consolidation of mature granulation tissue.[96,97]

The arrest in this inflammatory phase is not associated with successful control of local infection, and thus it has been proposed that diabetic individuals are more vulnerable to wound infection[98,99] due to the existence of a primary deficit in innate immune response mechanisms.[100,101]

Hyperglycemia, again, seems to act as the proximal trigger for an exaggerated inflammatory reaction (Figure 3). Hyperglycemia and its distal operators—advanced glycation end products (AGE), TNF-α and other pro-inflammatory signalers—exert profound cytotoxic effects in fundamental 'building-block' cells of describes tissue.[11,102] Compelling evidence describes an inflammation-prone, pro-oxidative and pro-degradative environment in the core of diabetic wounds.[103–106]

Uncontrolled pro-inflammatory cytokine secretion imposes a pro-catabolic balance in the wound bed that both increases peripheral insulin resistance and reduces injured tissue's anabolic response.[107,108] TNF-α downregulates fibroblast collagen synthesis in diabetic skin and upregulates the synthesis of metalloproteinases by amplifying the wound's proteolytic and pro-degradative profile.[109,110] Although some studies have ruled out hyper- or hypoglycemia's role in significantly disrupting PMNs cells' ability to enter into apoptosis,[111] others have pointed to poorly controlled glycemia levels as a major factor in the prolonged residence of apoptosis-resistant PMNs with active secretory functions,[111] which ultimately translates into an elevated proteolytic/degradative balance.[112,113] This pro-degradative environment reduces local availability of growth factors and their receptors, hindering the ability of fibroblasts and endothelial cells to participate fully in the healing process.[88,114] PMN are also considered a source of ROS and nitric oxide species within the wound bed, with remarkable cytotoxic and pro-degradative potential.[115,116] The increased rate of ROS is an indirect consequence of poorly-controlled glucose levels given that existing evidence provides a connection between

Figure 3. Simplified hyperglycemia-associated healing impairment



High glucose burden and fluctuating glucose spikes are toxic under acute and chronic conditions to a large constellation of cell populations.
AGE: advanced glycation end products ECM: extracellular matrix ERS: endoplasmic reticulum stress
PMNs: polymorphonuclear neutrophils RAGE: AGE receptor

the high-glucose–induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway and exaggerated NETosis.[117] Hyperglycemia also induces myeloid progenitor proliferation and expansion, as well as increased neutrophil production of S100A8/S100A9, which ultimately binds to AGE receptors (RAGE). This interaction translates to enhanced ROS production and myelopoiesis[118] that reinforces the triad of hyperglycemia, neutrophil infiltration and local ROS production.

Aside from PMN, M1 subclass macrophages predominate in diabetic wounds.[119] Although the molecular drivers behind this process are not yet fully understood, it has been demonstrated that human monocytes and macrophages undergo M1-like inflammatory polarization when exposed to high levels of glucose in both culture conditions and in hyperglycemic subjects.[120] This hyperpolarization to the pro-inflammatory arm represents a flaw in the transition to the M2 subclass and, therefore, to an anti-inflammatory and pro-healing profile. Additionally, diabetic wound macrophages exhibit defective efferocytosis, a mechanism for clearing apoptotic bodies in the wound bed.[121] This failure increases the local surge of proinflammatory cytokines, which perpetuates inflammation and increases the risk of it becoming chronic.[121] Another study has documented that hyperglycemia itself, without additional metabolic factors, induces a mixed profile of M1/M2 cytokines that nurture diabetes-associated inflammation and atherosclerosis.[122] The diabetic systemic low-grade inflammatory phenotype is able to introduce monocyte chromatin modification that, in turn, intensifies the persistent pro-inflammatory state.[123]

The proliferative phase in diabetic foot ulcer healing is frequently slow, torpid and asynchronous.[124] This may entail irregular and abnormal fibroblast recruitment, scarce or abnormal extracellular matrix protein secretion, limited cell-anchoring scaffold synthesis, poor or abnormal angiogenesis—including pathologic vascular remodeling, slow contraction of wound contours, torpid re-epithelialization and the inability to remain in remission after epithelial resurfacing.[11,125,126] From the molecular angle, compelling evidence has identified high glucose burdens and accompanying fluctuating glycemia spikes[127] as the proximal trigger of many cellular impairments that generically transform into fibroblasts, endothelial cells and keratinocytes in mitogenic and motogenic arrest; premature apoptosis and the onset of a senescent phenotype.[128–130] Multiple *de novo* circuitries, metabolic shunts, inflammatory-prone reactants and abnormal pathways in diabetics impact the wound-healing response and perpetuate the ulcer.[131,132]

Glycoxidation derivatives are intrinsically cytotoxic to productive cells in granulation tissue, and further amplify pro-inflammatory and pro-oxidative circuitry by binding to RAGE.[133,134] Glycoxidative products accumulate in non-labile dermal collagen[132,134] leading to cutaneous cell toxicity and premature senescence, impairing fibroblast and endothelial cell physiology,[99] and consequently delaying granulation tissue formation and maturation.[11,133] Conclusively, within the wound, the triad of TNF-α, ROS and AGE can initiate apoptosis of fibroblasts and vascular cells, thereby prolonging inflammation, reducing growth factor availability and opening the gate for the onset of the so-called 'wound senescent cell society'.[130,135–137] It is not surprising therefore, that repair-committed cells in diabetics move through proliferative arrest, senescence, and apoptosis (Figure 3).[136]

Re-epithelialization failure in diabetics and the tendency toward local recidivism are significant challenges for clinicians, wound care providers, and basic scientists. It has been suggested that an incomplete program of keratinocyte activation and differentiation[138] is fundamental for the presence of mitotically active—but not migrating—epithelial cells along the wound's leading edge.[73,76] High glucose has shown to exhibit a toxic effect on keratinocytes, reducing proliferation, replicative life span,[139] and migratory responses.[140] The fact is that, as long as the wound is not resurfaced, the threat of infection and amputation remains.

Diabetes and infection susceptibility The relationship between DM type 2 (DMT2) and immunity is an expanding research field in which new puzzle pieces are continuously discovered, often increasing in complexity and stimulating controversy within the field. This research incentive is fueled by the understanding that diabetes increases the risk of certain infections[141] and infection-related mortality.[142]

DMT2 is currently considered an immunometabolic disease, given the role of T-lymphocyte activation in inflammation and in the onset of insulin resistance.[143] The robust pathogenic loops linking insulin secretion, peripheral insulin resistance, immuno-inflammation and DMT2 are beyond the scope of this analysis, and have already been thoroughly reviewed.[144–146] The links are well defined: inflammation leads to peripheral insulin resistance and, in turn, insulin resistance leads to inflammation.[147] However, it is important to note that inflammation does not necessarily represent immunocompetence. On the contrary, some experimental data suggest that diabetes-associated hyperinflammation amplifies damage from bacterial infections and leads to increased susceptibility to Gram-negative bacteria.[148] Since the actual cause of death of the mice in one study was hyperinflammation, the authors suggest that this rather counterintuitive finding may respond to diabetes-associated RAGE overexpression, which preconditions a chronic inflammatory scenario that ultimately may be lethal when presented with the additional challenge of a bacterial infection.[138] Cumulative evidence documents a significant correlation between infection and rate of glycemic control.[21,149,150] It follows that heightened susceptibility to infections would be associated with insufficient glycemic control.[151,152] This observation is particularly relevant in the scope of our review to cellulitis, DFU, the devastating conditions of necrotizing fasciitis, and Fournier's gangrene.[22]

Hyperglycemia and insulin deficiency are considered the two major etiopathogenic pillars of diabetes-associated immuno-deficiency (Figure 4) and susceptibility to infections.[153–155] Hyperglycemia as a primary trigger of pro-inflammatory cytokine spillover[145] results in local and systemic inflammation, and peripheral insulin resistance.[156,157] Increased levels of various inflammatory markers and mediators—including white blood cell count, C-reactive protein, pro-inflammatory cytokines and plasminogen activator inhibitor-1—are elevated in insulin resistant subjects and DMT2-affected patients.[158,159] Although it may appear contradictory, evidence suggests that peripheral blood mononuclear cells (PBMC) from DMT1 and DMT2 patients secrete lower constitutive-[160] and lipopolysaccharide (LPS)-stimulated[161] levels of TNF-α, IL-1 (α and β) and IL-6, as compared with matched controls. The same cytokine secretion impairment was confirmed for in vitro models where PBMCs from healthy donors

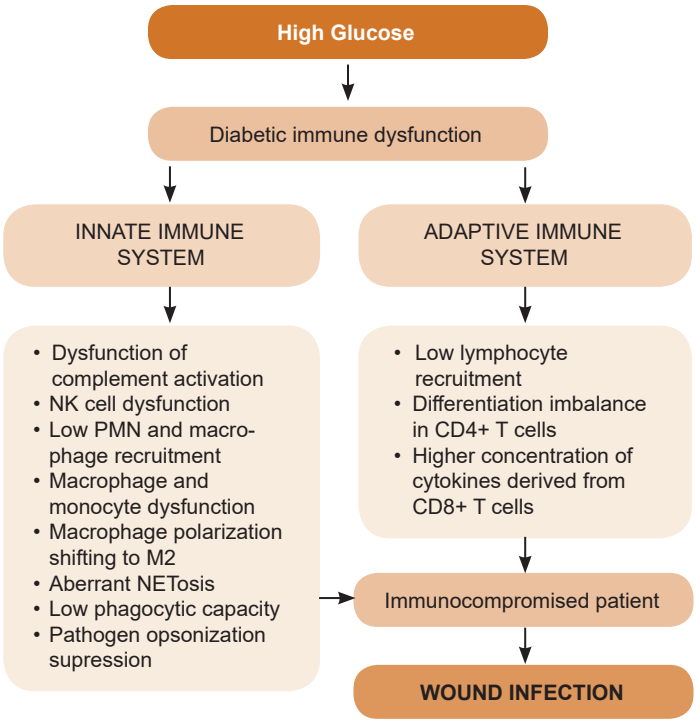
were exposed to high glucose levels[162] or dextrose octreotide.[163] This study demonstrates that glucose exposure dampened IL-2, IL-6 and IL-10 levels in a concentration-dependent manner while conversely inducing the expression of TGF-β1 which may explain immune failure.[162]

Increased glycation leads to a reduction of IL-10 secretion by myeloid cells.[32] A recent study demonstrated that PBMC steady-state expression of IL-1β appeared significantly increased while IL-6 expression reduced 3.45-fold in a cohort of DMT2 patients, compared with healthy control subjects.[164] This is a counterintuitive observation considering the canonic links between IL-6, glucose metabolism, DMT2 and their complications.[165–168]

Aside from these findings, a wealth of classic studies associated elevated endovascular levels of pro-inflammatory cytokines with insulin resistance and DMT2.[169,170] Subclinical blood elevation of some of these markers anticipates the onset of DMT2,[171,172] and the progression of multi-organ complications.[173,174] More recent studies[175] have identified and expanded epigenetic explanations as to why hyperglycemia induces long-lasting inflammatory upheaval, even days after glucose normalization. The fact is that hyperglycemia-associated chronic inflammation is epigenetically sculpted and is one of the biochemical insignias of metabolic memory.[176] Therefore, unraveling the roots of this plethora of conceptual controversies requires additional studies.[177–179]

Incorporation of AGEs to non-diabetic–derived cells has conclusively shown elevations in cytokine secretion,[100] which led to the hypothesis that AGE is somehow involved in diabetics' increased basal cytokine secretion.[100] Glycation also reduces expression of class-I major histocompatibility complex on the surface of myeloid cells, which accounts for impaired cellular immunity.[32] The presence of high glucose concentrations leads to elevated constitutive (steady state) cytokine production in resting cells, which becomes insufficient upon PBMC stimulation. Cell stimulation provoked by cytokine insufficiency is thought to be a critical factor in impaired immunity and vulnerability to invading pathogens among diabetics.[32,162,163,180] However, an alternative line of thought has suggested that stimulating the immune system will imperil—rather than protect—diabetics from acute Gram-negative bacterial infections, and that dampening hyperinflammation (with dexamethasone, for example) may restore the innate immune response to such infections.[148]

Figure 4. Diabetes impairs immune response and predisposes patients to wound infection



Hyperglycemia disrupts both the innate and the adaptive immune systems, making diabetic subjects susceptible to infections.
NETosis: neutrophil extracellular traps release
NK cells: natural killer cells

Other studies have addressed leukocyte recruitment and pathogen recognition abilities. Animal models demonstrate that diabetes impairs CD45+ leukocyte and CD8+ T cell recruitment, and reduces adhesion molecule expression and cytokine production upon microbial invasion of different organs.[181,182] More conflicting evidence exists, however, regarding pathogen recognition via toll-like receptors (TLR). One line indicates that TLR expression is reduced in diabetic mice.[182,183] In contrast, studies in human samples attest that TLR expression is low in diabetic subjects with complications and deficient glycemic control, whereas it is high in patients with well-controlled glycemia without complications.[184] Overall it has been concluded that TLR pathway impairments lead to diminished recognition of bacteria and may be one of the mechanisms implicated in diabetic susceptibility to wound infection.[183]

More coincidental revelations stem from the characterization of neutrophil dysfunction documented in diabetic individuals or in healthy donors exposed to high glucose burdens. The so-called 'oxidative burst' plays a critical role in neutrophils' antimicrobial defense and is reduced upon high glucose concentration due to poor ROS and superoxide anion productions.[185,186] Other studies have documented that sustained hyperglycemia leads to neutrophils' functional decline and that the mechanisms behind this decline include increased adhesive capacity, as well as diminished chemotaxis, phagocytic activity and bactericidal capacity.[102,187,188] This is a conflicting observation given the solid evidence supporting the concept that hyperglycemia is a major trigger of inflammation and hyper-oxidation in diabetes.[189–191]

Other studies show lower neutrophil degranulation,[192] decreased phagocytic capabilities,[193] immunoglobulin-mediated opsonization inhibition[194] and limited NET response ability.[195] These conflicts could be due to the type and origin of the experimental designs of, and samples used in, the studies; however, the overall interpretation of these data has led to the conclusion that hyperglycemia dampens PMN chemotaxis and phagocytic activities.[21] Diabetes impairs the physiology of macrophages and other innate immune cells. As stated above, diabetic patients are highly susceptible to bacterial infections, and often have impaired wound healing. However, despite years of research, the

molecular mechanism underlying macrophage dysfunction in diabetes is not fully understood .[196]

Under high glucose conditions, in vitro and in vivo models have shown reduced complement receptors, adhesion capacity, phagocytosis and antibacterial activity.[197] Diabetic hyperlipidemia and hyperglycemia introduce epigenetic modifications to macrophages that promote the onset of an inflammatory phenotype.[198] Thus, high glucose levels induce inflammatory polarization of human macrophages in vitro whereas AGEs significantly prime and promote M1 macrophage markers expression and IL-6 and TNF-α secretion.[199] Accordingly, M1 macrophages have strong microbicidal and antigen-presenting capacities, produce pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), and ROS; whereas M2 macrophages are considered pro-resolution response cells, producing anti-inflammatory mediators (IL-10 and TGF-β) and consequently resolving inflammation.[200] In light of these observations, it is controversial to posit M2 macrophage polarization in diabetic mice (db/db) as an explanation for their susceptibility to bacterial infection.[196] As a matter of fact, M2 cells are scarce within the wound environment where they are obviously necessary.[201] Irrespective of the controversial issue of in-wound recruited macrophage polarization, a recent study confirms that in diabetic mice, macrophage phagocytosis and bactericidal activities are reduced upon long-term exposure to high glucose burdens.[196] For these authors, long-term high-glucose treatment reduced macrophage glycolytic capacity and glycolytic reserve, in turn, impairing phagocytic capability.

NK (natural killer) cells derived from diabetic individuals also demonstrate defects in activating NKG2D and NKp46 receptors related to NK degranulation failure.[196] Hyperglycemia is also associated with a reduction in C4-fragment opsonization, which inhibits classical or lectin pathways of complement activation,[202] in impaired neutrophils' bacterial killing capacity.[100,203] By using peripheral blood lymphocytes from diabetic animal models and human samples, studies have concluded that uncontrolled diabetes increases chromatin condensation, DNA fragmentation and lymphocyte death.[204]

Unlike the effect of hyperglycemia on immune cell activity in DMT2, the impact of insulin deficiency in DMT2 immunoresponsive cells against pathogens has not been widely studied.[170] Given that these cell functions are energy-dependent processes, proper insulin-regulated glucose metabolism is necessary. Insulin-driven metabolic processes are not merely associated with immune-cell ATP generation and utilization. Glucose and lipid metabolism influences cellular phenotype, potential cellular reprograming during patterns of recognition, and ultimately activation status.[205] In activated T-lymphocytes, insulin stimulates glucose uptake, oxidation, pyruvate flux and pyruvate dehydrogenase activity, amino acid transport, lipid metabolism and protein synthesis.[206] Recent findings implicate insulin in shaping the immune response by modulating cell differentiation and polarization.[207,208] Thus, in addition to its role in substrate metabolism, insulin is also an anti-inflammatory and immunomodulatory hormone[209,210] via immune cells' metabolic regulation.[211]

Recent studies substantiate the importance of insulin in normal innate immune response. An insulin deficit is associated with alveolar macrophages' phagocytic impairment as well as poor cytokine secretion in alloxan-treated rats, reverted after insulin intervention.[212] Insulin treatment of diabetic mice bone marrow-derived macrophages restored production of critical pro-inflammatory cytokines upon LPS exposure.[213]

Conclusively, the apparently 'trivial' blood glucose derangements in diabetes reduce bactericidal and wound healing capacities in innate immune operators, a phenomenon that, according to latest evidence, is related to transcriptional aberrations in gene coding for macrophage differentiation and lymphocyte migration and proliferation at the hematopoietic stem/progenitor cell level.[214] Thus, therapeutic manipulation of immune-metabolomic loops is a promising therapeutic road.

Infection of diabetic foot ulcers: biofilm and its interaction with the wound matrix Typically, once an ulcer develops, it is colonized with microorganisms that may lead to a state of clinical infection.[113] About 15%–25% of DM patients develop foot ulcers during their lifetime and half of these become infected, a recurrent complication in diabetics.[215,216] Infection can spread to soft tissues and bone making it the main causal factor of lower extremity amputation in most countries.[217] Thus, the management of DFI represents a high cost for the health system, a decrease in the quality of life of diabetic population, and a great research incentive.[217]

DFI is defined as the presence of an inflammatory response and tissue damage that can drive the clinical spectrum from superficial cellulitis (mild infection) to chronic osteomyelitis (severe infection), with host–microorganism interaction being crucial in determining progression.[218,219] This interaction is defined by Casadevall and Pirofsk as the "damage response framework model",[220] and proposes that infection outcomes are dependent on mutual contributions of both the microbe and the host.[220] In comparative terms, infection occurs when invading organisms overwhelm the host's defenses.[221] In contrast, colonization is defined as the presence of proliferating bacteria without an overt host immunological reaction.[26] The reported critical limit is 10^5 colony-forming units per gram of tissue,[222] indicating the presence of a 'critical' degree of colonization marking the point at which host defenses are no longer able to contain the infection.[223] Nevertheless, preexisting diabetic neuropathy, peripheral vascular disease, impaired leukocyte function[224] and a deteriorated innate immune system make DFI clinical diagnosis difficult while simultaneously worsening its prognosis.[225,226] Under these circumstances, onset of classic signs of infection may not occur,[227] even when there is a high bacterial load.[226] Thus, the invading pathogen may progress and infect with no clinical translation.

Staphylococcus aureus is a major pathogen of human skin. This is also the most common pathogen identified in patients with acute superficial DFU.[23,228,229] This pathogen, in its interaction with the host's diabetic wound environment, is able to amplify certain glucose-regulated genes that ultimately increase its virulence, indicating that hyperglycemic conditions facilitate pathogen adaptation and survival, ultimately worsening patient prognosis.[230]

Chronic ulcers usually exhibit polymicrobial infections including a mixture of aerobic/anaerobic and Gram positive/negative bacteria.[227,231] Some of the heterogeneous groups of bacterial species identified in DFU patients have been compiled

in Table 1.[26,232–234] Bacterial predominance differs between studies. Nevertheless, recent literature point to Gram-negative bacteria as the predominant group.[23,229,235,236] The reported prevalence included *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli*.[237,238] Usually, these results are influenced by several factors including infection severity, demographic characteristics, glycemic control, and ongoing or previous antibiotic treatments, as well as bacterial identification method.[26]

Table 1: Bacterial species identified in diabetic foot ulcers

Aerobic and anaerobic facultative bacteria	Anaerobic bacteria
Staphylococcus epidermidis	*Clostridium species*
Staphylococcus saprophyticus	*Peptostreptococcus species*
Pseudomonas aeruginosa	*Dialister pneumosintes*
Klebsiella pneumoniae	*Bacteriodes fragilis*
Escherichia coli	*Anaerococcus prevotii*
Streptococcus mutans	*Anaerococcus tetradius*
Streptococcus pyogenes	*Eggerthella lenta*
Bacillus subtilis	*Fusobacterium mortiferum*
Proteus species	*Veillonella dispar*

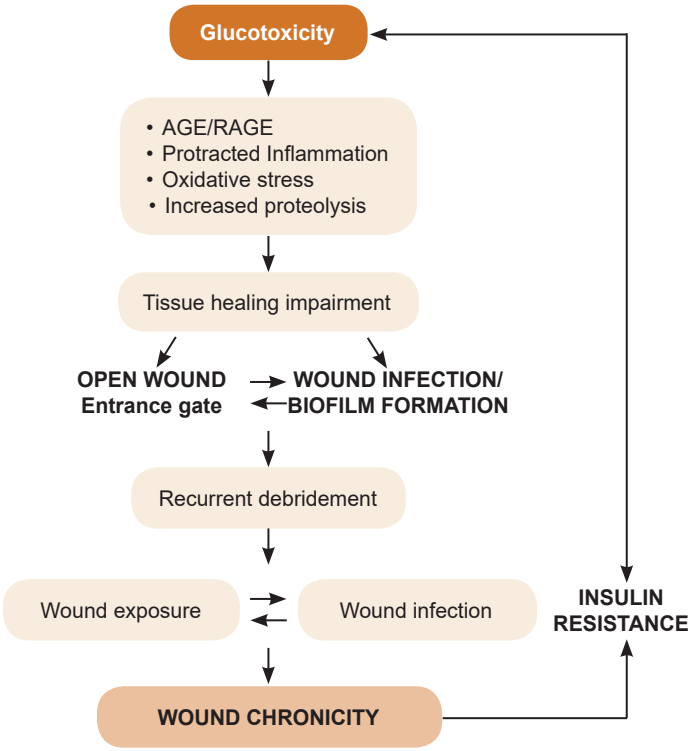
*Adapted from [26] and [234]

A traditional debate in this field is the relationship between pathogenic potential and microbial bioburden, and how it impacts the host. Here, it is important to highlight the diversity of the microorganism population and its potential interactions, as these may turn into cooperative pathogenic loops that enhance antimicrobial resistance and imprint a particular signature on each individual ulcer.[239] The symbiotic microbial interaction within the ulcer's ecosystem confers a virulence profile that is far more important than the microorganism concentration in and of itself.[222,240] Long-standing ulcers are more predisposed to infection which, in addition to impairing the healing response, may reduce peripheral insulin sensitivity.[21,241,242] Thus, the longer the wound is maintained, the greater the risk for infection. Another contribution of infected ulcers is their pathogenic effect at the organism level. These ulcers act as pro-inflammatory organs superimposed onto a host, pouring pro-inflammatory reactants, oxygen free radicals and bacterial toxins into central circulation, amplifying tissue injury and general homeostasis (Figure 5).[243]

Finally, it is noteworthy that DFI is frequently associated with existing biofilm in the context of ulcers. Biofilm is a niche for symbiotic microorganism interactions that essentially act as a protector shield for bacterial populations.[244–246] How biofilm-making microorganisms interact with immunocompromised diabetics and their subsequent pathophysiological consequences are relevant research topics.

The term biofilm was coined by the scientific community at the end of the 20th century, which indicates that this is an emerging and expanding research area.[247–250] DFU pathogens can exist as planktonic form (free-living) or as a biofilm (sessile-living).[246] Both phenotypic states may play important roles in impairing healing and causing infection of both acute and chronic wounds.[251] Biofilm acts as a collective entity endowed with superior antimicrobial resistance when compared with its individual constituents. Several in vitro experiments indicate that antibiotic resistance in biofilm bacteria is up to 1000 times higher than in planktonic bacteria.[252]

Figure 5. Wound–infection feedback loop



AGE: Advanced glycation end products RAGE: AGE receptor

The ability of a microorganism to build up biofilms is an important virulence factor and an advantageous organizational step. As stated above, biofilm offers a protective environment or physical barrier to biological and antimicrobial substances, facilitating microorganism attachment to surfaces or to each other, ultimately enabling survival and antibiotic resistance.[236,245] 'Inoffensive' non-pathogenic bacteria, incapable of promoting chronic wound infection, may symbiotically interlink with pathogenic biofilm and act synergistically to cause a chronic infection.[24] This structured community of microorganisms can be classified as mono- or polymicrobial, encased in extracellular polymeric substances (EPS) or exo-polymeric substances.[253]

The community is a mixture not only of bacterial cells, but also of fungi, viruses, proteins, extracellular DNA and other biogenic factors that increase virulence and reduce treatment success.[246] This and other virulence factors are possible through cell-to-cell communication via quorum sensing (QS).[246,254] QS is a form of cellular communication mediated by small molecules that depend on cell density. The species of bacteria that reaches critical-mass concentration produces large amounts of small signaling molecules that modify gene expression. Indeed, bacterial exchange coordination activities are based on this mechanism, according to population size.[246] QS, together with the exchange of genetic material by bacteria in the biofilm, give rise to different microorganism phenotypes that ultimately affect ulcers as can be seen in anti-microbial treatment results.[23] The fact that bacteria are not motile in the biofilm context and have lower metabolic and proliferative activities than their planktonic counterparts,[255] makes appropriate antibiotic selection difficult. Many antibiotics used for DFI treatment are only effective against actively dividing cells.[256]

Over the last few years, the concept of biofilm in dynamic reciprocity between wound-bed cells and the host has attracted increasing interest. Correspondingly, it has been proposed that biofilms are responsible for over 90% of all chronic wounds. An electron microscopy study assessing wound tissue biopsies suggested that about 60% of chronic wounds have a biofilm compared to 6% of acute wounds.[249] It is likely that at least half of all chronic wounds develop a biofilm.[248,249] This result indicates the contribution of biofilm to impaired wound healing even when molecular mechanisms underlying biofilm-induced chronification remain poorly understood.[257,258]

The various mechanisms by which biofilm obstructs the healing response include failures in granulative tissue formation and the re-epithelialization trajectory.[259] Accordingly, it is likely that these events are consequences of anti-proliferative signals derived from pathogens and a persistent inflammatory environment[257] that aborts fibroblasts and keratinocyte mitogenic, motogenic and secretory functions.[260] In line with this notion, Trøstrup demonstrated that *P. aeruginosa* induces a state of cellular quiescence reminiscent of premature senescence.[62] *P. aeruginosa* also secretes a plethora of proteases resulting in collagen, fibrinogen and elastin degradation, inhibition of PMNs and complement systems, and basement membrane degradation.[261,262] Similarly, proteases secreted by *S. aureus* also degrade collagen and elastin. The ability to degrade surface-associated adhesins enables bacterial phenotype transition from adhesive to invasive.[263] Inhibition of neutrophil phagocytosis and chemotactic activity is also associated with bacterial wound infection.[264] In this steady inflammatory milieu, PMN-derived elastase and other degradative enzymes increase wound tissue damage, expand wound size and perpetuate chronicity.[62] In-depth studies examining *P. aeruginosa* and host interaction showed that TLR activation is inhibited by pathogen-derived elastase, which allows evasion of host immunosurveillance.[265] *P. aeruginosa*-derived rhamnolipids inhibit human beta-defensin secretion by challenged keratinocytes, which contributes to pathogen survival and colonization in compromised epithelia.[266] Furthermore, it has been proposed that biofilm and lipopolysaccharide EPS of Gram-negative bacteria inhibit complement activation, further contributing to evasion of the host's innate defense system.[267,268] Microorganism-derived PAMP, together with platelet-derived factors stimulate the influx of PMNs and other immune cells, spreading the wound's pro-inflammatory reactants, increasing the level of ECM-degradative proteases, and consequently curtailing the proliferative phase.[112,269,270] Additionally, infiltrated inflammatory cells in response to bacterial invasion via proinflammatory cytokines and AGE/RAGE axis activation produce large amounts of ROS that act as local causal factors for premature cell senescence.[271,272] In other words, the pathogen manages to prevent otherwise normal PAMP-induced innate immunity activation; and graphically speaking, the DFU turns into a battlefield in which the pre-debilitated diabetic host's immune system is overwhelmed by biofilm-entrenched microorganisms, thus perpetuating wound arrest.[62,273]

Biofilm identification is complex and dependent on more than simple wound cultures obtained and evaluated using traditional microbiological techniques. More sophisticated and expensive techniques such as light and scanning electron microscopy are required to evaluate biofilm in a wound. Therefore, biofilm presence is often overlooked.[274,275] New molecular techniques including DNA micro-arrays, multiplex real-time polymerase chain reaction and functional metagenomics offer a unique opportunity to characterize biofilm microbiome.[218] These technologies facilitate analysis of a microorganism's resistance potential and virulence factors.[26]

Conclusively, underlying diabetic complications predispose increased risk of developing DFI and other peripheral tissue infections as compared to the risk in healthy populations. Biofilm-forming microorganisms counteract the host's defenses, prolong DFU inflammation, deteriorate host anabolism and ultimately increase the risk of amputation (Figure 5).[24,258,276]

CONCLUDING REMARKS

Wound healing is a complex biological process consisting of precisely-predetermined overlapping phases integrated in a sequential cascade aimed at morpho-functional restoration in a physiological time window. Successful reparative response requires concerted and cooperative integration of both systemic and local signaling networks and driving forces. DM is an archetypal disease in which a variety of both local and systemic factors combine to disturb most healing phases. Thus, DFUs serve as a model for chronic wounds, an often-devastating diabetic complication, and the first cause of lower-limb amputations worldwide.

Underlying the ulcer's onset and expansion is a complex interplay of pathogenic vicious circles that turn DFUs into a pro-inflammatory, pro-oxidant, pro-apoptogenic and pro-senescence-inducing organ, superimposed onto a host with an already-debilitated immune response. The failure of diabetic patients' peripheral immunosurveillance, as in the subsequent elicitation of effector mechanisms, is the foremost contributing factor to DFU infections.

Diabetic dysimmunity is likely a major determining factor on patient outcomes. It seems there is still a long way ahead before we achieve a uniform, comprehensive understanding of the actual immune profile of diabetic individuals. Further studies are needed to disentangle critical contradictions and contemporary conceptual paradoxes. Some of the current critical controversies are highlighted in this paper. Of note, however, is the divergent experimental data on whether hyperglycemia reduces pro-inflammatory cytokines and whether this cytokine dampening accounts for immune failure, as compared with other major ongoing questions in DMT2.

We still do not know how to manipulate hyperinflammation or how to reinstate leukocyte physiology once it is disrupted. At the moment, infection remains a dismal complication of these wounds, protracting pre-existing inflammation, dismantling local immune response, amplifying fibroblast and keratinocyte arrest and further disrupting the host's internal homeostasis. Given that DFUs are recurrently seeded by biofilm, eliminating infection is a challenging task. This review confirms that, despite the efforts to understand DFU pathophysiology, infection pathology and its interaction with the host, DFU prevalence is rising while any reduction in amputation rates remains modest. Therapeutically promising targets include: 1) Identification and pharmacological manipulation of epigenetic drivers in wound-prolonged inflammation and chronification, even in the ideal scenario of a non-infected ulcer; 2) Ablation of the wound cell-senescent drivers, which could contribute to restoring an acute wound-like closure trajectory; and 3) Characterization of actors and pathways underlying hyperglycemia and insulinopenia-

induced diabetic immune failure, and subsequent pharmacological interventions to rebuild the immune system. These academic imperatives must go hand-in-hand with diabetic self-care educational programs, as well as systematic foot and neurological examination by qualified specialists.

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Carbapenamase-Producing *Acinetobacter baumannii* in China, Latin America and the Caribbean: A Systematic Review and Meta-Analysis

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ABSTRACT

INTRODUCTION Carbapenem-resistant *Acinetobacter baumannii* is a complex health problem, causing difficulties in clinical–therapeutic management worldwide. It is of particular concern in Latin America, the Caribbean and China, where it is an emerging health problem. Carbapenemases produced by these organisms inactivate carbapenem antibiotics. Monitoring circulating genotypes' geographic dispersion contributes to more effective control measures. However, exhaustive studies on carbapenem-resistant *A. baumannii* are scarce.

OBJECTIVES Study the production of carbapenemases in clinical isolates of *A. baumannii* resistant to carbapenem antibiotics and the geographic distribution of the sequences circulating in China, Latin America and the Caribbean.

DATA ACQUISITION We followed PRISMA indications. We carried out a systematic search in Pubmed, BVS and CKNI on papers on *A. baumannii* and carbapenemases published during 2015–2020 in English, Spanish and Chinese, and selected 29 cross-sectional studies that met the search criteria. Studies were evaluated using JBI Critical Appraisal tools, and quantitative data were collated for meta-analysis using the Metaprop library in Stata15.

DEVELOPMENT OXA-type carbapenemases were detected in all studies; among *A. baumannii* resistant to carbapenem antibiotics, predominant types were OXA-23, OXA-24, OXA-54 and OXA-72; metallobetalactamases were identified less frequently than OXA carbapenemases. Only one clinical isolate producer of Class A carbapenemases (KPC) was identified in Colombia. In total, 41 sequence types were identified; in Latin America and the Caribbean the most common types were: ST79, ST25, ST1 and ST15; in China, the sequences ST195, ST208, ST191, ST368 and ST369 were the most prevalent. ST2 was found in both regions.

CONCLUSIONS The most prevalent carbapenemases and sequence types vary by region, indicating different ancestral strains. Microbiological surveillance, antibiotic use optimization, adequate infection treatment and timely control strategies are essential for carbapenem-resistant *A. baumannii* prevention and control in geographies such as Latin America, the Caribbean and China where such resistance is an emerging health problem.

KEYWORDS Acinetobacter baumannii, carbapenemase, genotype, epidemiology, Latin America, Caribbean region, China

INTRODUCTION

The genus *Acinetobacter (Acinobacter spp.)* is made up of several species. These gram-negative bacilli are among the most common nosocomial pathogens worldwide. *Acinetobacter baumannii* is the most clinically relevant species, due to its ability to develop various mechanisms that lend themselves to antibiotic resistance.[1] A member of the beta-lactam class (the same class of antibiotics as penicillins and cephalosporins), carbapenemic antibiotics are the last resort in treating *A. baumannii* infections.

There has been a worldwide increase in carbapenem resistance observed in clinical isolates of *A. baumannii*.[2] The Latin American Antimicrobial Resistance Surveillance Network found *A. baumannii* to have high resistance to carbapenems in 15 countries in the region during 2014–2016. The percentage of resistant isolates varied from 8% to 89%.[3] In China in 2016, 71.4% of *A. baumannii* isolates were resistant to carbapenems.[4] WHO published a list of priority pathogens in 2017, including *A. baumannii, Pseudomonas aeruginosa,* and carbapenem-resistant *Enterobacteriacae spp*. as critical priorities.[5]

IMPORTANCE

This meta-analysis shows the different types of carbapenemases in *A. baumannii* in China, Latin America and the Caribbean, and the geographic distribution of the circulating sequence types. The data provide useful information for antibiotic resistance surveillance in the regions chosen for analysis, where this is an emerging health problem.

The main mechanism of carbapenem resistance in *A. baumannii* (CRAB) strains is carbapenemases production. The most common of these are Ambler's class D oxacillinases (OXAs).[2] Six subgroups of OXAs have been identified in *A. baumannii*: the species' intrinsic carbapenemase OXA-51–like, and the acquired carbapenemases OXA-23–like, OXA-24–like, OXA-58–like, OXA-143–like, and OXA-235–like.[6] Class B metallobetalactamases (MBLs) are also a major threat because they are often located in mobile genetic elements, easily transferable between bacteria. Four types of MBLs are frequently detected in *A. baumannii*: imipenemase (IMP), Verona imipenemase (VIM), Seoul imipenemase (SIM), and New Delhi betalactamase (NDM).[7]

Molecular characterization of *A. baumannii* isolates is very useful in identifying the source of an outbreak and in helping to control its spread. Multilocus sequence typing (MLST) is highly discriminative and has been applied successfully to several bacterial patho-

gens, including *A. baumannii.* Additionally, this typing allows for comparisons between laboratories and provides a powerful tool for conducting epidemiological studies worldwide.[8]

The emergence of carbapenem-resistant forms of *A. baumannii* is a complex global problem, difficult to manage both clinically and therapeutically. Monitoring carbapenemase genotypes and molecular epidemiology studies contribute to more effective control measures. However, comparative analyses of circulating forms in different geographies are scarce. This work is a systematic review of published information on carbapenemase production and sequence types characterized in *A. baumannii* isolates in China, Latin America and the Caribbean (LAC).

DATA ACQUISITION

We followed the SPIDER scheme in preparing this study.[9] We carried out a systematic review of all relevant publications in 2015–2020, adjusted according to recommendations contained in PRISMA guidelines (Preferred Reporting Items for Systematic Reviews and Meta-Analyses.)[10]

Search strategy and article selection The search was carried out in the following databases: PubMed (Medline), BVS (Regional Portal, Virtual Health Library), and CNKI (Chinese database). We used the following keyword combinations: "carbapenem resistance" OR "carbapenemase producing" combined with "Acinetobacter baumannii" combined with the names of the following countries: "Argentina", "Bahamas", "Belize", "Bolivia", "Brazil", "Chile", "China", "Columbia", "Costa Rica", "Cuba", "Ecuador", "Guatemala", "Guyana", "Haiti", "Honduras", "Jamaica", "Mexico", "Nicaragua", "Panama", "Paraguay", "Peru", "Salvador", "Surinam", "Trinidad", "Uruguay", and "Venezuela". We selected articles in three stages: by title, by abstract content, and finally, according to information contained in the full text.

Inclusion criteria We included studies with the following characteristics: 1) observational studies with a cross-sectional design; 2) analysis of *A. baumannii* clinical isolates in adult populations; 3) reports on CRAB; 4) detection of class A, B and D carbapenemases by molecular methods such as reverse-transcriptase polymerase chain reaction testing (RT-PCR) or MLST; 5) carbapenemase genotype analysis in LAC or China published in 2015–2020 in English, Spanish or Chinese.

Exclusion criteria Studies with one or more of the following characteristics were excluded: 1) contained no information on CRAB; 2) studied isolation in pediatric populations; 3) did not report on class A, B and D carbapenemase classes; 4) duplicated other research; and 5) studies that reported experiments in non-human subjects or were review articles, conference abstracts, meta-analyses or systemic reviews.

Review articles and meta-analyses were only considered in this review's discussion section. Screening for inclusion was carried out individually by two team members. When there were differences of opinion, these were discussed with the principal investigator. Endnote X9 and Excel were used to manage references.

Study quality evaluation Study quality was evaluated by two researchers using the JBI Critical Appraisal Tools for Prevalence Studies.[11] This tool includes nine items, each of which was scored as either 'Yes' (when the requirement was met), as 'No' (when the requirement was not met) or as 'Not Clear' (if it was unknown whether the requirement was met) or as 'Not Applicable'.[11] Studies were considered high quality when their score was ≥80% of the maximum possible score (8 or more items scored "Yes"), average quality when their score was 70%–79% (6–7 items scored 'Yes'), and low quality when their score was <70% (6 or fewer items scored 'Yes').

Data extraction Two team members carried out data selection and extraction individually, as well as bias risk analysis. We organized data on carbapenemase genotypes in China, Latin America and the Caribbean into a matrix (Table 1).

Table 1: Data extraction characteristics

Term	Definition
Registration	Title, author, publication year
Study	Study type and investigation period
Region	Latin American and Caribbean countries and Chinese provinces
Sample	Sample size (CRAB number)
Intervention	Detection method: RT-PCR or MLST
Genotype	Number of carbapenem genotypes
Sequence type	Detected clones or genetic lines

CRAB: Carbapenem-resistant *A. baumannii*; MLST: Multilocus sequence typing; RT-PCR: Reverse-transcriptase polymerase chain reaction

Data analysis and statistics We carried out a quantitative study (meta-analysis) on *A. baumannii* carbapenemase genotypes and a qualitative study (qualitative descriptive synthesis) for sequence types, due to the great diversity of sequence types and differences between geographical areas. Two investigators undertook data analysis for the quantitative study. Statistical analysis was performed using the Metaprop module of Stata version 15 (StataCorp LLC U.S.A),[12] and obtained estimates of the combined prevalence of predominant *A. baumannii* carbapenemases in different regions, as well as their 95% confidence intervals (95% CI), which are represented in corresponding forest graphs (Figure 2).

We used either fixed-effect or random-effect models according to statistical heterogeneity between studies, which was assessed using the Cochran I2 statistic (a value of 0% indicates no heterogeneity; 25%, 50% and 75% are considered to have low, medium and high heterogeneity, respectively). Egger's weighted linear regression test, combined with a funnel plot, was used to assess publication bias. An assessment of 'no publication bias' was made when the regression line started from the origin of the ordinate axis (Y) (publication bias increases as the line moves away from the Y coordinate's origin). Statistical significance was assessed at 0.1 and not 0.05.[13]

DEVELOPMENT

Literature search, selection and validation We initially selected 334 articles, which were reduced to 318 after eliminating duplicates. Of these, 261 were excluded for failure to meet inclusion criteria or because they were outside the scope of this review. Finally, we fully reviewed a total of 57 articles, and 29 were selected that met all established criteria, which were then included in the meta-analysis (Figure 1).

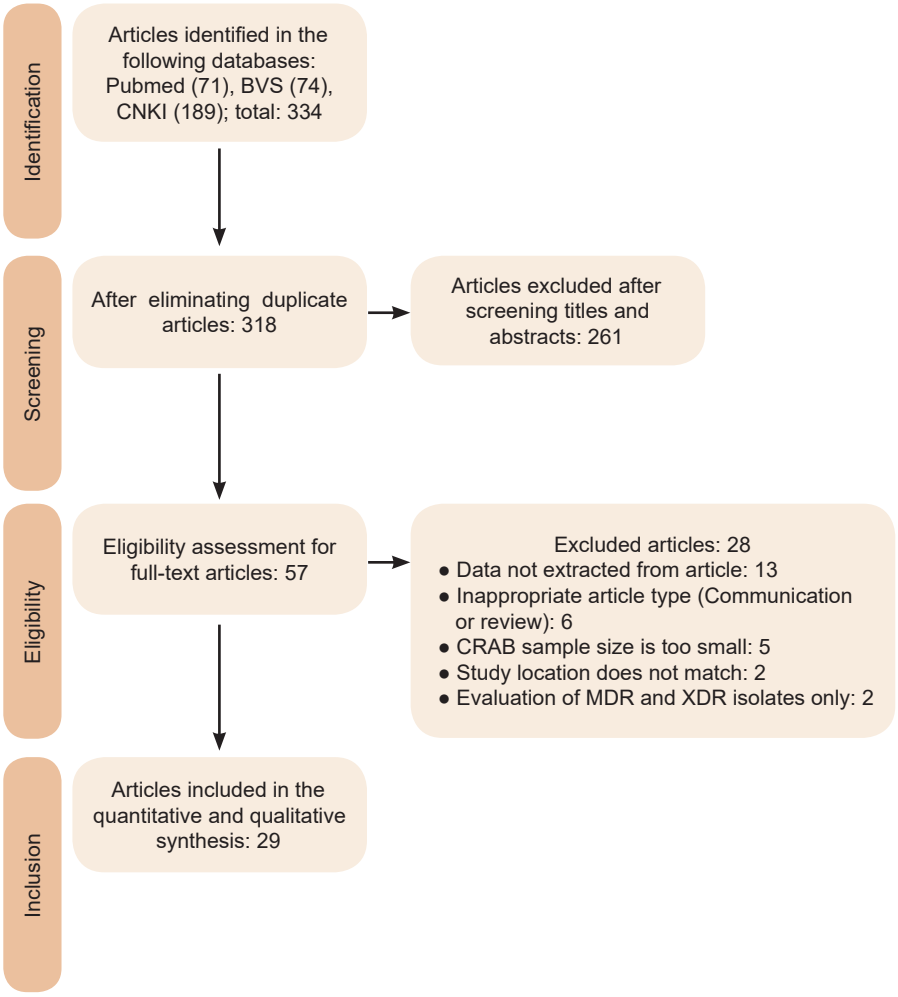
Medium-high scores were obtained upon evaluation of the cross-sectional observational studies (Table 2). In this study, 95% is

used as expected prevalence in calculating sample size, as carbapenemase production is the CRAB's dominant cause. Considering three studies on molecular isolate typing in hospitals,[14–16] the confidence level equal to 95% and precision equal to 5%, the estimated minimum CRAB sample size was 73. Consequently, we excluded 14 articles due to small sample size. Another 14 studies on carbapenemase genoptyping were excluded for only reporting detection of Class D carbapenemases.

Carbapenemase characteristics Of the 29 studies included in this systematic review, 12 were from LAC, involving 11 countries and 17 were from 11 Chinese provinces. OXA-type carbapenemases were detected in all studies. In LAC, OXA-23, OXA-24, OXA-58 and OXA-72 genotypes were predominant, with respective prevalences of 0.73 (0.54–0.89), 0.06 (0.00–0.17), 0.03 (0.01–0.06) and 0.02 (0.00–0.06). In China, OXA-23, OXA-24 and OXA-72 were the most common, with respective prevalences of 0.91 (0.84–0.96), 0.03 (0.01– 0.08), 0.02 (0.00–0.05).

Metallobetalactamases were less frequent than OXAs and were detected in only two countries in the Latin American and Caribbean region, with NDM 0.01 (0.00–0.01) as the predominant genotype, and in six Chinese provinces, with four predominant genotypes: NDM 0.02 (0.00–0.04), VIM 0.07 (0.00–0.21), IPM 0.02 (0.00–0.06) and SIM 0.01 (0.00–0.03) (Table 3). Only one clinical isolate Class A carbapenemase producer (KPC) was found, and it was isolated in Colombia.

Figure 1: Literature selection results (PRISMA)



CRAB: Carbapenemase resistant *Acinetobacter baumannii*; MDR: Multi-drug resistant; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; XDR: Extensively drug-resistant

According to the I2 values, high heterogeneity was observed among studies and we consequently used a random-effects model for the meta-analysis. Egger tests (p >0.1) and funnel plots show the characteristic shape of the asymmetric dispersion (Table 3 and Figure 2).

Molecular typing characteristics In 17 of the 29 studies, MLST was performed on CRAB isolates. In total, 41 sequence types (STs) were identified: 16 in LAC and 26 in China. Clear geographical differences were observed in predominant ST frequencies: ST79, ST25, ST1 and ST15 were more common in LAC; while ST195, ST208, ST191, ST368 and ST369 were found more frequently in China. ST2 was found in both (Table 2 and Figure 3).

DISCUSSION

Carbapenemase types Antibiotic overuse has led to an increase in multi-drug–resistant *Acinetobacter.* More than 50% of *Acinetobacter spp.* isolates in the United States, South America, India and China are resistant to carbapenem antiobiotics.[46] This study verifies the high prevalence of OXAs in CRAB isolates in China, Latin America and the Caribbean. OXA-51–like carbapenemases (including OXA-51, 64, 65, 66, 68, 69, 70, 71, 78, 79, 80 and 82) occur naturally in *A. baumannii*.[47] OXA-24–like carbapenemases (including OXA-24, 25, 26, 40 and 72) have been found in both plasmid and chromosomal structures; OXA-58–like and OXA-23–like carbapenemases are encoded by plasmids,[48] which increases the probability of horizontal transmission. The plasmid-encoded carbapenemases OXA-23, OXA-24 and OXA-58 were the most frequently isolated carbapenemases in the two regions analyzed in this study. These results justify the non-inclusion of OXA-51 type carbapenemases, as their resistance to CRAB is intrinsic, and thus has little impact on amplifying carbapenem resistance in this species.

Carbapenemase OXA-72 was first identified in 2004 in an *A. baumannii* isolate from Thailand,[49] and subsequently detected in Brazil, Mexico, Ecuador, Peru, China and Europe. [27,43,45,50,51] PXA-231 and OXA-253 were identified in Brazil and Peru, respectively. Both belong to the OXA-143–like group. OXA-231 and OXA-253 were reported the first time in *A. baumannii* isolates from Brazil (in 2007 and 2014, respectively) and still appear mainly in that country.[52,53] The dissemination of this subgroup requires monitoring, as it has been described more recently in Iran (2017), Colombia (2017) and Peru (2018). [45,54,55]

Table 2: Studies included in the systematic review

Author, year, reference	Year isolated	Detection method	Place	Number of CRAB isolates	Carbapenem type (number detected)			Sequence type (n/N)	The study met all selection and quality criteria[&]	The study did not meet these quality criteria:		Study quality
					Class A	Class B	Class D			Adequate sample size	Data analysis with sufficient coverage of identified sample	
Chen F. (2018)[17]	2011	PCR	Hu Nan	34	NA	IMP-5(22)	OXA-23(33) OXA-24(29)	NA	No	x	x	Medium
Chen Y. (2018)[18]	2013–2017	PCR	Guang Dong	66	ND	NDM-1(3)	OXA-23(60) OXA-24(19) OXA-58(3)	NA	No	x	–	High
Chen Y. (2017)[19]	2011–2013	PCR/MLST	Shang Hai	56	NA	ND	OXA-23(56)	ST208(28/56) ST191(12/56) ST540(7/56)	No	x	x	Media
Han L. (2017)[20]	2013	PCR	Shan Xi	45	ND	ND	OXA-23(44)	NA	No	x	–	High
Huang ZY. (2019)[21]	2016	PCR/MLST	Hu Nan	67	NA	VIM(54)	OXA-23(63) OXA-58(1)	ST195(28/67) ST368(9/67) ST829(8/67) ST210(2/67) ST90(2/67) ST136(2/67)	No	x	x	Medium
Jiang L. (2018)[22]	2017	PCR/MLST	Guang Dong	122	NA	VIM(7) SIM(2)	OXA-23(115)	ST195(10/28) ST208(9/28) ST1633(3/28) ST345(1/28) ST381(1/28) ST457(1/28)	No	–	x	High
Zhao L. (2018)[23]	2015–2016	PCR	An Hui	145	ND	IMP-4(4)	OXA-23(134) OXA-24(1)	NA	Yes	–	–	High
Song X. (2017)[24]	2013–2014	PCR	Shan Dong	32	ND	VIM(3)	OXA-23(28)	NA	No	x	–	High
Chen J. (2017)[25]	2015–2016	PCR	Jiang Xi	64	ND	VIM(56) NDM-1(17) SIM(12)	OXA-23(56) OXA-24(2)	NA	No	x	–	
Huang G. (2016)[26]	2012–2014	PCR/MLST	Chong Qing	248	NA	NA	OXA-23(163)	ST368(102/248) ST195 (31/248) ST191 (29/248) ST369 (29/248) ST208 (21/248) ST381 (7/248) ST136 (2/248) ST229 (1/248) ST457 (1/248)	Yes	–	–	High
Chen Y. (2018)[27]	2014–2016	PCR/MLST	Liao Ning	78	NA	ND	OXA-23(33) OXA-72(45)	ST2(9/78)	No	–	x	High

Author, year, reference	Year isolated	Detection method	Place	Number of CRAB isolates	Carbapenem type (number detected)			Sequence type (n/N)	The study met all selection and quality criteria[&]	The study did not meet these quality criteria:		Study quality
					Class A	Class B	Class D			Adequate sample size	Data analysis with sufficient coverage of identified sample	
Ning N. (2017)[29]	2009–2014	PCR/MLST	Bei Jing	101	NA	NA	OXA-23(95) OXA-40(1)	ST191(32/101) ST195(31/101) ST208(15/101) ST368(6/101) ST469(6/101) ST218(2/101) ST373(2/101) ST383(2/101) ST429(2/101) ST369(1/101)	No	–	x	High
Lu Q. (2019)[30]	2013–2015	PCR	Guang Xi	61	NA	NDM-1(1)	OXA-23(44)	NA	No	x	–	High
Zhang Y. (2019)[31]	2017	PCR/MLST	An Hui	28	ND	ND	OXA-23(25)	ST1779(8/28) ST1789(6/28) ST195(5/28) ST191(2/28) ST368(2/28) ST369(2/28)	No	x	–	High
Wu H. (2017)[32]	2015–2016	PCR/MLST	Shan Dong	55	NA	ND	OXA-23(55)	ST208(21/55) ST369(14/55) ST195(11/55) ST451(6/55) ST381(1/55)	No	x	x	Medium
Li P. (2015)[33]	Not mentioned	PCR	Beijing	145	NA	NA	OXA-23(134) OXA-58(1)	NA	No	–	x	High
Bado I. (2018)[34]	2010–2011	PCR/MLST	Uruguay	73	ND	ND	OXA-23(58) OXA-58(2)	ST79(20/73) ST958(1/73)	Yes	–	–	High
Camargo CH. (2016)[35]	2009–2013	PCR/MLST	Brazil	71	ND	ND	OXA-23(68) OXA-72(2)	ST79(16/71) ST1(16/71) ST15(20/71)	No	x	–	High
Castilho SRA. (2017)[36]	2010	PCR	Brazil	51	NA	NA	OXA-23(31) OXA-58(2)	NA	No	x	x	Medium
Castillo Y. (2019) [37]	2008–2013	PCR	Perú	46	ND	ND	OXA-23(44) OXA-24(1)	NA	No	x	–	High
Gonzalez-Villoria AM. (2016)[38]	2006–2013	PCR/MLST	México	192	NA	ND	OXA-24(70) OXA-23(57) OXA-58(23)	ST758(9/22) ST417(2/22)	No	–	x	High
Rodríguez CH (2017)[39]	2016	PCR/MLST	Argentina	100	NA	ND	OXA-23(100)	ST1(45/100) ST25(34/100) ST79(15/100)	No	–	x	High
Opazo-Capurro A. (2019)[40]	1990–2015	PCR/MLST	Chile	56	NA	ND	OXA-23(17) OXA-58(17)	ST162(4/56) ST15(3/56) ST109(2/56) ST318(1/56)	No	x	x	Medium
Ovalle MV. (2017) [41]	2012–2014	PCR	Colombia	97	KPC(1)	NDM(3) VIM(1)	OXA-23(87) OXA-24(1)	NA	Yes	–	–	High
Quiñones D. (2015) [42]	2010–2012	PCR	Cuba	220	NA	NDM (1)	OXA-23(130) OXA-24(35) OXA-58(5)	NA	Yes	–	–	High

Author, year, reference	Year isolated	Detection method	Place	Number of CRAB isolates	Carbapenem type (number detected)			Sequence type (n/N)	The study met all selection and quality criteria[&]	The study did not meet these quality criteria:		Study quality
					Class A	Class B	Class D			Adequate sample size	Data analysis with sufficient coverage of identified sample	
Brisolla LC (2019) [44]	2008–2014	PCR/MLST	Brazil	107	NA	NA	OXA-23(104) OXA−231(2) OXA−72(1)	ST730(43/107) ST317(28/107) ST1(10/107) ST79(8/107) ST107(2/107) ST986(1/107) ST175(1/107) ST22(1/107)	No	–	x	High
Levy-Blitchtein S. (2018)[45]	2014–2016	PCR/MLST	Perú	78	ND	ND	OXA-23(11) OXA-24(55) OXA-72(10) OXA-253(2)	ST2(7/16) ST79(6/16) ST1(2/16) ST3(2/16) ST108(1/16)	Yes	–	–	High

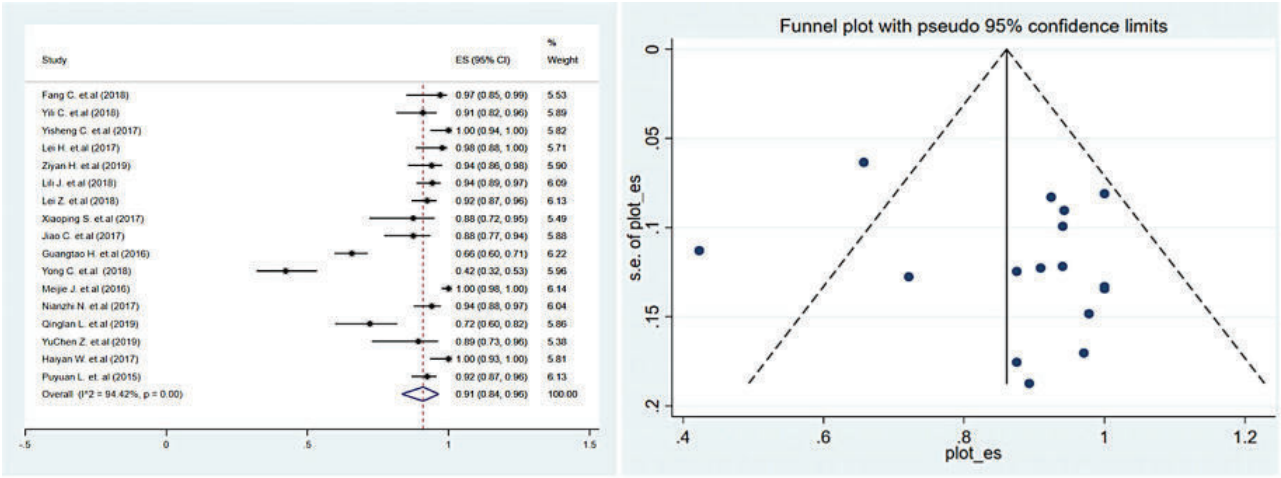
All were cross-sectional studies.
[&]: Quality criteria: 1. The sample was appropriate to address the target population, 2. The sample was obtained using an adequate method, 3. The sample size was adequate 4. Participants and the context are described in detail 5. Data analysis was carried out with sufficient coverage of the identified sample 6. Effective methods were used to identify diseases or health problems 7. The sample was measured using standard and reliable methods for all participants 8. The statistical analysis was appropriate 9. Response rate was adequate or low response rate was adequately managed.
n: number of times the sequence was found; N: total isolates studied; NA: Not applicable (detection not performed); ND: Not detected;
X: did not meet the requirement; –: met the requirement.

Table 3: Meta-analysis of carbapenemase genotypes in China, Latin America and the Caribbean

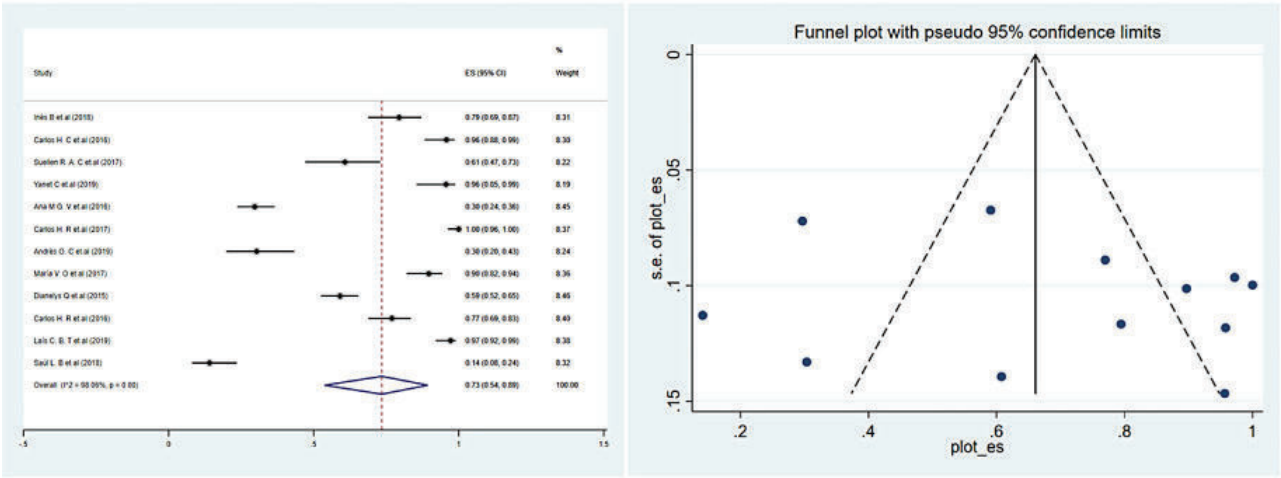
Place	Subgroups		Number of studies	n/N	Prevalence (95% CI)	Heterogeneity, I2 (%)	Heterogeneity p value	Egger's test
Latin America and the Caribbean	Class D	OXA-23	11	804/1217	0.73 (0.54–0.89)	98.06	>0.001	0.42
		OXA-24	11	162/1217	0.06 (0.00–0.17)	96.96	>0.001	0.36
		OXA-58	11	49/1217	0.03 (0.01–0.06)	85.97	>0.001	0.87
		OXA-72	11	42/1217	0.02 (0.00–0.06)	88.20	>0.001	0.94
	MBLs	NDM	9	4/933	0.01 (0.00–0.01)	0.00	>0.05	0.40
		VIM	9	1/933	–	–	–	–
		IMP	9	0/933	–	–	–	–
		SIM	9	0/933	–	–	–	–
China	Class D	OXA-23	17	1290/1499	0.91 (0.84–0.96)	94.42	>0.001	0.36
		OXA-24	17	51/1499	0.03 (0.01–0.08)	92.34	>0.001	0.11
		OXA-58	17	5/1499	–	–	–	–
		OXA-72	17	45/1499	0.02 (0.00–0.05)	91.07	>0.001	0.66
	MBLs	NDM	14	21/1005	0.02 (0.00–0.04)	74.58	>0.001	0.57
		VIM	14	120/1005	0.07 (0.00–0.21)	97.36	>0.001	0.60
		IMP	14	26/1005	0.02 (0.00–0.06)	85.77	>0.001	0.21
		SIM	14	14/1005	0.01 (0.00–0.03)	57.92	>0.001	0.54

MBLs: Metallobetalactamases; n: Number of isolates with the genotype; N: Total number of carbapenem-resistant *A. baumannii* isolates
Number of studies: number of studies in which carbapenemases were identified

Figure 2: Forest plot and funnel plot for OXA-23 carbapenemase prevalence in China, Latin America and the Caribbean



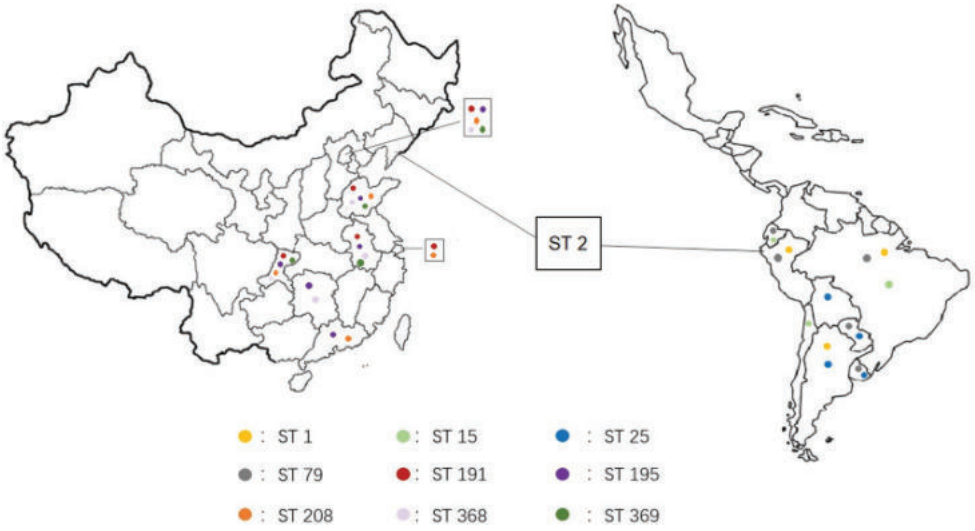
(A)



(B)

A: OXA-23 carbapenemase prevalence in China
B: OXA-23 carbapenemase prevalence in Latin America and the Caribbean

Figure 3: *Acinetobacter baumannii* sequence type (ST) geographic distribution in China, Latin America and the Caribbean



The data obtained in this review show that MBLs have a very low prevalence and are mainly of the NDM, VIM and IPM types. Five Chinese provinces reported MBLs; Hu Nan and Jian Xi provinces, in particular, have very high prevalence of this type of carbapenemases. In LAC, only Cuba and Colombia detected MBLs, both of which had very low prevalence rates. A meta-analysis of Iranian isolates reported a prevalence of 21.9% and 6.2% of OXA-24 and OXA-58 carbapenemase isolates, respectively, and higher prevalences of MBLs (IMP, 16.7%; VIM, 12.3% and NDM, 2.7%)[56] than those found in LAC and China. A study in Egypt reported a prevalence of 95.7% IMP, 7,1% VIM and 42.9% GIM in *A. baumannii* isolates.[57] The emergence and spread of MBL-producing *A. baumannii* strains has been reported in the United States, Canada, Europe, Japan, Australia, Africa and the Middle East.[58] The differences in OXA and MBL prevalence between countries is likely due to pressures of antimicrobial selection, horizontal transfer of carbapenemase genes by mobile genetic elements (plasmids) between species, propagation of clones carrying these genes or to a combination of all these factors.

Detected sequence types MLST is considered the gold standard for detecting bacterial sequence types and is highly useful in epidemiology. This review includes 17 studies based on MLST. Due to the great diversity in sequence types and the differences between countries and provinces, we only carried out a descriptive qualitative study on these articles (Figure 3). In the case of LAC, these studies mainly originated in South America (namely in Brazil, Peru, Argentina, Colombia, Chile, Bolivia, Ecuador, Paraguay and Uruguay). The most common sequence types in the region were ST79, ST25, ST1 and ST15. ST79 had the widest geographic spread, and was detected in Ecuador, Peru, Brazil, Paraguay, Uruguay and Argentina,[34,35,39,43,45] which implies greater current dissemination than had been reported earlier (in a review carried out in 2018).[59] ST25 was detected in Bolivia, Paraguay, Uruguay and Argentina,[39,43] and had been previously identified in Honduras (2012) and Brazil (2013).[60,61] ST1 was found in isolates from Peru, Brazil and Argentina,[39,44,45] and was the predominant Argentinian isolate. ST15 was detected in Ecuador, Chile and Brazil; it was associated with carbapenemase production (mainly OXA-23–like carbapenemases), and has had rapid dissemination, mainly in South America.[62]

Eight provinces in China conducted multilocus sequence studies using MLST; ST195 and ST208 were the most common. These two sequence types have been found in other regions of the world and tend to become the dominant STs once introduced.[63–65] A study of the clinical and molecular characteristics of CRAB-produced bacteremia in China showed clones ST195 and ST208 to be predominant, and bacteremia resulting from *A. baumannii* clone ST195 was associated with higher lethality.[66] Clones ST191, ST368 and ST369 are moderately prevalent in China and are more commonly found in Asia than the rest of the world. Hospital infection by *A. baumannii* clones ST191 and ST369 has also been reported in China and South Korea.[67,68] The STs that are the most common in China are rarely reported in LAC; only ST369 has been reported in Mexico in 2020.[69] This may be the result of differing clonal ancestries in both regions and selection pressure from different antimicrobial use habits.

ST2 was found in both regions (Peru in LAC and Liao Ning in China). ST2 is prevalent in some countries in Pacific Asia and Europe,[70–74] but has not been reported with any frequency in LAC in the last five years. Although this study found ST2 to be associated with OXA-72, other studies have described ST2 *A. baumannii* clones as mainly being producers of OXA-23.[74–76] ST2's dissemination in LAC merits consideration.

This study has provided estimates of the prevalence of different types of carbapenemases in *A. baumannii* in LAC and China, and the geographical distribution of different circulating CRAB STs and supports adjustments to resistance surveillance programming and antimicrobial management.

Study limitations There were some Latin American and Caribbean countries and Chinese provinces that produced no studies that met our selection criteria. Consequently, there is no information on carbapenemase-producing CRAB strain genotypes in these regions. Additionally, most studies do not detect Class A carbapenemases. This exclusion may have led to an overrepresentation of other carbapenemase classes and this preferential study of certain classes may have introduced bias. Finally, while the Egger test in not statistically significant and publication bias is unlikely, there is considerable heterogeneity between studies, as suggested by the asymmetric and sparse shape of the funnel plots. This could be due to insufficient sample size in the included studies, or to some variation (either geographical or temporal) in the strains or other methodological aspects such as differences in sample inclusion and exclusion criteria, among other factors.

CONCLUSION

A. baumannii resistance to carbapenem antibiotics is a global threat, and there is increasing diffusion of carbapenem-producing *A. baumannii* isolates and increasing geographic ST variation. Enzymes produced by regional isolates are generally very simi-

lar, while differences in STs are observed between China, Latin America and the Caribbean. Different regions have different epidemic CRAB strains, and it is important to consider local epidemiology in order to best tailor patient treatment. Studying circulating enzymes, clones and genetic lines facilitates understanding of region-specific resistance mechanisms.

Multidisciplinary collaboration is necessary (microbiologists, clinicians, epidemiologists and specialists in preventive medicine with experience in infection control), and will allow for early detection and study of resistance using molecular methods; adequate treatment, taking into consideration the patient's clinical status, common circulating strains, and infection characteristics; and adoption of epidemiological measures aimed at multi-drug–resistant infection prevention and control, reducing transmission at community- and hospital levels. A multidisciplinary approach can provide better results in managing a complex, multifactorial problem with major implications for public health.

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Innate Immune Stimulation Should not be Overlooked in Post-exposure Prophylaxis and Early Therapy for Coronavirus Infections

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ABSTRACT

We discuss the suitability of innate immune stimulation in acute respiratory infection post-exposure prophylaxis. The induction of innate immunity can be used to reduce susceptibility to immune-evasive pathogens (coronavirus, influenza virus, respiratory syncytial virus and rhinovirus). After the emergence of multiple SARS-CoV-2 variants, scientists are debating whether new variants could affect vaccine efficacy and how antigens could be redesigned to compensate. In addition, there is insufficient vaccine production to cover universal demand, and equitable vaccine distribution is a global challenge. Given these factors, non-specific immune stimulators may be suitable for a quick first response in the case of a suspected or early respiratory infection. Our group completed several HeberNasvac studies in healthy volunteers and patients with respiratory infections, and is currently starting large clinical trials in patients with early SARS-CoV-2 infections. This nasal formulation of hepatitis B vaccine has demonstrated its capacity to stimulate innate immunity markers (TLR3, TLR7 and TLR8 in tonsils) at the virus' entry site, in systemic compartments (HLA class II in monocytes and lymphocytes) and in the activation of dendritic cells, lymphocytes and other cell lines in vitro and ex vivo. In addition, research generated by the current pandemic may obtain results useful for treating other acute respiratory infections, which have long been main drivers of mortality among older adults and in early childhood.

KEYWORDS Immunity, innate; toll-like-receptors; SARS-CoV-2; COVID-19; respiratory tract infections; Cuba

INTRODUCTION

Innate (or nonspecific) immunity plays a key role in shaping antiviral defenses. RNA viruses are detected by toll-like receptors (TLR), among other innate immune receptors, specifically TLR7 and TLR8, while TLR3 recognizes double-stranded RNA (dsRNA) intermediates formed during viral replication. To avoid detection, coronaviruses (CoVs) and other RNA viruses that cause respiratory infections have developed evasive strategies.[1]

Older adults and people with underlying health conditions are at higher risk of developing severe clinical manifestations of COVID-19. This could be associated with less responsive innate immunity ligands, altered immune responses—both in quality and quantity—changes in the cytokine milieu and lower expression of co-stimulatory signals.[2] Thus, intrinsic host factors may contribute to disease severity and mortality.[3]

Intranasal (IN) stimulation of innate immunity receptors using TLR agonists decreases mortality in mice using the mouse model of lethal infection with SARS-CoV and influenza A virus (IAV).[4,5] Pre- and post-exposure prophylaxes following non-specific immune stimulation with Hiltonol (a TLR3 agonist), before and after administering a lethal dose of mouse-adapted SARS-CoV, resulted in a 100% survival rate.[4] A second study, using commercial TLR3, TLR7/8 and TLR9 agonists, reduced mortality rates in mouse-adapted SARS-CoV—infected 12-month-old mice when they were administered up to 3 days before the lethal SARS-CoV challenge. The TLR3 agonist either completely or partially prevented death in 22-month-old IAV-infected mice following intravenous or intraperitoneal administration, respectively, highlighting the effect of innate immune stimulation even in very old animals.[5] In vitro mouse-adapted SARS-CoV replication inhibition in human epithelial cells treated with a TLR3 agonist, and the reduction in survival of polyinosinic:polycytidylic acid (poly(I:C)-treated TLR3 knockout mice further confirmed the role of TLR3 signaling in the induced protection.[5]

IMPORTANCE

Stimulation of innate immunity against SARS-CoV-2 opens new options for post-exposure prophylaxis and early therapy for other respiratory viruses. This article describes HeberNasvac used as a mucosal innate immunity stimulator and discusses its clinical potential.

DEVELOPMENT

HeberNasvac (coded CIGB2020 when used as a non-specific immune stimulator) is a mucosal and parenteral therapeutic vaccine; a liquid formulation containing a recombinant hepatitis B core and surface antigens HBcAg and HBsAg, produced at the Genetic Engineering and Biotechnology Center (CIGB) in Havana, Cuba. It contains 100 µg of each antigen in 1.0 mL of saline-phosphate buffer. In 2015, Cuba's national regulatory authority approved its use in the country for chronic hepatitis B therapy, and it has also been subject to clinical trials in Japan, Bangladesh and other Asian countries.[6,7] The proteoliposomal and nucleoprotein composition of HBsAg and HBcAg, respectively, organized in a virus-like structure, facilitates its ability to stimulate dendritic cells[8] and activate B and T cells,[9] which results in a strong adaptive Th1 immune response.[10]

The clinical and pharmacological results of HeberNasvac in hepatitis B patients[6–10] show evidence of innate immune stimulation at the nasopharyngeal and oral mucosa, the main entry sites of SARS-CoV-2. We explored its effect on the local RNA receptor expression (TLR3, TLR7 and TLR8) and other innate immunity markers (Table 1).

A phase 1/2 open-label, randomized clinical trial was conducted in 46 hospitalized patients ≥60 years old, of both sexes, with symptoms of respiratory infection (78.3%), or asymptomatic,

Evidence (Ref. or RPCEC id.)	Study design	Objective	Results
Background: in vitro and in vivo studies			
Immune modulatory and antiviral effects of HeberNasvac[7]	In vitro study of PBMC and pulsed DCs from vaccinated CHB patients.	Evaluation of cytokines in sera, stimulated PBMC and DCs from CHB patients, as well as the antiviral effect of the product.	Significantly higher levels of IL-1β; IL-6; IL-8; IL-12 and TNFα than in unvaccinated CHB patients (control) ($p < 0.05$). Antiviral effect associated with cytokine stimulation.
Immune modulatory and antiviral effects in vitro and in animal models[8–10]	In vitro stimulation of dendritic cells, B & T cells.	Assessing HeberNasvac capacity to increase the markers of innate immunity at molecular and cellular levels and its impact on adaptive immune modulatory response.	Dendritic cells pulsed in vitro induce stronger adaptive immune response & subvert tolerance when injected in tolerogenic transgenic mice.[8] B & T cells activation.[9] Th1 pattern of response after IN administration and Th1 immune modulating effect.[10]
Multi-agonist effect on TLRs, MyD88/ TRIF pathways and antiviral effects[14]	In vitro studies stimulating HepaRG cells with HBcAg and HeberNasvac.	Assessment of the capacity of HeberNasvac and their antigens to increase markers of innate immunity at molecular and cellular levels linked to a functional antiviral assay. *Treatment:* Cells were treated in vitro and in specific cases reinfused to animals.	Multi-TLR agonist effect in TLR2, TLR3, TLR7, TLR8, TLR9 cells. Increased stimulation of adaptors MyD88; TRIF, NF-kb & IRF3, and molecules involved in antigen presentation (HLA class I, HLA class II, B7.1; B7.2, IFNa and IFNb and other cytokines).These genes increased their expression (>2x) with statistical signification. Innate immune stimulation triggered strong antiviral effect in infected HepaRG cells (similar to Entecavir's effects).
Human studies			
Safety and local (IN & SL) effects on innate immune markers[11] RPCEC00000306	Open-label, controlled and randomized phase 1/2 clinical trial in post-exposure prophylaxis of patients with acute respiratory infections or SARS-CoV-2(+).	Assessment of safety and local and systemic immune modulatory effects in a cohort of patients (symptomatic, 78.3%) or asymptomatic but exposed to SARS-CoV-2 positive patients. *Treatment:* 24 patients received HeberNasvac, 3 IN doses on days 0, 7 and 14, and a SL dose daily, for 14 days. 22 patients did not receive the product (control group). All patients received the standard treatment.	Safe and well tolerated in patients with acute respiratory infections. Higher proportion of patients with increased TLR-related genes in tonsils compared to the non-treated controls. Day 8: TLR3, 44.4% vs. 0%**; TLR7,66.6% vs. 0%*** and TLR8, 50.0% vs. 8.3%* Higher MFI % of HLA-class II marker in PBMC's monocytes (55%; $p = 0.023$) and lymphocytes (20%; $p = 0.008$) in treated vs. non-treated groups. Preliminary evidence of reduction in number of days with symptoms in treated patients. Mean (SD): 10.12 (9.43) in treated vs. 13.75 (10.80) in controls.
SARS-CoV-2 pre-exposure prophylaxis: safety and effect[13]	Open-label, single arm pilot study in health-care workers and their household contacts.	Assessment of the prophylactic effect in 20 highly exposed volunteers by detecting infection or disease worsening in the SARS-CoV-2(+). *Treatment:* 20 volunteers received 3 IN doses on days 0, 7, and 14 SL administrations every day.	In a 6 months follow-up period, 4 out of 20 volunteers were SARS-CoV-2(+). The infected patients were older, with comorbidities. Clinical evolution, CT scans, and symptoms evidenced mild progression: 3 patients developed slight cough and 1 patient (55 years old, with diabetes and hypertension) required noninvasive oxygen therapy (2L), symptoms vanished on day 5. Safety: the treatment was safe and well tolerated, 340 doses, 1.18% were associated with an AE (nasal drops and sneezing), no systemic AE reported, all AE were mild and did not required treatment.
Immune- modulating effect in healthy volunteers[13]	Open-label, single arm pilot study in volunteers treated with one standard IN administration.	Assessment of the cytokine production of PBMC from 10 healthy volunteers receiving 1 HeberNasvac dose. *Treatment:* 1 IN administration, extraction after 24 hours.	Significant increase in PBMC-induced production of cytokines compared to pre-immune values. IFNg (4.5X); TNFa (9.1X); TGFb (5.4X); IL-2 (2.3X); IL-10 (3.1X). Early cytokine induction (24 hours after stimulation).

AE: Adverse Events; CHB: chronic hepatitis B; DC: dendritic cells; HBcAg: Hepatitis B core antigen; IN: intranasal; MFI: mean fluorescence intensity; PBMC: peripheral blood mononuclear cells; RPCEC: Cuban Public Registry of Clinical Trials; SL: sublingual; TLR: Toll-like receptors. The value of $p < 0.05$ (*); 0.01 (**) and 0.001 (***) correspond to statistically significant; very significant or highly significant differences.

but close contacts of SARS-CoV-2 patients.[11] The study was approved by the Center for State Control of Medicines, Equipment and Medical Devices (CECMED) and listed in the Cuban Registry of Clinical Trials, RPCEC00000306-En.[12] Twenty-four patients received HeberNasvac in the study group by both intranasal (IN) and sublingual (SL) routes (100 µg of each antigen in a volume of 1 mL). The product was administered daily by SL route for 14 days and by IN route at days 1, 7 and 14. The 22 patients allocated to the control group did not receive HeberNasvac. Both groups received the standard treatment defined by the Cuban Ministry of Health. The adverse events were studied only in vaccinated volunteers. A total of eight different adverse events were detected in five patients (20.8% of treated patients). Local adverse events consisted in nasal drops (0.58%), sneezing (0.3%), and otalgia (0.3%). Fever (0.3%) and asthenia (0.3%) were reported as systemic adverse events. None of the adverse events was classified as serious, half of them were unrelated, 25% possibly related, and 25% related to the product (nasal drops and sneezing). Most adverse events disappeared without treatment (62.5%) or after a pharmacological treatment in the case of fever and otalgia (32.5%). One patient required treatment due to pneumonia, which was related to the basal condition according to causality analysis. In summary, the administration of HeberNasvac simultaneously by IN and SL routes was safe and well tolerated.

The first PCR test, conducted five days after beginning treatment, detected one positive patient in the treatment group and two in the control group. A subsequent serological study using ELISA detected 50% participants positive to SARS-CoV-2 in the treated group and 41% in the control group, suggestive of a previous infection. There was no difference in the evolution of respiratory symptoms. Among the three PCR-positive patients, one from the control group developed pneumonia, diarrhea, edema, and fatigue and after discharge; fatigue and shortage of breath remained for 42 days. The other two patients (one in each group) remained with mild symptoms.[11]

No significant differences appeared in the hematological and blood chemistry analyses between patients treated with HeberNasvac and the control group. Preliminary evidence indicated a reduction in the symptomatic phase in treated patients; a mean of 13.75 (standard deviation, SD 10.80) symptomatic days in the control group vs. 10.12 (SD = 9.43) days in the treated group.[11] IN/SL HeberNasvac administration stimulated innate immune markers at local and systemic compartments; a significant proportion of treated patients had higher gene expression of RNA receptors (TLR3, TLR7 and TLR8) in their oropharyngeal mucosa (tonsils) as well as a significant increase of HLA class II expression in lymphocytes and monocytes from peripheral blood mononuclear cells (PBMC), characterized by 20% and 55% increases in mean fluorescence intensity (MFI), respectively[11] (Table 1).

HeberNasvac safety and efficacy in SARS-CoV-2 pre-exposure prophylaxis was studied in two open-label, single-arm pilot studies in a high-incidence area of Dhaka, Bangladesh. The first assessed the drug's prophylactic effect in 20 highly-exposed volunteers (healthcare workers and their household contacts) by detecting infection or disease. Results showed a limited number of infected volunteers after six months' follow-up and mild disease progression in those who tested SARS-CoV-2–positive.[13] The second study was conducted in ten healthy volunteers treated with one standard IN administration of HeberNasvac to determine immunomodulatory and antiviral cytokine expression.

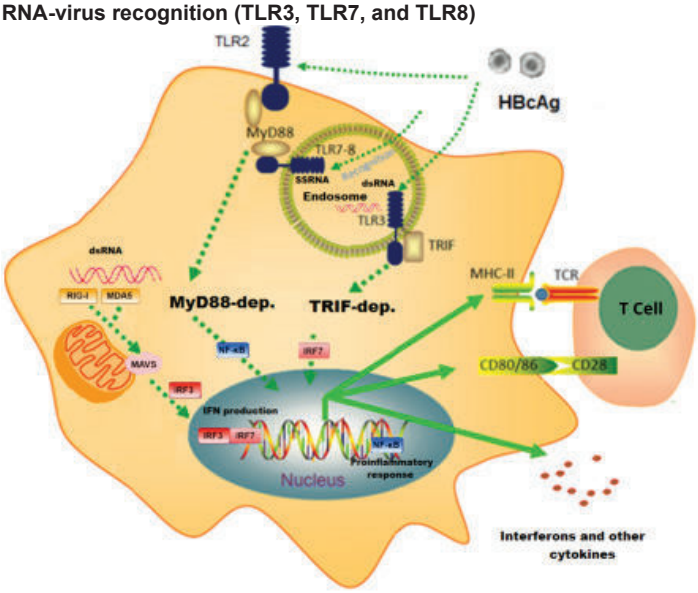
Several immunomodulatory and antiviral cytokines were induced very early, as soon as 24 hours after IN administration[13] (Table 1). A third study in 15 recently-infected SARS-CoV-2 patients immunized as described by Fleites[11] showed no severe COVID-19, and no patient required ventilation.[Aguilar JC, Akbar SMF, clinical trial report, unpublished data] A group of patients with mild or moderate COVID-19 proceeded to severe clinical manifestations of infection during the study. However, these patients remained stable and were discharged from hospital, leading to the approval of a larger clinical trial.

Immunomodulatory effects found in clinical trials were consistent with in vitro immunomodulatory and antiviral effects detected at cellular and molecular levels in previous studies with peripheral blood mononuclear cells (PBMC) and other cells in culture; chronic hepatitis B (CHB) patients and animal models of immune tolerance[7–9] (Table 1). In particular, HBcAg showed a multi–toll-like receptors (multi-TLR) agonist effect, stimulating in vitro dendritic cells and lymphocytes, as well as human hepatoma (HepaRG) cells (infected or not), increasing expression of TLR2, TLR3, TLR7, TLR8 and TLR9 and their signal transduction pathways; MyD88 (Myeloid Differentiation Factor 88), TRIF, IRF3, NF-kB (NF-kappaB).[11,14] The high RNA content associated with HBcAg—15% to 25% RNA/protein, w/w— stimulates TLR3, TLR7 and TLR8 expression,[11,14,15] implicating stimulation of both MyD88-dependent and TRIF-dependent pathways. This merits the induction of class I/II HLA co-stimulatory molecules B7.1 and B7.2 (CD80/86), type I interferons and other cytokines. Taken together, a set of molecules involved in antiviral immune defense is innervated, consistent with the immunomodulatory and antiviral effects found both in animal models and in vitro.[8–10]

HeberNasvac administration device (dropper vs. spray), posology and dosing are now being optimized in a second study approved by CECMED, the Cuban regulatory authority, for this new use (RPCEC00000326-En, ongoing).[12] Two other protocols have been registered and are pending CECMED approval: a phase 2/3 clinical trial in SARS-CoV-2–positive patients during early stages of infection, aimed at preventing progression to severe COVID-19 (RPCEC00000353-En),[12] and an intervention study on post-exposure prophylaxis for acute respiratory infections (ARI) in older adults (aged >60 years) to include persons exposed to SARS-CoV-2, and household contacts of confirmed cases (RPCEC00000356-En).[12] These clinical trials should be completed in 2021–2022.

Recent transcriptomic studies of SARS-CoV-2–in vitro infected cells recommend the use of TLR3 and TLR7/8 agonists for early immune stimulation.[16] Studies in newborn calves infected by bovine coronaviruses have shown the down-regulation of the TLR3 gene, compared to the expression of the same gene in newborn calves infected by bovine rotavirus, further indicating that TLR3 is a target during CoV infection[17] and that stimulation of these TLRs may be appropriate for early COVID-19 therapy and SARS-CoV-2 pre- and post-exposure prophylaxis. The multi-TLR agonistic effect of HBcAg stimulates both MyD88-dependent and independent pathways, resulting in synergistic antiviral responses such as those previously described for the combination of TLR

Figure 1: Pathways of TLR-mediated activation induced in differentiated HepaRG cells in culture by HBcAg, the antigen of HeberNasvac comprising the RNA ligands of the TLRs involved in RNA-virus recognition (TLR3, TLR7, and TLR8)



HBcAg particles, alone or as part of HeberNasvac, stimulate expression of TLRs and other innate immunity markers in cells from oropharyngeal scrapings, as well as several cell types in culture, i.e. dendritic cells, B and T cells and hepatocytes in culture.[8,9,11,14] Signaling through MyD88-dependent and TRIF-dependent pathways results in higher expression of IFN and other proinflammatory cytokines, HLA class I/II stimulation and costimulatory molecules. HBcAg multi-agonist effect is used as a tool to overcome the evasive mechanism described for SARS-CoV-2 and other respiratory pathogens.[1]

ligands.[18,19] The agonistic effect of HBcAg on TLR2 and TLR7 has already been described;[20,21] however, we reported for the first time the simultaneous innervation of TLR3/TLR7 and TLR8 genes,[11,14] a rare quality for a single compound.

HeberNasvac's effect on innate immunity markers was detected as early as day four of treatment and continued up to day eight. Three daily sublingual (on days one to three) and one intranasal (on day one) administrations may be as effective as a schedule of seven administrations for increasing the proportion of patients with higher expression of the receptors and mediators required to detect SARS-CoV-2 and prevent pathogenesis. Such a rapid innervation of the innate immune system is also an attractive property.[13]

Other groups are studying the stimulation of the cytoplasmic RNA receptor RIG-I as a prophylactic and therapeutic protection against SARS-CoV-2 infection in mice.[22] Post-exposure prophylaxis or early therapy in SARS-CoV-2 infections rapidly activate innate immunity, triggering a fast and effective adaptive response, counteracting the virus' evasive mechanisms.[1]

Another SARS-CoV-2 mechanism to evade innate immunity (considered a virus evolutive advantage) was recently described in the variant of concern (VOC) B.1.1.7 (alpha).[23] In this variant, subgenomic RNA overexpresses open reading frames (Orf) proteins Orf9b and Orf6, two well-known innate immunity antagonists,[23] making VOC alpha more effective than the original Wuhan wildtype SARS-CoV-2 variant in suppressing the host's innate immune response in airway epithelial cells. Orf9b and Orf6 interact with TOM70, a mitochondrial protein required for mitochondrial antiviral-signaling protein (MAVS) activation, an RNA-sensing adaptor. The emergence of new VOCs alpha and delta, both of which show increasing transmissibility and severity, further supports the role of immune evasion on pathogenesis, and calls attention to innate immune stimulators for post-exposure prophylaxis and early therapy. HeberNasvac acts to a) improve SARS-CoV-2 RNA detection by stimulating TLR3, TLR7 and TLR8 expression,[11,14] and b) through direct antiviral/immunomodulatory activities as an efficient inducer of interferons and related cytokines.[7–10] TLR-mediated activation is induced by HBcAg, the antigen of HeberNasvac comprising the RNA ligands of the TLRs involved in RNA virus recognition (TLR3, TLR7, and TLR8). Figure 1 describes the pathways of TLR-mediated activation induced in differentiated HepaRG cells by HBcAg, the antigen of HeberNasvac comprising the RNA ligands of the TLRs involved in RNA virus recognition (TLR3, TLR7, and TLR8).

Few products are being evaluated as prophylactic and therapeutic immunomodulators in SARS-CoV-2 infection. The Bacillus Calmette–Guérin (BCG) vaccine was introduced early in the pandemic in several countries as an innate immunostimulant; however, shortages of BCG vaccines and their limited availability for pediatric immunization prevented widespread use.[24] Other immunomodulators being studied are interferons (IFN);[25] their intranasal/inhaled administration has received ongoing attention since the early days of the COVID-19 pandemic. A novel nasal IFN formulation (Nasalferon) received regulatory approval for emergency use in SARS-CoV-2 pre- and post-exposure prophylaxis in Cuba.[26] Since the beginning of the pandemic, two parenterally-administered products, HeberFERON (IFN alfa 2b and gamma) and Heberon (IFN alfa 2b), were included in the Cuban guidelines for SARS-CoV-2 treatment.[27]

Local stimulation of the innate immune system by SL administration using preparations of inactivated bacteria has been used to prevent recurrent respiratory infections.[28] IN administration of the adenovirus influenza vaccine prevents death in mice infected with lethal respiratory pathogens.[27] The US Food and Drug Administration (FDA) approved a phase 2 trial of the nasal influenza vaccine candidate T-COVID (Altimmune, Gaithersburg, MD, USA), a recombinant adenovirus influenza vaccine, for use in early SARS-CoV-2 infection therapy.[29]

In patients with severe COVID-19, HLA-DR expression on monocytes and myeloid dendritic cells (mDCs) is reduced. Plasmacytoid DCs, mDCs and CD14+ monocytes of COVID-19 patients are less responsive to stimulation with bacterial (TLR2/TLR4/TLR5) and viral (TLR3/TLR7/TLR8) ligand cocktails compared to healthy controls, indicating that innate immune cells in COVID-19 patients are functionally impaired.[30]

HeberNasvac's immunostimulatory effect supports its use in post-exposure prophylaxis and therapy for SARS-CoV-2 and, most likely, for other respiratory infections. Commercial agonists stimulating TLR3, as Poly (I:C) and TLR7/8 (CL097M-012), also innervate antiviral, inflammatory and humoral pathways, decreasing viremia and resulting in clinical improvement in dengue-infected monkeys;[31] in cancer, the relevance of TLR3 stimulation is currently under study.[32] Taken together, the stimulatory properties of HeberNasvac, and HBcAg in particular, may open new prophylactic and therapeutic opportunities.

A quarter century ago, the molecular mechanisms of the innate immune system were practically unknown. Adjuvants and immunomodulators were selected by trial and error,[33] and some were described as IFN inducers. Today we understand some of these mechanisms, and we know that innate immunity can be trained.[34] Long-term stimulation of innate immune responses (trained immunity), by certain live vaccines, cytokines, toll-like receptors and adjuvants, induces heterologous protection against infection through epigenetic, transcriptional and functional reprogramming of innate immune cells. The induction of trained immunity can be applied to reduce susceptibility to and severity of SARS-CoV-2 infection.[34,35]

Products with HBcAg's demonstrated properties—now initiating phase 2/3 trials—may induce an adaptive immune response if administered soon after infection by viral respiratory pathogens such as SARS-CoV-2. These innate immunostimulatory and modulatory mechanisms are currently used in the development of novel treatments for non-respiratory viruses, as has been the case with HeberNasvac antigens in developing a therapeutic vaccine for HIV, the formulation of which includes HBsAg, HBcAg and the HIV multiepitopic vaccine candidate CR3;[36] and the development of a nasally-administered vaccine against SARS-CoV-2 named Mambisa that is under study in protocols RPCEC00000345-En and RPCEC00000382-En.[12]

CONCLUSIONS

Innovative innate immunostimulators such as HeberNasvac may become useful tools for pre- and post-exposure prophylaxis and early therapy in SARS-CoV-2 and other respiratory infections. The knowledge generated by the SARS-CoV-2 pandemic will be useful in confronting respiratory infections in general, which remain a main cause of mortality worldwide. Due to the emergence of SARS-CoV-2 variants, we should be prepared for prolonged coexistence with this pathogen. Innate immunostimulators for nasal or sublingual use, suitable for pharmacy distribution and self-administration, may become a first therapy after symptom onset, and suitable for prophylactic treatment of at-risk contacts, especially among older adults and persons with comorbidities.

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Caution Ahead: Traffic Accidents in Cuba

Conner Gorry MA

The call came from Calixto García University Hospital: Alfredo shattered his leg in a head-on collision driving from Havana to the beach. After emergency surgery and several weeks of intensive physical rehabilitation, Alfredo is back on two feet.

Messages flooded in, my phone vibrating off the table: Manolo and his son were being examined at Havana's Carlos J. Finlay Military Hospital after his Lada was nearly totaled in a late-afternoon crash. Both father and son are fine (the Lada, however, is not).

Then there was that other call, the one I most fear since I've lived the experience once previously here in Cuba: a friend was killed in a traffic accident—she was 30 years young and with a future so promising, her tragic death strains comprehension.

All these events happened in the past six weeks and they all had two factors in common: each occurred on a Sunday and all of them were preventable.

According to data released in November 2021 from Cuba's National Commission on Traffic Safety (CNSV), Sunday is the most lethal day to be on Cuban roads, with one fatality for each 11 accidents, while Friday, between 3 PM and 6 PM is the most accident-prone, with 20% of all accidents occurring at this time.[1]

Analysis provided by CNSV found the most common causes of traffic accidents in Cuba for the first nine months of 2021 were: irresponsible drivers and pedestrians; technical problems with the vehicles involved; and above all, inattentive drivers who did not have proper control of their vehicles at the time of the accident. Drilling down further, data revealed that speeding and right-of-way violations were involved in most accidents, while 17% of drivers were legally drunk according to breathalyzer tests measuring blood alcohol content (BAC) at the time of the accident in question.[2]

Both the number of accidents—5612—and total fatalities—350—increased in 2021

While official sources accentuated the positive regarding the grim reality of traffic accidents in the country—highlighting the decrease in accidents between 2015 and 2020 and the 13% drop in people injured in road accidents from 2020 to 2021[3]—the bigger picture is more sobering.

First, both the number of accidents 5612 and total fatalities (350) increased in 2021, representing a jump of 1%–2% from the previous year.[3] More importantly, the period under study for 2021 was between January and September, as opposed to the full 12-month calendar year for 2020—meaning there were more accidents and deaths in 25% less time. The context in which these accidents occurred is also troubling: not only was the country closed to tourism during the time studied, but nearly all non-essential interprovincial travel was suspended and a country-wide curfew of 9 PM was in place. All this reveals that accidents, including fatal ones, increased in a shorter timeframe, during which traffic volume was drastically reduced.

Other factors contributing to the problem according to CNSV include unlicensed drivers—54% of accidents involved drivers with no license, representing over 16,000 drivers in total.[3,4] The majority of them were driving electric motorcycles, vehicles that prior to 2021 did not require license plates either, meaning a driver fleeing the scene of an accident would be almost impossible to locate. Obtaining and renewing drivers' licenses, plus vehicle inspections and registration, were all suspended due to COVID-19 throughout the period studied. Since motor vehicle services re-opened in mid-November, the expectation is that the trifecta of unlicensed drivers, technical defects and vehicles without plates will gradually improve. However, the backlog, combined with the infamously sluggish bureaucracy, means the impact will not be immediately felt.

There are other, more complicated and costly reasons behind traffic accidents as well, lack of infrastructure investment and improvements chief among them. Street lighting in Cuba is dangerously deficient, even on main city thoroughfares and four-lane highways, making night driving especially challenging. Roadway signage, though vastly improved over the past five years, is still lacking and sometimes confusing, which can lead to indecisive or conversely, abrupt maneuvers behind the wheel—both perilous.

Road conditions themselves are also a major hazard as anyone who has driven here knows: drivers routinely swerve brusquely to avoid the ubiquitous potholes common to Cuban streets, while motorcycle riders must keep their eyes pegged to the asphalt directly in front of them or risk being tossed in the air like a popped kernel of corn. Poor geometric design on certain roads, including sight distance, curve radius, horizontal and vertical alignments and super-elevation, can also affect road safety.[5] Currently there are over 2000 ongoing studies related to improving sight distance, and 22,000 traffic signs are being installed.[3] Both initiatives should help. Unfortunately, Cuba's ongoing economic crisis, compounded by the pandemic and strengthened US sanctions, pits roadway investment and maintenance against other, more immediate priorities.

The CNSV underscored the role of driver and pedestrian responsibility as a major cause of traffic accidents. Jaywalking and stepping into oncoming traffic is an all-too-common practice here. Indeed, data shows that 45% of accidents were caused by pedestrians and 38% of the injured in all accidents in the period studied were 66 years or older.[3] Speeding, drunk driving and texting/talking on a cell phone while at the wheel are other dangerous practices that must be addressed. The fact that accidents (of all types) have been the fifth cause of death in Cuba for several years running[6] lays bare the urgency of the problem.

Although there is little good news on Cuba's traffic safety front, the benefit of such data is that it allows for policy analysis and action to reverse the deadly trend that saw 23 accidents, 15 people injured and one killed every day in Cuba between January and September, 2021.[3] While so much is out of our control in these pandemic times, navigating our roads carefully, soberly—whether driving, walking or cycling—is clearly within our command.

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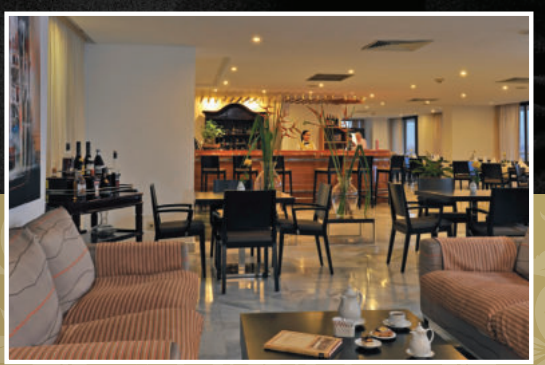

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