MEDICC Review

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CUBA HEALTH 2022

Universal Health on the Health Agenda 2030
in the Context of COVID-19

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- Climate change and public health
- Safety and quality of medical care
- Emerging and re-emerging diseases
- Antimicrobial resistance
- New forms of international cooperation in health
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- Health and wellbeing tourism
- Medical education...and more.

Conference languages: Spanish and English
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Cover photo:
Face masks, still required in all public spaces in Cuba, drying on the window as the family dog surveys the street, Havana.

Photo: Jorge Luis Baños, IPS.

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Politics, Profits & Pandemics: Earth's Worst-Case Scenario

The year 2020 was one for the record books: an estimated 90 million people were driven into extreme poverty; it tied for the hottest year on record, with soaring global temperatures and heat waves resulting in thousands of fatalities; and in any given month, 19% of our planet's land area was stricken by severe drought, affecting yield potentials for staple crops like corn, wheat and soybeans. Meanwhile, glacier retreat, biodiversity loss and rising sea levels continued apace.[1] Women, people over 65 and babies, agricultural workers and the poor were—and still are—the hardest hit.

Global crises including the COVID-19 pandemic, a worldwide recession and geopolitical tensions in both hemispheres contributed to the bleak backdrop against which the United Nations held its 26th annual Climate Change Conference (COP26; November 2021). The key takeaways? We are nowhere close to cutting greenhouse gas emissions to maintain a livable climate and there is an egregious gap in financing and political will to reverse this disastrous course; Paris Agreement targets to help reach this goal are falling appallingly short; and low- and middle-income countries are the most vulnerable to human-induced climate change—despite high-income countries producing the most greenhouse gases.[1,2]

The results from COP26—non-binding and without teeth—were dispiriting. UN Secretary-General António Guterres said "the approved texts are a compromise…unfortunately the collective political will was not enough to overcome some deep contradictions."[3] Guterres put it more bluntly at the follow-up meeting of the International Panel on Climate Change (IPCC) in February 2022, stating that the "IPCC report is an atlas of human suffering and a damning indictment of failed climate leadership."[4,5]

Guterres does not overstate the case. Today, 26% of the global population does not have access to safe drinking water;[6] over 25% have no access to basic sanitation services and 29% have no access to basic hygiene—including the possibility of washing with soap and water at home. In Central and Southern Asia, 42% cannot wash at home with soap and water and in Sub-Saharan Africa, 75% of people don't have this capability.[7] One in four people around the globe (1.9 billion) are moderately or severely food insecure[8] and 267 million people in coastal communities are at imminent flood risk due to severe weather events and rising sea levels.[9]

> **The steady destruction of our biosphere optimizes conditions for deadly pathogens to flourish in water, food and air**

We are in a situation where "no country is meeting the basic needs of its residents at a globally sustainable level of resource use and no country is on track to do so."[10] The alarm has sounded and the warning signs (i.e. evidence-based science) are clear. It is incumbent upon all of us to make—and demand—urgent change.

One lesson hammered home by the current pandemic is that we do not have the luxury of time, compromise or half measures. The Earth's climate system is already dangerously vulnerable; if we continue to consume and pollute at the current rate, gains made on food and water security, carbon offsetting and global warming will be lost.[1] The worst-case scenario of an uninhabitable planet is no longer relegated to science fiction.

Another lesson from the pandemic is that planetary and human health are intimately intertwined. The steady destruction of our biosphere optimizes conditions for deadly pathogens to flourish in water, food and air, and exacerbates vector-borne diseases like dengue, Zika and malaria, as well as zoonotic viruses, including SARS-CoV-2. Given the ecological reality and the uncertain climate resiliency of our planet, it's not surprising experts agree that COVID-19 will not be the last, nor the worst, pandemic.[11]

The planet's health, *our* health, will brook no delay. Every day, the gap widens between what needs to be done to address climate change and what is actually being done. To bridge that gap, leaders need to be held to tenets of good governance, including pandemic preparedness, evidence-based policymaking and collaboration over confrontation; population and planetary health should be prioritized in policy, practice and research; and access to basic health services must finally be guaranteed as a fundamental human right—across the globe.

> **"The idea that some lives matter less is the root of all that is wrong with the world." – Paul Farmer**

Implementing such changes will take vision, political will and financing. Each must be bold, robust and unequivocal. COVID-19 economic recovery packages need to be equitable, transdisciplinary and green—moving forward, the only sustainable economies will be environmental economies, where ecological and social outcomes carry as much weight as GDP growth in policy design, implementation and practice. Already, the reduction in carbon dioxide emissions achieved to date are sliding in the wrong direction and in danger of being nullified altogether by pandemic recovery packages emphasizing short-term economic gains over long-term planetary stability.[1] They need not be mutually exclusive.

Over-exploitation of the Earth's resources, where economic activity outstrips environmental thresholds, usually at the expense of the most vulnerable, is no longer viable. Denying low-and middle-income countries a voice in setting the global development agenda, is no longer viable. Resigning ourselves to a reality where half the world's population does not have access to essential health services was never acceptable and now more than ever, more starkly than ever, is not viable.[12]

> Leaders need to be held to tenets of good governance, including pandemic preparedness, evidence-based policymaking and collaboration over confrontation; population and planetary health should be prioritized in policy, practice and research; and access to basic health services must finally be guaranteed as a fundamental human right—across the globe…Denying low-and middle-income countries a voice in setting the global development agenda, is no longer viable.

To reverse course, the lessons of the COVID-19 pandemic must be heeded. The pandemic showed that scientific collaboration, global coordination, streamlined regulatory processes like seamless trials and intersectoral cooperation are possible. Unfortunately, as the pandemic worsened, these positive actions were eclipsed by nationalist (and in some cases neo-colonialist) policies, finger pointing, mis- and disinformation by governments and the media, mixed messaging by health authorities and fear mongering. Inequities were laid bare, within and among countries, and the woeful inadequacies of health systems exposed. It is no coincidence that health is the single indicator that cuts across all five main actions of the Paris Agreement; still, just 0.5% of overall funding from multilateral climate finance is allocated specifically to protect or improve human health.[13]

Stronger, better-funded regional and international health authorities are essential. Bodies like WHO, PAHO and the nascent European Health Union are best equipped to tackle this challenge, but face legitimacy issues related to confusing messaging,[14] political posturing by rich, powerful countries that affects financing and buy-in by others, marginalization of lesser developed countries in agenda setting and the intrinsic problem of lack of enforcement. From the Alma Ata Declaration in 1978 to the Paris Agreement in 2015 and most recently the COVAX initiative, history has shown that simply coaxing member nations into compliance to improve population and planetary health does not work.[15] The current pandemic made this abundantly clear, with WHO Director-General Tedros Ghebreyesus calling vaccine nationalism and the lack of political will to safeguard the health of all nations “a catastrophic moral failure.”[16]

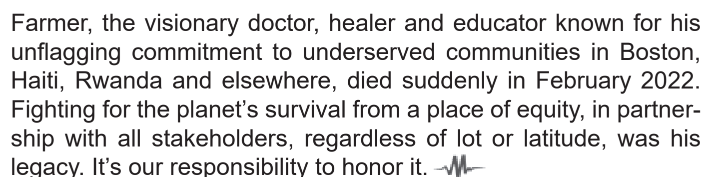
Funding for capacity-building, bolstering health systems’ infrastructure, emergency preparedness and technology/knowledge transfer to combat climate change and the disease burden it creates is an urgent priority in Africa, Asia, Latin America and the Caribbean. Under-representation of these regions in policy and decision-making on a global scale is not acceptable; empowering regional bodies such as the African Union and the Association of Southeast Asian Nations, while harnessing indigenous and local knowledge for improved stewardship are first steps.

For companies like Pfizer–BioNTech, there is zero motivation to change the model and 22 reasons to maintain the status quo: in 2021, they posted $22 billion in net profits, twice that of the previous year

Reviving the possibility of waiving intellectual property patents to benefit health in the Global South must be on the table. Powerful pharmaceutical companies, and their host countries, cannot be permitted to dictate policy on issues of planetary and population health. At the close of 2021, only 14% of people in low-income countries had received one COVID vaccine dose—in fact, more boosters had been administered in high-income countries by that time than total doses in all low-income countries combined. What’s worse, vaccine giants—Pfizer–BioNTech and Moderna—were profiting at a rate of $65,000 *per minute*. And this after receiving more than $950 million (Moderna) and $800 million (Pfizer-BioNTech) in public funds to develop their vaccines.[17,18] For companies like Pfizer–BioNTech, there is zero motivation to change the model and 22 reasons to maintain the status quo: in 2021, they posted $22 billion in net profits, twice that of the previous year.[19]

Finally, planetary and population health will continue to deteriorate if all policy, multilateral agreements and collective action is not based on, and their outcomes measured by, fundamental concepts of equity. In short, “the idea that some lives matter less is the root of all that is wrong with the world” and we must condemn all policies that are predicated on this premise. This principle, championed by Dr Paul Farmer throughout his unorthodox life and career serving in some of the most medically underserved and impoverished contexts on the planet, must be the road map forward.

Farmer, the visionary doctor, healer and educator known for his unflagging commitment to underserved communities in Boston, Haiti, Rwanda and elsewhere, died suddenly in February 2022. Fighting for the planet’s survival from a place of equity, in partnership with all stakeholders, regardless of lot or latitude, was his legacy. It’s our responsibility to honor it.



The Editors

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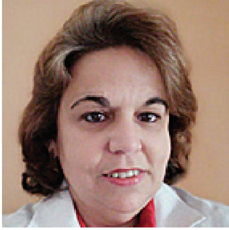

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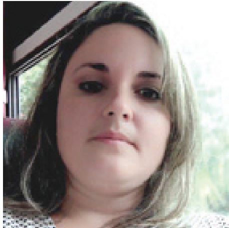

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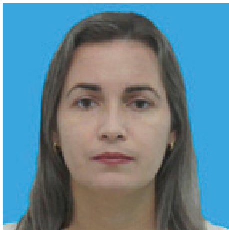

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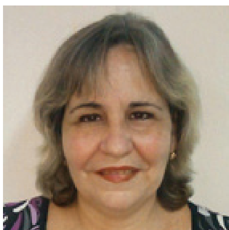

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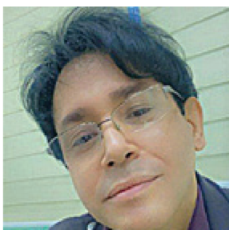

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Hematological Alterations in Patients Recovered from SARS-CoV-2 Infection in Havana, Cuba

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ABSTRACT

INTRODUCTION COVID-19 sequelae, or the short-, medium-, and long-term manifestations of the disease are under continuous study. There are currently few reports on the evolution of hematological variables following a demonstrated absence of SARS-CoV-2 after infection.

OBJECTIVE Identify hematological alterations in Cuban adults recovered from SARS-CoV-2 infection, and their relation with disease severity.

METHODS We selected 348 persons recovered from COVID-19 residing in Havana, Cuba with an RT-PCR study negative for SARS-CoV-2 performed two weeks after hospital discharge; a structured survey was administered to obtain clinical–epidemiological data. Three groups were established according to COVID-19 clinical criteria: asymptomatic, mild/moderately symptomatic, and severely symptomatic, which, in turn, were divided according to hospital discharge date and blood sample collection date. We performed hemograms with differential leukocyte counts and compared results among groups. We then measured the associations between hematological variables, personal medical history, and relevant lifestyle habits (smoking).

RESULTS All hematological variables were within normal reference limits, although men from the group of severely ill patients had increased total leukocytes, neutrophils and lymphocytes, and decreased hemoglobin and eosinophils, which was also evident in those with a recovery time of 31–90 days.

CONCLUSIONS The relation between hematological variables and degree of clinical severity offers evidence as to persistence of systemic alterations (possibly inflammatory) associated with viral infection. Their identification and characterization can facilitate personalized patient followup and rehabilitation.

KEYWORDS COVID-19, SARS-CoV-2, hematology, leukocytosis, neutrophils, eosinophils, Cuba

INTRODUCTION

COVID-19, caused by the SARS-CoV-2 virus, is most frequently characterized by fever, malaise, cough, sore throat and muscle aches, occurring in approximately 95% of patients who develop symptoms following an incubation period of 4–5 days.[1–5] Severe presentations of the disease begin a week after symptom onset and present as dyspnea accompanied by hypoxemia, and can progress to respiratory failure, a clinical picture consistent with criteria for acute respiratory distress syndrome (ARDS).[5,6]

Laboratory, chemical and hematological variables have been characterized in multiple publications as predictors of clinical severity.[7–13] Variations in hematological and blood chemistry values during the disease's active phase have been noted, especially in hospitalized patients and patients who progress to severe forms of the disease. The most common findings include lymphopenia, elevated D-dimer levels, and elevated levels of lactate dehydrogenase, C-reactive protein, and ferritin.[14,15]

IMPORTANCE Hematological alterations in patients recovered from SARS-CoV-2 infection confirm the persistence of inflammatory processes associated with viral infection and reveal the existence of pathological processes following, and possibly associated with, COVID-19.

Some of these variables are associated with poor prognoses, including increased total white blood count, increased neutrophil/lymphocyte ratio (NLR),[16,17] eosinopenia, prolonged prothrombin time, increased liver enzyme levels, and increased levels of interleukin-6 (IL-6) and procalcitonin.[8,15] Increased eosinophil levels prior to clinical discharge have been reported as indicators of improved outcomes.[8–21] However, there are few reports as to the status of these variables in individuals who are in stages of convalescence or recovery.

The Cuban Action Protocol for COVID-19 establishes how to manage recovering COVID-19 patients in primary health care (PHC),[22] emphasizing multidisciplinary collaboration and personalized followup, aimed at detecting complications or sequalae to adopt the most appropriate treatment, aid in rehabilitation and improve quality of life.

This study's objective was to identify hematological variations in adult Cubans considered clinically recovered from SARS-CoV-2 infection.

METHODS

Design and study group definition We carried out a cross-sectional analytical, observational study from June 25 through July 25, 2020, in all 15 municipalities of the Cuban capital, Havana. The study universe was comprised of all Cubans aged >18 years who were infected with SARS-CoV-2 from March 11 through June

11, 2020, with negative RT-PCR (real-time polymerase chain reaction) results two weeks after hospital discharge. Those who traveled outside their local community polyclinic's geographic health area during the study period and those whose records showed either discrepancies between databases or a lack of information (provided by patients or attending physicians) were excluded. A final sample was obtained based on the sole criterion of willingness to participate in the study.

We established three study groups, according to the disease's clinical characteristics and evolution: the asymptomatic group, composed of those who developed no symptoms or clinical signs of COVID-19; the mild–moderate group, of those patients who presented clinical signs of COVID-19 or reported symptoms, without presenting clinical complications; and severe group, denoting those who presented severe symptoms, with complications such as pneumonia, ARDS, cardiac arrhythmias, venous thrombosis and disseminated intravascular coagulation, who required intensive care due to alterations in respiratory rate, blood oxygen saturation, partial pressure of arterial oxygen or pulmonary infiltrates >50% in 24–48 hours, septic shock or multiple organ failure or dysfunction.

Clinical groups were, in turn, divided according to the period between hospital discharge date and the date biological samples were taken. For study purposes, this variable was defined as 'recovery time' and was divided as follows: time-group 1 (≤30 days); time-group 2 (31–60 days); time-group 3 (61–90 days), and time-group 4 (>90 days). We thus ensured that comparisons were made between individuals with similar recovery times.

Data collection and biological sampling Each municipality established a schedule according to their territorial extension and number of reported cases.[23] Working groups were created, bringing together researchers and specialists from multiple polyclinic health areas of different municipalities, and databases of these areas were obtained from the Municipal Hygiene and Epidemiology Divisions, facilitating identification and selection of individuals who met research criteria. They were then visited by genetic counselors and primary healthcare physicians who provided them with information necessary to aid in their decision as to whether to participate in the research.

Interviews were conducted during the morning hours in selected polyclinics. During the interviews, participants received a brief explanation as to the study's purpose and characteristics, signed informed consent forms, and participated in the structured survey. The survey included general personal data, medical history, relevant lifestyle habits and aspects related to the disease and its evolution, as well as the treatment received. This information was used to make definitive assignments to the corresponding study groups, since the databases consulted only provided relevant clinical information that was collected at the time positive diagnosis was made.

Biological samples were also taken during these interviews. People of advanced age, or those who had physical limitations were visited and interviewed in their homes. Blood samples (3 mL) were taken by polyclinic laboratory personnel at least two hours after eating. Samples were extracted via peripheral venous puncture, maintaining aseptic and antiseptic measures, guaranteeing patient safety and sample quality. Samples were stored in Vacutest tubes with the anticoagulant EDTA-K2 (Deltalab, Spain) at 8 ºC and protected from light until processing.

Variable definitions The following hematological variables were analyzed: hemoglobin concentration, platelet cell count, total leukocytes, lymphocytes, neutrophils, eosinophils, basophils and monocytes. Reference values for each variable by age and sex were adopted according to standardized equipment values and international units.[24] Medians and interquartile ranges (IQR) were collected for each variable, according to clinical group and recovery time. Results were classified as normal, low and high, and we used the semiological nomenclature corresponding to each condition.

Hematological studies All hematological studies were performed at the National Medical Genetics Center (CNGM) clinical laboratory (Havana, Cuba), using the BC-6800 Automatic Hematology Analyzer (Mindray, Spain) that performs a differential count of five leukocyte subpopulations.[25] All laboratory techniques were performed according to established operational regulatory procedures and followed good clinical laboratory practices.[26]

Data analysis and processing IBM SPSS, version 22, was used for statistical analysis, and GraphPad Prism, version 7.00 (GraphPad Software, San Diego, USA) was used to prepare the figures.

Odds ratios and 95% confidence intervals (95% CI) Odds ratios with their confidence intervals were calculated to evaluate the association of smoking and the most prevalent variables in medical histories with each hematological variable.

Ethical considerations This study is part of a research project approved by CNGM's Scientific Council and Medical and Research Ethics Committee, and by the Cuban Ministry of Health's Innovation Committee. All participants provided written informed consent. Individuals with cognitive disabilities were represented by their parents, guardians or legal representatives. Participant confidentiality was maintained through data encryption and limited access to information. Individual study results were communicated to patients and their attending physicians to facilitate better patient care and followup.

RESULTS

From March 11 through June 11, 2020, 1183 confirmed positive cases of SARS-CoV-2 infection were diagnosed in Havana.[24] After applying selection criteria, we obtained a sample of 348 individuals, divided into the three clinical groups (Table 1).

Most patients (56.6%; 197/348) had mild–moderate COVID-19 symptoms, and only 7.5% (26/348) presented with severe forms of the disease. Distribution by sex was similar in the three clinical groups, and there were more women in the sample (58.3%; 203/348) (Table 1). Patients who suffered severe forms of the disease tended to be older, and this group also had the highest percentage of pre-existing comorbidities and chronic diseases (data not shown).

Recovery for the vast majority of patients (92%; 320/348) was 31–90 days (time-groups 2 and 3). In the other two time-groups (≤30 days and >90 days) there were few or no cases in at least one clinical group, limiting comparative analysis by clinical severity (Table 1).

Table 1: Characteristics of patients recovered from SARS-CoV-2 infection, by clinical group

Variable	Clinical group			Total N (%) 348 (100.0)
	Asymptomatic n (%) 125 (35.9)	Mild–moderate n (%) 197 (56.6)	Severe n (%) 26 (7.5)	
Age, median (IQR)	44 (30−56)	49 (38−58)	57 (49−70)	49 (34−57)
Sex				
Female	72 (57.6)	116 (58.9)	15 (57.7)	203 (58.3)
Male	53 (42.4)	81 (41.1)	11 (42.3)	145 (41.7)
Pre-existing conditions				
Asthma	18 (14.4)	43 (21.8)	6 (23.1)	67 (19.2)
Cancer	2 (1.6)	4 (2.0)	0	6 (1.7)
Ischemic heart disease	4 (3.2)	4 (2.0)	3 (11.5)	11 (3.2)
Diabetes mellitus	12 (9.6)	24 (12.2)	4 (15.4)	40 (11.5)
Chronic obstructive pulmonary disease	2 (1.6)	4 (2.0)	3 (11.5)	9 (2.6)
Chronic kidney disease	1 (0.6)	1 (0.5)	2 (7.7)	4 (1.1)
Hypercholesterolemia	4 (3.2)	11 (5.6)	2 (7.7)	17 (4.9)
AHT	44 (35.2)	79 (40.1)	17 (65.4)	140 (40.2)
Obesity*	25 (20.0)	44 (22.3)	9 (34.6)	78 (22.8)
Hematological disorders	0	3 (1.5)	0	3 (0.9)
Liver disorders	2 (1.6)	2 (1.0)	0	4 (1.0)
Lifestyle habits				
Smoking	28 (22.4)	20 (10.2)	2 (7.7)	50 (14.4)
Alcohol	65 (52.0)	86 (43.7)	11 (42.3)	162 (46.6)
Coffee	92 (73.6)	146 (74.1)	17 (65.4)	255 (73.3)
Recovery time				
<30 days	0	2 (1.0)	0	2 (0.6)
31−60 days	51 (40.8)	52 (26.4)	9 (34.6)	112 (32.2)
61−90 days	69 (55.2)	122 (61.9)	17 (65.4)	208 (59.8)
>90 days	5 (4.0)	21 (10.7)	0	26 (7.5)

AHT: Arterial hypertension; IQR: Interquartile range; n: Number of patients; %: Percentage related to column total;

*Obesity was determined by calculating body mass index (BMI) from patient weight and height, as reported by patients. Individuals with BMIs >30 were classified as obese.

Hematological parameter results Medians and IQRs of hematological variables by clinical group (asymptomatic, mild–moderate and severe) for those in two of the recovery groups (31−60 day time-group and 61−90 day time group) are shown in Figure 1. With the exception of hemoglobin concentrations in men with a severe form of the disease, values are within reference ranges. However, appreciable differences were found in values of patients who presented severe disease forms when compared to the other two clinical groups (asymptomatic and mild–moderate), in both the 31–60 day time-group and the 61–90 day time-group. The neutrophil/lymphocyte ratio in both time-groups and the hemoglobin concentration in women in the 61–90 day time-group are exceptions; these two variables present similar values in all three clinical groups.

Patients with severe disease presentations had higher numbers of total leukocytes, neutrophils, lymphocytes and monocytes, and lower numbers of eosinophils. These differences were greater in the 31−60 day time group, when comparing the severe group's leukocyte and neutrophil levels with those of the asymptomatic group and the mild–moderate symptomatic group. Differences in lymphocyte and monocyte levels were remarkably large between severe and asymptomatic groups.

Severely-ill patients in the 61−90 day time-group also had higher total leukocyte and neutrophil levels than asymptomatic and mild–moderate symptomatic patients.

In both time-groups, eosinophil levels were somewhat lower in severe patients. Basophil levels were normal and similar in all three clinical groups and at all recovery times (data not shown). On the other hand, platelet counts were higher in the severe group at both recovery times, although striking differences were only observed in the 31−60 day time-group.

Positive hematological variable results analysis Median and IQR variable values fell within reference ranges (except hemoglobin concentrations in men); however, a relatively high percentage of individuals had values outside the reference ranges. Table 2 shows the absolute frequencies and percentages of individuals with values above or below the reference range for each variable according to clinical group and recovery time. In 31−60 day time-group, 15.2% (17/112) presented with anemia, distributed among three clinical groups, compared to 2.4% (5/208) of those in the 61−90 day time-group who suffered from anemia.

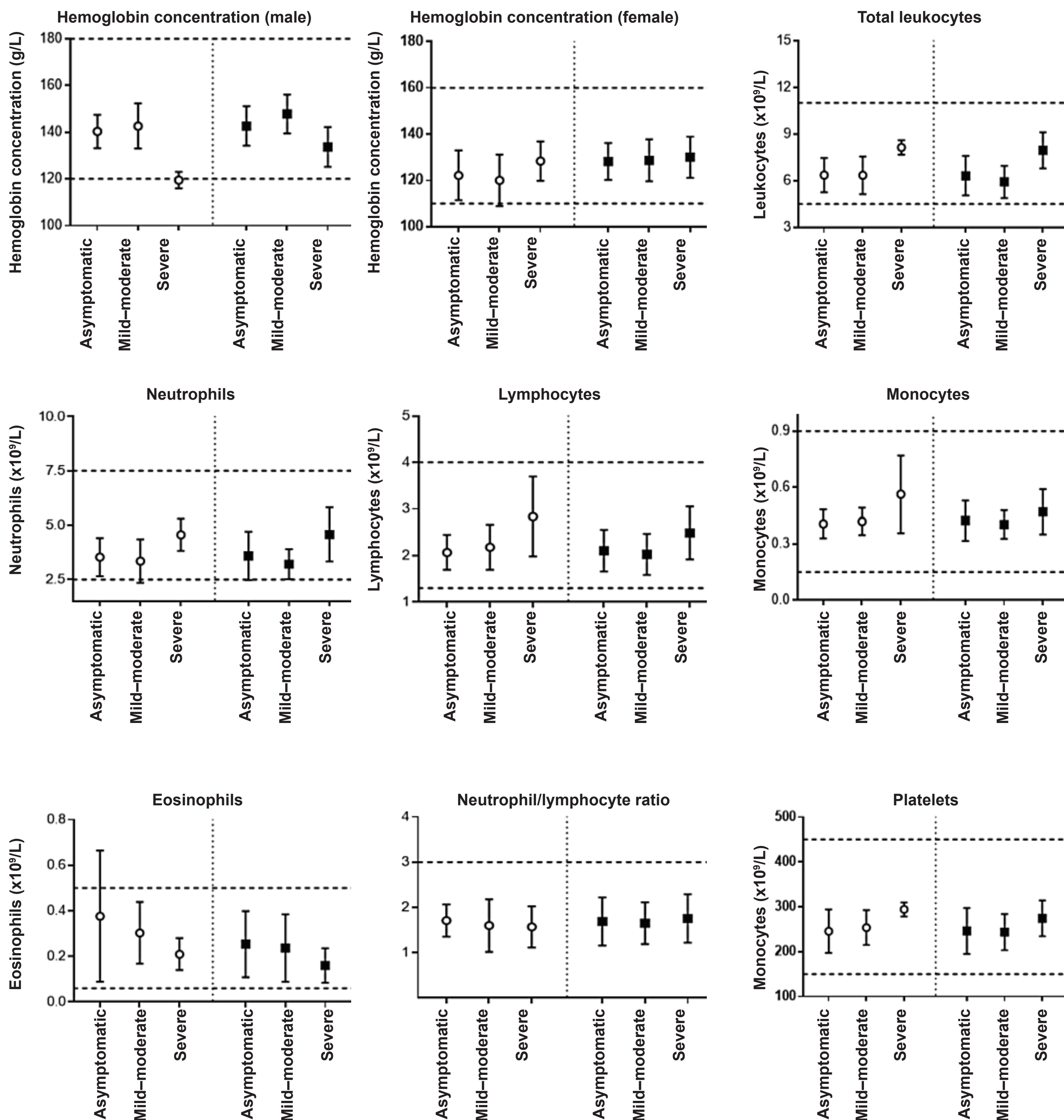
Persons with >90 days of recovery time who presented with asymptomatic SARS-CoV-2 infection had normal hematological values. Only 2 of the 21 individuals with mild–moderate COVID-19 presented leukopenia with neutropenia, and another 2 presented with eosinophilia (values not shown).

Association analysis We calculated odds ratio values (95% CI) to asses association between medical history (bronchial asthma, diabetes mellitus, arterial hypertension, obesity and smoking) with changes in hematological variables (Table 3). Among obese individuals, neutropenia was 2.28 times more frequent than for those in other weight groups. Smokers, however, were less likely to have high eosinophil levels.

DISCUSSION

Scientific publications and case reports on COVID-19 have increased exponentially since the appearance of the first cases in 2019. Reports have characterized COVID-19's infectious agent, the various presentations of the disease and the pathophysiological mechanisms behind the disease's complications;[5,27] treatments and their efficacy;[28,29] and more recently, vaccine development and clinical trials.[30] However, scarcity of labora-

Figure 1: Characteristics of patients recovered from SARS-CoV-2 infection, by clinical group and recovery time (31−60 days and 61−90 days)



*Median values and interquartile ranges for each variable are shown. The horizontal dotted lines represent reference values according to the international unit system. Vertical dotted lines separate patient groups according to recovery time.

○ Recovery time: 31−60 days

■ Recovery time: 61−90 days

Table 2: Absolute frequencies and percentages of patients recovered from SARS-CoV-2 infection, by hematological variable reference values, according to clinical groups and recovery time*

Hematological condition	Recovery time 31−60 days			Recovery time 61−90 days		
	Asymptomatic n = 51	Mild–Moderate n = 52	Severe n = 9	Asymptomatic n = 69	Mild–Moderate n = 122	Severe n = 17
Hemoglobin						
Normal	42 (82.3)	42 (80.8)	8 (88.9)	69 (97.1)	118 (96.7)	16 (94.1)
Low	6 (11.8)	10 (19.2)	1 (11.1)	2 (2.9)	2 (1.6)	1 (5.9)
High	3 (5.9)	0	0	0	2 (1.6)	0
Platelets						
Normal	45 (88.2)	52 (100)	9 (100)	62 (89.9)	113 (92.6)	17 (100)
Low	5 (9.8)	0	0	7 (10.1)	7 (5.7)	0
High	1 (2.0)	0	0	0	2 (1.6)	0
Leukocytes						
Normal	45 (88.2)	39 (75.0)	7 (77.8)	55 (79.7)	102 (83.6)	14 (82.4)
Low	6 (11.8)	10 (19.2)	0	12 (17.4)	19 (15.6)	0
High	0	3 (5.8)	2 (22.2)	2 (2.9)	1 (0.8)	**3 (17.6)**
Neutrophils						
Normal	44 (86.3)	36 (69.2)	8 (88.9)	53 (76.8)	92 (75.4)	13 (76.5)
Low	7 (13.7)	13 (25.0)	0	16 (23.2)	28 (23.0)	2 (11.8)
High	0	3 (5.8)	1 (11.1)	0	2 (1.6)	**2 (11.8)**
Lymphocytes						
Normal	45 (88.2)	51 (98.1)	7 (77.8)	63 (91.3)	111 (91.0)	15 (88.2)
Low	5 (9.8)	1 (1.9)	1 (11.1)	4 (5.8)	11 (9.0)	1 (5.9)
High	1 (2.0)	0	1 (11.1)	2 (2.9)	0	1 (5.9)
Monocytes						
Normal	50 (98.0)	52 (100)	8 (88.9)	68 (98.6)	121 (99.2)	16 (94.1)
Low	0	0	0	0	0	0
High	1 (2.0)	0	1 (11.1)	1 (1.4)	1 (0.8)	1 (5.9)
Eosinophils						
Normal	34 (66.7)	42 (80.8)	7 (77.8)	52 (75.4)	114 (93.4)	13 (76.5)
Low	3 (5.9)	0	2 (22.2)	5 (7.2)	8 (6.6)	2 (11.8)
High	14 (27.4)	10 (19.2)	0	12 (17.4)	17 (13.9)	2 (11.8)
NLR						
<3	50 (98.0)	49 (94.2)	8 (88.9)	60 (87.0)	114 (93.4	13 (76.5)
≥3	1 (2.0)	3 (5.8)	1 (11.1)	9(13.0)	8 (6.6)	4 (23.5)

* Only the results of the 31−60 day time group and the 61−90 day time group, equivalent to 320 individuals of the total 348 (92%) are shown. In the other two time-groups, at least one clinical group appeared as an empty category.

n: Number of patients; NLR: Neutrophil/leukocyte ratio. Values with notably high percentages compared to other groups are highlighted.

tory studies of patients recovered from the disease makes it difficult to compare this study's results.

Our study participants were mostly reintegrated into their family, community and working lives, although they complied with clinical–epidemiological followup through community health services, in accordance with the Cuban protocol for convalescent patient care.[22] The fact that the medians and IQRs of most hematological values fell within reference limits indicates that most study participants had normal values at the time of sampling.

However, it is noteworthy that patients who presented with severe forms of the disease had higher levels of leukocytes, neutrophils and monocytes, decreased hemoglobin concentrations, and decreased eosinophil levels. Severely ill patients were also more likely to have leukocytosis, neutrophilia, eosinophilia, and a higher percentage of them had NLR values greater than 3 at two and three months after clinical discharge. From these results, it could be interpreted that the immunological and inflammatory mechanisms triggered in these patients and that motivated the torpid evolution of the disease continued to be stimulated over time, even after the infection had disappeared.[10,31–33] This is similar to reports by Sherina,[34] which confirm persistence of an immune response eight months after SARS-CoV-2 diagnosis. On the other hand, the group of patients who presented with severe disease also presented with a greater number and likelihood of associated comorbidities, which makes their care more complex and a slower evolution more likely. The influence of age and comorbidities on hematological alterations cannot be ruled out, even when statistical analyses have not shown a clear association.[6,35]

Studies in convalescent patients or in patients recovered from infection have referred mainly to the presence of symptoms, pulmonary alterations, immune responses and, to a lesser extent, changes in hematological parameters. Shaw[36] reported persistence of pathological images in chest tomography and demonstrated that disease consequences can persist for over a month after clinical discharge, including the appearance of other infections and alterations in laboratory findings such as progressive lymphopenia and neutrophilia. Similar results were reported by Sonnwber,[37] who described persistence of symptoms in a group of recovered patients, with decreased capillary pO_2 in 37% of study participants, as well as increased levels of C-reactive protein (12%), IL-6 (6%), procalcitonin (9%), D-dimer (27%) and ferritin (17%), 100 days after the infection was diagnosed. In the present study, only the results of a complete leukogram are available; determinations of specific and other non-specific inflammatory response variables were not performed, preventing comparison of these results with those of other authors.

Zhao[33] found decreased lymphocytes and increased neutrophils in COVID-19 patients in the recovery stage up to four weeks after hospital discharge (similar to influenza infection). They also found increased neutrophil levels in critically ill patients, consistent with our study's results. They conclude that COVID-19 patients have

Table 3: Odds ratios values (95% confidence intervals) between pre-existing conditions and altered hematological variables

Altered hematological variable	Pre-existing condition				
	Asthma	Diabetes mellitus	Arterial hypertension	Obesity	Smoking
Hemoglobin low	1.635 (0.471−5.671)	1.390 (0.314−6.165)	0.493 (0.210−1.159)	1.043 (0.374−2.905)	0.783 (0.255−2.406)
Hemoglobin high	-	1.187 (0.030−1.154)	0.443 (0.073−2.688)	1.158 (0.128−10.512)	0.244 (0.040−1.499)
Leukocytes low	1.101 (0.507−2.393)	3.508 (0.819−15.027)	0.834 (0.456−1.527)	2.336 (0.956−5.708)	1.036 (0.438−2.452
Leukocytes high	0.462 (0.135−1.581)	1.444 (0.182−11.495)	0.940 (0.292−3.024)	1.462 (0.313−6.814)	0.833 (0.177−3.921)
Platelets low	0.655 (0.248−1.730)	1.390 (0.314−6.165)	0.596 (0.255−1.391)	0.639 (0.253−1.613)	3.906 (0.515−29.647)
Neutrophils low	2.131 (0.967−4.695)	2.502 (0.860−7.279)	1.042 (0.612−1.776)	**2.285 (1.079−4.839)**	1.197 (0.552−2.595)
Neutrophils high	0.465 (0.113−1.911)	0.442 (0.089−2.205)	0.837 (0.221−3.175)	2.351 (0.290−19.091)	1.352 (0.165−11.047)
Lymphocytes low	0.950 0.343−2.631)	0.659 (0.214−2.026)	0.846 (0373−1.922)	1.168 (0.424−3.220)	0.872 (0.286−2.656)
Lymphocytes high	-	0.0644 (0.073−5.652)	0.330 (0.060−1.827)	0.978 (0.960-0.996)	0.836 (0.096−7.311)
Monocytes high	1.196 (0.137−10.407	0.250 (0.044−1.411)	0.330 (0.060−1.827)	0.281 (0.056−1.420)	-
Eosinophils low	2.490 (0.568−10.926	2.854 (0.373−21.810)	1.477 (0.586−3.720)	1.321 (0.434-4.026)	1.067 (0.304−3.747)
Eosinophils high	0.997 (0.485−2.046	0.914 (0.383−2.181)	1.564 (0.854−2.865)	0.774 (0.403−1.485)	**0.434 (0.216−0.870)**
NLR high	0.821 (0.318−2.121	0.415 (0.156−1.099)	0.600 (0.273−1.319)	1.012 (0.394−2.602)	0.962 (0.318−2.909)

NLR: Neutrophil/leukocyte ratio
Confidence intervals that did not include the value 1, which represents no association, are highlighted.

decreased antiviral immunity and increased anti-inflammatory responses, which are maintained during the recovery stage.

Rodriguez[31] reported that increases in NLR during the disease's acute phase undergo a slow reversal during recovery. These results are similar to those of our study and could reinforce the hypothesis that inflammatory and immunological processes are stimulated in patients with more severe forms of the disease.

Although variables related to medications used during the disease's acute stage were not analyzed in this study, treatments for patients were uniform and governed by approved protocols established for the entire country, even for patients who had asymptomatic forms of the disease. These protocols include the use of steroids in intensive care units.[22] Medical literature reports an association between leukocytosis and steroid treatments, especially with high doses. These treatments can cause extreme and persistent leukocytosis, which may be associated with monocytosis, neutrophilia, lymphopenia and eosinopenia, conditions seen in the severe-illness group.[38,39]

The low levels of hemoglobin concentration in men, mainly in the 31−60 day time group, also seem to be related to the processes associated with infection. It is noteworthy that individuals with a longer recovery time (both men and women) had higher hemoglobin concentrations and a lower likelihood of anemia. One possible conjecture is that a longer recovery time led to the removal of factors—including direct alterations to iron metabolism or distribution—that were still present in shorter recovery periods.[40–42]

Identifying the causes or factors related to the eosinophil alterations found in this study is difficult, as these alterations were found not only in the severe group, but also the asymptomatic group, which included younger people and high percentages of eosinophilia. In this case, eosinophilia's various causes (and presence of intestinal parasites or allergies, common in this age group) must be ruled out by complementary testing.

Fraisse[21] suggests that COVID-19 may be either directly or indirectly responsible for eosinophilia, resulting from recovery mechanisms activated by a hyperstimulated immune system during the so-called 'cytokine storm.' These mechanisms could be influencing the high percentages of eosinophilia observed in our study. Liu[43] links eosinophilia to therapies combining lopinavir and interferon alpha 2b, and Mateos González[19] reported a relationship between eosinophilia and prescribed COVID-19 treatments involving lopinavir, ritonavir, azithromycin or low-molecular-weight sodium heparin.

One of the striking study results is the paradoxical negative association between smoking and high eosinophil levels. This directly contradicts previous reports by Hartl,[44] who found high levels of eosinophils in smokers in a study of over 11,000 Australians, and Caspard,[45] who reported elevated levels of peripheral blood eosinophils in current and former smokers with asthma, compared with never-smokers.

Exposure to tobacco and cigarette smoke causes alterations to airways and lung parenchyma, with direct cellular damage to the alveolar epithelium and other cells, leading to a localized inflammatory response that recruits other immune system cells, including eosinophils.[46] The negative association between smoking and high eosinophil levels found in this study may be due to direct acute damage caused to lung tissue by the virus, damage exacerbated by smoking, resulting in localized eosinophil recruitment to the lungs and lower circulating eosinophil levels in peripheral blood.

One limitation of this study is its cross-sectional characterization of recovered individuals, and the lack of comparison to the acute phase of the disease. There is also no information as to the status of the measured variables before infection, so some of the study's results could be related to pre-existing disease or

concomitant inflammatory or infectious processes (which could be undiagnosed). It is also not possible to rule out infections or other inflammatory processes in the period between the acute phase of the disease and the time of the study. Additionally, only complete leukograms were considered, and we did not examine other specific or non-specific inflammatory response variables. Another limitation of this study was the use of a structured survey to obtain information, which is prone to errors and recall bias, especially in individuals with severe forms of the disease.

However, the results presented provided evidence which can aid in developing improved strategies for followup care of patients recovering from COVID-19 in Cuba and their multidisciplinary management.

CONCLUSIONS

In persons recovered from SARS-CoV-2 infection, hematological changes and their relation to disease clinical severity suggest persistence of systemic changes—possibly inflammatory—associated with viral infection. Identification and characterization of such changes facilitate personalized COVID-19 followup care and rehabilitation.

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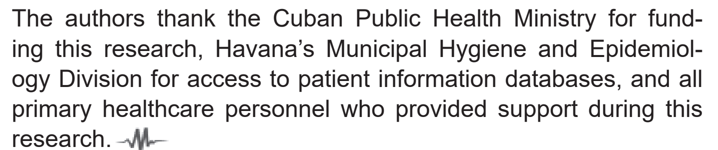

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Evaluation of SARS-CoV-2 Rapid Antigen Tests in Use on the Isle of Youth, Cuba

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ABSTRACT

INTRODUCTION The use of various diagnostic techniques is increasingly common in pandemic scenarios. It is important to update evaluations of their metric properties in different times and settings.

OBJECTIVE Evaluate metric properties of a SARS-CoV-2 rapid antigen test relative to a reference standard.

METHODS We carried out a prospective evaluation study of the SARS-CoV-2 rapid antigen test as compared to the RT-PCR test, which is considered the reference standard. Our sample was comprised of 778 individuals, and we calculated sensitivity, specificity, predictive values, prevalence and validity indices.

RESULTS Of the total 778 samples, 70 were true positives, 658 were true negatives, and 27 were false negatives when compared to RT-PCR test results. We obtained a sensitivity of 75.3% (95% CI = 65.96–84.50); a specificity of 96.1% (95% CI = 94.53–97.59); 72.2% for positive predictive value, and 96.6% for negative predictive value. The estimated prevalence was 11.9% and the validity index was 93.6%.

CONCLUSIONS The index values validate use of the SARS-CoV-2 rapid antigen test until prevalence falls below 2.5%, since as SARS-CoV-2 infection prevalence decreases, so does the predictive value of the PCR result.

The use of the SARS-CoV-2 rapid antigen test on the Isle of Youth, Cuba, was decisive in the pandemic's clinical–epidemiological management.

KEYWORDS SARS-CoV-2, COVID-19, antigens, validation study, sensitivity and specificity, Cuba

INTRODUCTION

The COVID-19 health emergency, now recognized as a global pandemic, had its turning point on February 23, 2020, when the city of Wuhan—where the first cases were reported—was put into quarantine.[1] The virus continues to spread at an accelerated pace, due to efficient transmission not only by symptomatic, but also by asymptomatic and presymptomatic persons as well.[1,2] New and more contagious variants appeared after this study was concluded, and the proportion of symptomatic patients has also increased.

The world's scientific community has raced to find solutions to the problems posed by the SARS-CoV-2 virus in diagnosis, treatment and prevention.[3] Thus, over the course of the pandemic, efforts in various fields have yielded more or less effective therapies, including monoclonal antibodies, as well as preventive vaccines based on various platforms. Numerous and diverse diagnostic tests have also appeared on the market, each aimed at identifying infected patients as soon as possible.

Control strategies for diagnostic testing have been based on molecular detection of viral RNA in respiratory samples; and in most available commercial assays, by reverse-transcriptase polymerase chain reaction (RT-PCR). RT-PCR is used as a reference technique due to its sensitivity and specificity for detecting respiratory viruses.[2] However, rapid serological marker tests also support diagnosis, based on detection of antigens and antibodies: the former are qualitative tests that only express one result (positive or negative), while the latter yield a quantitative measurement of circulating immunoglobulins (IgG), the amount depending on the individual patient's infectious phase.[4]

IMPORTANCE

This study provides recent information on fundamental attributes of a rapid antigen test for SARS-CoV-2 infection in an epidemiological scenario with particular geographic and epidemiological characteristics.

South Korea, calling on extensive experience in other epidemic outbreaks like SARS1 in 2015, is one of the countries that carried out the largest number of tests per population, in the process demonstrating that one of the best strategies for controlling the epidemic was massive testing. This strategy has been implemented by a number of countries in their fight against the COVID-19 pandemic.[5] And "test, test and test" has been WHO's recommendation to countries worldwide in handling the COVID-19 pandemic.[6]

The values that manufacturers of diagnostic tests include in their products come from patients in reference hospitals under ideal conditions which should not be extrapolated to other populations or real-world situations.[6] Therefore, tests should be chosen based on reliability and validity, measured in terms of their sensitivity, specificity, and predictive values (positive and negative) obtained from open populations.[7,8]

WHO has included in its Emergency Use Listing of COVID-19 diagnostic tools some general considerations about rapid antigen tests, as well as several assays;[9] however, objective quantitative evaluations of their metric properties under field conditions are still needed. This is our primary goal in evaluating the rapid COVID-19 antigen test used on the Isle of Youth, Cuba, an area whose geographical location and clinical–epidemiological man-

agement strategy had an impact and marked a milestone in the management and containment of the disease.

The Isle of Youth is a Special Municipality south of Cuba's main island and is bordered to the north by the Gulf of Batabanó, to the east by waters shared by Matanzas province, and to the south and west by the Caribbean Sea. These unique characteristics mean that the island can only be reached by air or sea. At the beginning of the pandemic, navigation was restricted to cargo transport with strict control at points of entry, and travelers flying in were under close surveillance upon arrival and were isolated for several days in designated quarantine centers. The same procedures were applied to anyone arriving by sea, which limited movement to areas of highest risk and minimized access to the territory.

Compliance with epidemic control measures was easier in a territory with only 83,479 population and a population density of 37.9 inhabitants per km^2. Although a localized epidemic in this context would translate into high incidence and lethality rates, containment was less complicated due to both the low number of inhabitants and the restriction of access routes into the territory; so, everything that occurs on the island would be controllable, theoretically, once the transmission chain was interrupted.

The Isle of Youth's demographic characteristics—together with timely diagnosis of SARS-CoV-2 infection using the rapid antigen test—allowed epidemiological control actions to be carried out practically in real time, which positively impacted the COVID-19 epidemic's containment, with much more favorable results than those exhibited by Cuba as a whole at that time.

The objective of this study was to evaluate metric properties of a SARS-CoV-2 rapid antigen test relative to a reference standard under field conditions.

METHODS

Study design and participants We carried out a prospective study to evaluate the SARS-CoV-2 rapid antigen test, comparing it with the RT-PCR reference standard.

Procedures All persons presenting with COVID-19–suggestive symptoms detected during January–April 2021 (who gave their consent to participate) were included in the study (778 in total). They were identified in doctor's visits aimed at detecting cases or as a result of epidemiological controls—a strategy in which passive, active and specialized surveillance was combined during the epidemic. All patients underwent both a rapid antigen test and RT-PCR testing. This period corresponded to a local transmission phase in the area during the second wave of the epidemic in Cuba.

Statistical analysis examined point and interval estimates at 95% of the tests' operational characteristics with respect to the reference standard (sensitivity, specificity, predictive values, and validity indices; the latter defined as the total percentage of coin- cidences between tests).

Study test descriptions

Reference test (gold standard) RIDAGENE SARS-CoV-2 (R-Biopharm, Germany) was used in this study; it is a real-time multiplex RT-PCR for direct quantitative detection of SARS-CoV-2 RNA from oropharyngeal or nasopharyngeal swabs from individuals with symptomatic respiratory infections.

Rapid test The SARS-CoV-2 rapid antigen test used (SD BIOSENSOR, INC., South Korea) is a rapid immunochromatographic test for qualitative detection of specific SARS-CoV-2 antigens present in the nasopharynx. The manufacturer's clinical evaluation of the test's sensitivity and specificity were 96.52% and 99.68%, respectively.

Sampling Two nasopharyngeal exudate samples were collected from each patient whose clinical manifestations were suggestive of COVID-19. Appropriate protective measures were used during collection, and sampling was carried out by trained personnel. One sample was used to detect the SARS-CoV-2 antigen (processed in situ in laboratories designated for this purpose on the Isle of Youth, following manufacturer instructions), and one was used for RT-PCR, kept at –20 °C, and sent to Cuba's National Reference Laboratory at the Pedro Kourí Tropical Medicine Institute (IPK) in Havana for processing.

Ethics This study was approved by the municipal ethics commission and written informed consent was obtained from all participants.

RESULTS

We obtained rapid antigen tests and RT-PCR tests for SARS- CoV-2 for all 778 individuals. Of the total 778 sample-pairs, 70 (9.0%) were positive and 658 (84.6%) negative by both tests (Table 1).

Sensitivity was high (75.3%; 95% CI = 66.0–84.6) but lower than specificity (96.1%; 95% CI = 94.5–97.6) (Table 2). According to the positive predictive value, we can estimate a 72.2% probability of viral infection if the test is positive. If, on the other hand, the test is negative, there is a probability of 0.97% of not being actually infected. Estimated prevalence was 11.9% and the validity index was 93.6%. Concordance between the two tests, given by the validity index, was remarkably high, as are negative predictive value and specificity (Table 2).

Table 1: Contingency table for calculating evaluation indicators for BIOSENSOR's SARS-CoV-2 rapid antigen test

Antigen test	RT-PCR		Total
	Positive results	Negative results	
Positive results	70	27	97
Negative results	23	658	681
Total	93	685	778

RT-PCR: reverse-transcriptase polymerase chain reaction

Table 2: SARS-CoV-2 rapid antigen test evaluation criteria; Isle of Youth, January–April 2021

Criteria	SARS-CoV-2 Rapid Antigen Test	95% CI
Sensitivity	75.3%	66.0–84.6
Specificity	96.1%	94.5–97.6
PPV	72.2%	62.7–81.6
NPV	96.6%	95.2–98.1
Validity index	93.6%	91.8–95.4
Positive results by RT-PCR	11.9%	9.6–14.3

CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value; RT-PCR: reverse-transcriptase polymerase chain reaction; N = 778

DISCUSSION

This study was carried out in an unfavorable epidemiological context, during the second wave of the COVID-19 epidemic on the Isle of Youth, when the incidence rate reached 54.5 cases per 10,000 population—the highest in the country.

Many factors favored eventual epidemic control, but rapid antigen testing facilitated testing practically in real time—the utility of which was documented 6 months later when the territory reduced its cumulative incidence rate to 7.7 cases per 10,000 population; an average of 10 cases per month, all of which were sporadic and most of which were detected at the territory's points of entry. These results made the Isle of Youth an interesting example among municipalities concerning pandemic control actions and results.

The rapid antigen test's sensitivity and specificity were lower than those reported by the manufacturer, likely due to differences between the ideal conditions in which manufacturers test and validate their products and conditions prevalent in the field. However, our results are similar to those of other evaluations, including those of a study carried out in Mallorca, Spain, which included patients with symptoms suggestive of infection in remission reported by family physicians, or patients with previous contact with infected individuals whose infections were confirmed by RT-PCR, in which the Panbio (Abbott Rapid Diagnostic Jena GmbH, Jena, Germany) test's overall sensitivity and specificity were 71.4% and 91.8%, respectively.[10]

A study of symptomatic patients in the Netherlands based on the Roche/SD Biosensor rapid antigen test showed higher sensitivity and specificity than this investigation, at 84.9% and 99.5%, respectively.[11]

BinaxNOW's rapid antigen test[12] had very high specificity in both adults and children (at 100%) and high sensitivity in adults with recent symptoms (96.5%).

Rapid antigen testing under tents in a plaza in an urban environment, specifically San Francisco's Mission District (California, USA)—a setting of ongoing community transmission—reported 100% sensitivity and 97% specificity in adults who were ≤7 days from symptom onset.[13] Another investigation using this test conducted in Oshkosh (Washington, USA) demonstrated a sensitivity of 78.6% and a specificity of 99.8% in 1188 symptomatic patients after ≤7 days of clinical evolution.[14]

In Germany, the performance of a rapid antigen test (RAT) VivaDiag SARS-CoV-2 Ag Rapid Test Device (VivaCheck Biotech [Hanghzou] Co., Ltd., Hangzhou, China) in everyday clinical practice was assessed in all hospitalized patients at the Helios University Hospital Wuppertal, as well as their accompanying relatives at the Children's Hospital, resulting in a sensitivity and specificity of 27.5% and 99.6%, respectively. Sensitivity varied by group and was higher in symptomatic patients (52.9%) than in asymptomatic patients (20.6%), while specificity, at 99.6%, was the same in both groups.[15] This cohort's remarkably low sen- sitivity contrasts with that reported by WHO for the same rapid test (at 75.1%), which was similar to that in our study.[16] A study of patient samples from three hospitals in Pinar del Río Province and IPK in Cuba using the rapid antigen test SD BIOSENSOR ROCHE DiagnosticGmbH found a sensitivity of 80% for symptomatic and 61% of asymptomatic patients, and a specificity of 92% for both groups.[17]

Although all studies report high specificity values in the tests' ability to detect antigens, the tests' overall performance varies and appears to be highly dependent on brand and context. In general, rapid antigen tests are much more specific than sensitive, and exhibit better metric performance in asymptomatic cases, as documented by authors in various countries (Austria, France, Brazil, Italy, Chile and India), whose research consistently shows specificity values of 97%–100%.[18–23]

The vast majority of studies discussed in this paper meet or at least approach WHO performance requirements for rapid SARS-CoV-2 antigen tests (sensitivity ≥80% and specificity ≥97%).[24,25] The results of our study support the rapid antigen test's diagnostic use, at least until SARS-CoV-2 prevalence dips below 2.5%.[11]

The use of rapid antigen testing is further recommended in settings where molecular testing is limited or unavailable, or where it is only available with long turnaround times.[9] This last circumstance was the case on the Isle of Youth, when it was necessary to send samples to IPK, and results could take up to 72 hours.

WHO has updated its information regarding PCR diagnostic testing in the context of the COVID-19 pandemic, and notes "as the prevalence of SARS-CoV-2 infection decreases, so does the positive predictive power of the PCR result." In this study, we estimated 11.9% prevalence in people suspected of infection; which is not considered 'low' but could explain the low positive predictive value. However, infection prevalence in the population during the study period is below the prevalence of expected cases, and if the test is performed on people with no suspicion of disease who had no infected contacts, any positive result has a high probability of being a false positive,[26] so rapid diagnostic tests are not recommended in populations in which expected disease prevalence is low.[27,28]

According to a report from the European Center for Disease Prevention and Control (ECDC), rapid testing is recommended to evaluate individuals regardless of symptoms in settings where the proportion of positive tests is suspected to be ≥10%.[27]

Several studies consulted[10,29,30] showed positive predictive values (PPV) of 100% in symptomatic cases; much higher than the results of this study. For example, Gili found an overall prevalence of 42%, a PPV of 88.0% and a negative predictive value (NPV) of 100%.[30] Thirion-Romero reported 44.5% prevalence, with a resulting 96.8% PPV and a 73.8% NPV. Both studies reported prevalences higher than those in our research.[31]

Other authors, including Bulilete,[10] found 12.7% prevalence in symptomatic cases with ≤5 days of clinical evolution, and an NPV of 97.5%; so at least 2 of every 100 cases would result in false negatives. Pollock also showed a 12.7% prevalence, with NPVs for both symptomatic and asymptomatic cases of 96.8%,[12] while Igloi[11] demonstrated a 19.2% prevalence and an NPV of 96.5%—similar to the NPV reported in this study. Of these studies, therefore, approximately 3 in 100 tests will

yield false negatives (and result in epidemiological consequences).

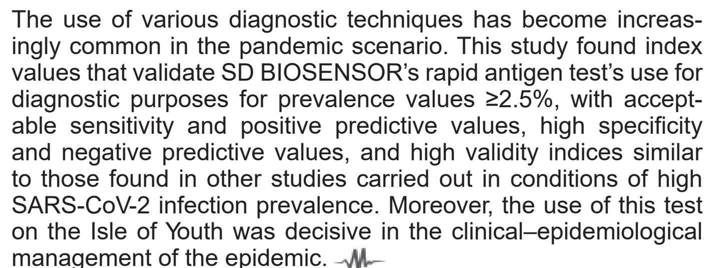
No diagnostic test is perfect. Despite the good metric properties of antigen-detecting techniques, diagnoses must be corroborated by molecular methods, monitoring compliance and other factors during preanalytical stages to minimize the risk of false negatives.

Study limitations The main limitation of this study was failure to explore test performance in different scenarios: asymptomatic and symptomatic, including analyses according to cycle threshold (Ct). The study was limited to symptomatic cases and did not stratify the sample according to different clinical evolution times or epidemiological categories, such as direct and indirect contact. Therefore, it was not possible to make any inferences as to the test's applicability in other contexts, like screening populations in circumstances where transmission is expected, in border control, or in workplaces, all of which have different vulnerabilities.

Further research is merited to explore test performance in asymptomatic and symptomatic individuals according to Ct values and epidemiological and clinical strata.

CONCLUSIONS

The use of various diagnostic techniques has become increasingly common in the pandemic scenario. This study found index values that validate SD BIOSENSOR's rapid antigen test's use for diagnostic purposes for prevalence values ≥2.5%, with acceptable sensitivity and positive predictive values, high specificity and negative predictive values, and high validity indices similar to those found in other studies carried out in conditions of high SARS-CoV-2 infection prevalence. Moreover, the use of this test on the Isle of Youth was decisive in the clinical–epidemiological management of the epidemic.



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Original Research

Community-Acquired Uropathogenic *Escherichia coli*, Antimicrobial Susceptibility, and Extended-Spectrum Beta-Lactamase Detection

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ABSTRACT

INTRODUCTION Urinary tract infection is the second-leading reason for consults in primary health care. Bacterial urinary tract infections are the most common, of which *Escherichia coli* is the main etiologic agent. Antimicrobial resistance and multidrug resistance complicate effective community treatment, especially if resistance is caused by extended-spectrum beta-lactamase production. WHO recommends that antimicrobial susceptibility be evaluated in different regions of the world at different times. Community-acquired *E. coli*'s susceptibility to colistin has not yet been studied in Cuba, and mcr-1 gene screening is necessary.

OBJECTIVE Evaluate community-acquired uropathogenic *E. coli* isolates' susceptibility to antibiotics, including colistin, and identify extended-spectrum beta-lactamase–producing bacteria.

METHODS We conducted a descriptive cross-sectional study that included 281 community-acquired uropathogenic *E. coli* isolates (153 from the Isle of Youth Special Municipality's Hygiene, Epidemiology, and Microbiology Center and 128 from Microbiology Laboratories of 7 institutions in Havana) from June 2016 through July 2018. We used the disk diffusion method to determine susceptibility to ampicillin, ampicillin/sulbactam, cefazolin, trimethoprim/sulfamethoxazole, ciprofloxacin, nitrofurantoin and fosfomycin. The disk elution method was used to determine susceptibility to colistin. The combined disk method was used to identify extended-spectrum beta-lactamases. Estimates were made regarding the frequency and percentages of antimicrobial susceptibility and resistance, as well as multidrug-resistance patterns.

RESULTS Of the 281 isolates, 68.3% (192/281) were resistant to ampicillin, 54.8% (154/281) were resistant to ciprofloxacin, and 49.5% (139/281) were resistant to trimethoprim/sulfamethoxazole. Resistance to colistin was not detected. On the other hand, 14.2% (40/281) were susceptible to the 8 antibiotics we evaluated, 22.1% (62/281) showed resistance to only 1 antibiotic, and 63.7% (179/281) were resistant to 2 or more antibiotics. In the extended-spectrum beta-lactamase determination, 34.5% (97/281) had inhibition zones ≤14 mm with cefazolin. Of those with inhibition zones, 64.9% (63/97) were positive in the phenotype test, and 35.1% (34/97) were negative. In extended-spectrum beta-lactamase–producing bacteria, 1.6% (1/63) were resistant to fosfomycin, and 3.2% (2/63) were resistant to nitrofurantoin. The most common multidrug-resistance pattern (22.9%; 30/131) was to ampicillin/sulbactam, ampicillin, cefazolin, ciprofloxacin, and trimethoprim/sulfamethoxazole.

CONCLUSIONS Uropathogenic *E. coli* resistance to the antibiotics most frequently used in community medical practice is quite common, and extended-spectrum beta-lactamase–producing bacteria is the mechanism for beta-lactam antibiotic resistance. Multidrug-resistance patterns include resistance to the antibiotics most used in community-acquired infections. Fosfomycin and nitrofurantoin are the most active in extended-spectrum beta-lactamase producing bacteria. All the isolates were susceptible to colistin.

KEYWORDS Uropathogenic *Escherichia coli*, urinary tract infections, microbial susceptibility tests, Cuba

INTRODUCTION

Urinary tract infections (UTIs) are one of the leading reasons for consults in primary health care. The US Centers for Disease Control and Prevention defines UTIs as common infections occurring when bacteria, often from the skin or rectum, enter the urethra and infect the urinary tract.[1]

The most common UTI cause is bacterial infection, and according to microbiology studies, *Escherichia coli* is the enterobacteria responsible for ≥80% of cases.[2] Sometimes symptoms do not disappear after treatment, possibly due to patient risk factors, the microorganism's virulence and pathogenicity, or bacteria's growing resistance to the antibiotics most often used in treatment.[3]

IMPORTANCE This work highlights the need to conduct sensitivity tests before treating urinary tract infections caused by *E. coli*, due to increasing antimicrobial resistance in Cuba.

The most common practice for treating community-acquired UTIs is prescribing treatment without prior microbial identification. However, due to increasing antibiotic resistance, this approach is not very effective.[4] Extended-spectrum beta-lactamase (ESBL) production is the mechanism most often associated with multidrug resistance in gram-negative bacilli, especially in *E. coli*,[3] and increased incidence in community isolates has been reported.[4]

The prevalence of multidrug-resistant uropathogenic *E. coli* varies by geographic region.[5] Monitoring its susceptibility to antibiotics helps detect variations in usual susceptibility patterns and assists

in properly treating UTIs by decreasing treatment failures due to antibiotic resistance.[3]

In 2015, a mechanism for transferable colistin resistance was first detected—mediated by the mcr-1 gene in *E. coli* and *Klebsiella pneumoniae* isolates collected from hospitalized patients, animals and raw meat.[6] For this reason, WHO recommends screening for this resistance mechanism, and the Latin American and Caribbean Network for Antimicrobial Resistance proposes evaluating country-specific *E. coli* community isolates' susceptibility to colistin.[7,8]

In Cuba, studies show a high prevalence of UTI-causing *E. coli* that are resistant to the most common antibiotics used in medical practice and community circulation of ESBL-producing isolates.[9,10] However, research including different health institutions and geographic areas in the country is lacking. Furthermore, there are no reports about uropathogenic *E. coli*'s susceptibility to colistin in community isolates as a preliminary step to mcr-1 gene screening.

The purpose of this research was to evaluate uropathogenic *E. coli*'s susceptibility to antibiotics (including colistin) in community-acquired isolates, and to identify ESBL-producing bacteria.

METHODS

Design and sample We performed a descriptive cross-sectional study including 281 community-acquired UTI-causing *E. coli* isolates; 153 were collected by the Isle of Youth Special Municipality's Center for Hygiene, Epidemiology, and Microbiology, and 128 were collected from microbiology laboratories in several Havana hospitals from outpatients with community-acquired infections (namely the Calixto García, Manuel Fajardo, Enrique Cabrera, Ramón González Coro, William Soler and Freyre Andrade hospitals, and the Pedro Kourí Tropical Medicine Institute's [IPK] Clinical Microbiology Laboratory). Samples were collected from June 2016 through July 2018 and were studied in the National Reference Laboratory for antimicrobial resistance surveillance in infection-related pathogens associated with health care, at IPK in Havana.

Antimicrobial susceptibility Antimicrobial susceptibility was determined using the Kirby-Bauer technique in Mueller-Hinton agar,[11] except for susceptibility to colistin, which was studied using the colistin disk elution method in cation-adjusted Mueller-Hinton broth, according to the protocol recommended by the National Infectious Disease Institute at Dr. Carlos G. Malbrán National Laboratory and Health Institute Administration (INEI-ANLIS), Argentina, in 2017,[12] and susceptibility to fosfomycin, which was studied using Müeller-Hinton agar supplemented with glucose-6-phosphate.[13] The antibiotics studied were ampicillin/sulbactam (gradient strips 0.016–256 μg/L), ampicillin (0.016–256 μg/L), cefazolin (30 μg), ciprofloxacin (gradient strips 0.0032–32 μg/L), fosfomycin (200 μg), nitrofurantoin (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg) and colistin (10 μg). Disks and concentration gradient strips were sourced from Liofilchem (Italy). Results were interpreted according to the Clinical and Laboratory Standards Institute's (CLSI) 2019 standards,[14] USA, except for colistin results, which were interpreted according to the Antimicrobial Resistance Surveillance Network's (WHONET-Argentina) protocol.[7]

E. coli isolates' microbial susceptibility were rated as follows: 'sensitive' (antimicrobial activity level is associated with a high probability of therapeutic success); 'intermediate' (a high probability of therapeutic success because exposure to the agent is increased by either dosage or concentration at the site of infection); or 'resistant' (antimicrobial activity level is associated with a high probability of therapeutic failure).[7,14]

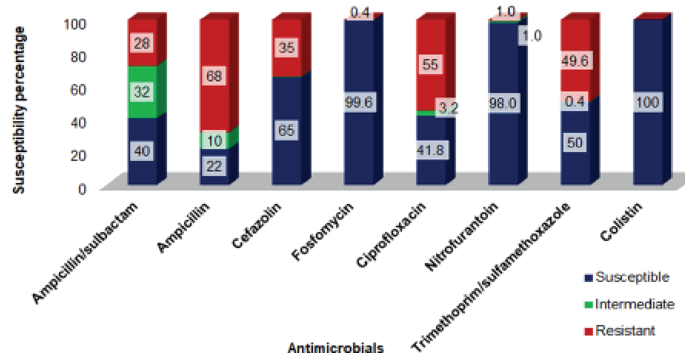
ESBL detection Cefazolin disks were used as predictors of susceptibility to oral cephalosporins based on the WHONET's 2017 protocol.[7] For all isolates with inhibition zones ≤14 mm for this antibiotic, the ESBL-producer phenotype was confirmed using the combined disk method: 1) ceftazidime (30 μg) and ceftazidime/clavulanic acid (30/10 μg), and 2) cefotaxime (30 μg) and cefotaxime/clavulanic acid (30/10 μg) (Liofilchem, Italy).[14] The *K. pneumoniae* ATCC 700603 strain was used as a positive control, and the *E. coli* ATCC 25922 strain was used as a negative control.

Statistical analysis Descriptive statistical measures (frequencies and percentages) were used to analyze isolates' antimicrobial susceptibility profiles, multidrug-resistance patterns and ESBL production.

RESULTS

Of the 281 uropathogenic *E. coli* isolates, 68.3% (192/281) were resistant to ampicillin, 54.8% (154/281) to ciprofloxacin and 49.5% (139/281) to trimethoprim/sulfamethoxazole. The percentages of isolates resistant to cefazolin (34.5%; 97/281) and to the ampicillin/sulbactam combination (28.1%; 79/281) were considered high, as they were above 20%. Figure 1 shows susceptibility and resistance percentages based on whether they were susceptible, intermediate or resistant.

Figure 1: Susceptibility to antimicrobials of *Escherichia coli* isolates causing community-acquired urinary tract infections (n = 281)



Pedro Kourí Tropical Medicine Institute, 2016–2018

The percentages of fosfomycin-resistant isolates and nitrofurantoin-resistant isolates were low (Figure 1). Resistance to colistin was not found, even though colistin was only evaluated in order to start mcr-1 gene screening (Figure 1). Percentages of resistant isolates were quite similar between Havana and the Isle of Youth (Table 1).

Of the isolates, 14.2% (40/281) were susceptible to the 8 antibiotics evaluated, 22.1% (62/281) were resistant to only 1 antibiotic, and 63.7% (179/281) were resistant to 2 or more antibiotics. Resistant isolates had 24 different antibiotypes, 10 of which include 1 or 2 antibiotic classes, and 14 include ≥3 classes. They were therefore considered multidrug-resistant (MDR) patterns.

Table 1: Percentage of uropathogenic *Escherichia coli*-resistant isolates, by geographic area

Antibiotic	Percentage of resistant uropathogenic *E. coli* isolates	
	Havana (n = 128)	Isle of Youth (n = 153)
	n (%)	n (%)
AMS	32 (25.0)	47 (30.7)
AMP	86 (67.2)	106 (69.3)
CFZ	37 (28.9)	60 (39.2)
FOS	0 (0.0)	1 (0.7)
CIP	78 (60.9)	76 (49.7)
SXT	71 (55.5)	68 (44.4)
NIT	2 (1.6)	1 (0.7)

AMP: Ampicillin; AMS: Ampicillin/sulbactam; CFZ: Cefazoline; CIP: Ciprofloxacin; FOS: Fosfomycin; NIT: Nitrofurantoin; SXT: Trimethoprim/sulfamethoxazole

Table 2: Multi-drug–resistant *Escherichia coli* strains in community-acquired urinary tract infections

Multi-drug–resistant isolates n (%)	Multi-drug resistance patterns
30 (22.9)	AMS, AMP, CFZ, CIP, SXT
24 (18.3)	AMP, CFZ, CIP, SXT
21 (16.0)	AMP, CIP, SXT
13 (9.9)	AMS, AMP, CFZ, CIP
10 (7.6)	AMP, CFZ, CIP
10 (7.6)	AMS, AMP, CIP, SXT
8 (6.1)	AMS, AMP, SXT
5 (3.8)	AMS, AMP, CFZ, SXT
4 (3.1)	AMS, AMP, CIP
2 (1.5)	AMP, CIP, SXT
1 (0.8)	AMS, AMP, CFZ
1 (0.8)	AMS, AMP, CFZ, CIP, SXT, NIT
1 (0.8)	AMS, AMP, CIP, NIT
1 (0.8)	CFZ, FOS, NIT
Total: 131 isolates	14 patterns

AMP: Ampicillin; AMS: Ampicillin/sulbactam; CFZ: Cefazoline; CIP: Ciprofloxacin; FOS: Fosfomycin; NIT: Nitrofurantoin; SXT: Trimethoprim/sulfamethoxazole

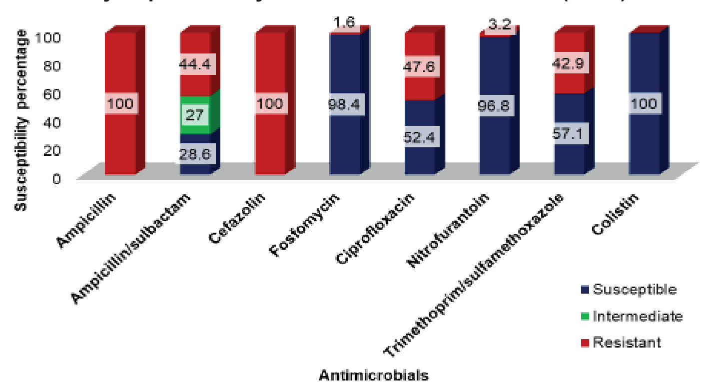
Of the MDR patterns, 4 were found in a single isolate, and 6 were repeated in 2 or more isolates. Most MDR patterns demonstrate resistance to ampicillin, ciprofloxacin and trimethoprim/sulfamethoxazole (Table 2).

In the ESBL determination, 34.5% (97/281) of isolates had inhibition zones ≤14 mm for cefazolin. Of those with inhibition zones, 64.9% (63/97) were positive in the phenotype test and 35.1% (34/97) were negative. All (63/63) of the positive ESBLs were resistant to cefazolin and ampicillin, 44.4% (28/63) were resistant to the ampicillin/sulbactam combination, 47.6% (30/63) to ciprofloxacin, and 42.9% (27/63) to trimethoprim/sulfamethoxazole. Fosfomycin and nitrofurantoin had the best in vitro activity in ESBL-positive uropathogenic *E. coli* isolates; 1.6% (1/63) of the isolates were resistant to fosfomycin, and 3.2% (2/63) to nitrofurantoin. We subdivided the susceptibility and resistance percentages based on whether they were susceptible, intermediate or resistant (Figure 2).

DISCUSSION

Physicians have historically prescribed antibiotics for UTIs without conducting microbiology studies to identify the causal bacteria, but emerging antibiotic resistance is increasingly limiting this practice.

Figure 2: Susceptibility of ESBL-producing *Escherichia coli* isolates causing community-acquired urinary tract infections to antimicrobials (n = 63)



Pedro Kourí Tropical Medicine Institute, 2016–2018

UTI treatment guidelines advise refraining from antibiotic use without susceptibility studies if the local resistance rate is greater than 20%.[5] For this reason, data on bacterial resistance patterns to antibiotics commonly used to treat UTIs should be frequently updated. These patterns vary by geographic region and may even change over time in a single area or within a single country.[15]

High rates of ampicillin resistance in uropathogenic *E. coli* isolates have been reported worldwide.[16,17] Several authors have reported ampicillin to be the antibiotic most affected by resistance in Cuba.[16–20] Poor in vitro efficacy and high resistance indicates that the use of ampicillin in the treatment of uncomplicated UTI without prior microbial identification should be avoided.[5]

Ciprofloxacin and trimethoprim/sulfamethoxazole are the most-commonly prescribed antibiotics in primary health care appointments in Cuba because of their recognized efficacy in treating UTIs, their availability on the market and their low cost, but indiscriminate use or misuse of both antibiotics has caused a decrease in bacterial susceptibility percentages in hospital-acquired and community-acquired infections.[21]

González[22] and more recently Cabrera[3] reported that >30% of community-acquired UTI-causing uropathogenic *E. coli* were resistant to ciprofloxacin in the province of Havana and to trimethoprim/sulfamethoxazole in the province of Mayabeque. In different parts of the world, this pathogen's resistance rates to both antibiotics exceeds 20%.[4,16]

ST131, an emerging hyperepidemic clone of UTI-causing *E. coli*—resistant to fluoroquinolones and co-resistant to other antibiotic classes—is often resistant to ciprofloxacin and can be found worldwide.[5] In Cuba, this clone was found in *E. coli* clinical isolates, so it could be responsible for the multidrug resistance in this study's isolates.[10]

Due to bacterial resistance to fluoroquinolone and to trimethoprim/sulfamethoxazole, using these antibiotics in community-acquired infections is not advised, and cephalosporin resistance limits treatment options. Using these antibiotics without previous susceptibility tests delays appropriate therapy, causes therapeutic failure and increases treatment costs.[5]

Although cefazolin (cephalosporin for parenteral use) is a very good predictor of susceptibility to oral cephalosporins in resistance

studies, its use for susceptibility estimates is not recommended. Instead, susceptibility or resistance to oral cephalosporins available in the local health area should be studied, because there is no oral presentation for cefazolin.[7]

In this study, less than 20% of isolates were resistant to cefazolin, consistent with what Marrero reported in the eastern province of Holguín;[19] therefore, oral cephalosporins are not recommended in treating community-acquired UTIs.

The combination of penicillins and beta-lactamase inhibitors, such as clavulanic acid, tazobactam and sulbactam, strengthens the action of these drugs by restoring their antimicrobial activity against bacteria that are resistant because of plasmid-mediated beta-lactamase production.[20] However, in this study, we found high frequencies of uropathogenic *E. coli* resistance to the ampicillin/sulbactam combination. This coincides with results of various studies in Cuba and other countries.[5,9,16,20] Therefore, use of this combination is limited in severe community-acquired infections where its in vitro efficacy has not been demonstrated.

Because of bacterial resistance, antibiotics that were used for a long time as first-line UTI treatment stopped being used without first performing susceptibility tests, and nitrofurantoin and fosfomycin were adopted to treat community-acquired UTIs.[23] Nitrofurantoin is a good option for treating uncomplicated community-acquired UTI because it achieves good concentrations in urine, has low resistance, and has been prescribed in Cuba and elsewhere as a urinary bacteriostatic agent for many years.[3,22] Fosfomycin has bactericidal action, is broad-spectrum, achieves high concentrations in urinary tracts, has low toxicity, and can be effective as a single dose for uncomplicated UTI treatment.[24]

Our results show good in vitro nitrofurantoin and fosfomycin activity against this pathogen. Previous studies in Cuba have shown uropathogenic *E. coli* susceptibility levels to both antibiotics at >90%.[3,22] However, nitrofurantoin's wide range of side effects (nausea, vomiting, abdominal pain, decreased appetite) leads to low treatment adherence, and in Cuba the oral presentation of fosfomycin trometamol is not available, so treating UTIs with those antibiotics in Cuban communities is unfeasible.

We did not find resistance to colistin, which could be because its use is limited to treating severe infections caused by gram-negative, multidrug-resistant bacilli. This reaffirms the need to continue using it appropriately.[8] Because community-acquired *E. coli* expressing the transferable colistin-resistant mcr-1 gene is circulating in the world, Cuba's microbiology laboratory network needs to actively screen for resistance to colistin in enterobacteria collected from community-acquired infections.

Evaluating community isolates' susceptibility to colistin has epidemiologic value in researching the mcr-1 gene and its variants, if one considers that WHO has issued an epidemiological alert notifying mcr-1 gene detection in *E. coli* community isolates in several countries, and has called on countries to implement and maintain the capacity for detecting, preventing and controlling transmission of microorganisms with transferable colistin resistance.[8]

Resistance patterns vary over time and by geographic region; therefore, it is advisable to maintain active local surveillance to update treatment guidelines as needed.[15] In this study, however, we did not find major differences between resistance profiles of isolates from Havana and those from the Isle of Youth.

MDR patterns coincide with the observations by Expósito,[18] who reported five MDR patterns in community-acquired UTI-causing *E. coli* isolates in Cuba's Guantánamo province, where most common resistance was to trimethoprim/sulfamethoxazole, ciprofloxacin, tetracycline and ampicillin. Guzmán reported higher resistance to ampicillin, cephalosporins, trimethoprim/sulfamethoxazole and ciprofloxacin in Venezuela.[16]

ESBL detection is used as a relevant clinical and epidemiological marker for reducing morbidity and mortality caused by ESBL-producing bacteria.[9] In the last few years, a growing number of reports show these microorganisms in community isolates.[15] It is important to determine their national, local and even institutional incidence in order to adjust antibiotic therapies, and to avoid treatment failures and increased resistance levels.

Cefazolin disks, a good ESBL predictor in antimicrobial susceptibility testing, helped detect isolates whose phenotypes included the ESBL mechanism. On these grounds, cefazolin disks are recommended for inclusion in Cuba's Microbiology Laboratory Network for researching community-acquired isolates, since ESBL-producing bacteria have been reported in Cuban communities.[9,10,20]

Multidrug-resistance in ESBL-producing bacteria is a significant health problem because these enzymes confer resistance to penicillins, cephalosporins and aztreonam; and 30% to 60% are resistant to beta-lactam antibiotics associated in their formulation with beta-lactamase inhibitors. ESBLs are coded in plasmids that carry resistance genes for other antibiotic classes, including quinolones, aminoglycosides and trimethoprim/sulfamethoxazole,[25] which were observed in the ESBL-positive strains isolated in our research.

It is a challenge to select antibiotic regimens for ESBL-producing bacteria because these must be adjusted to possible causative agents, and during selection, doctors must consider local epidemiology, which differs from hospital to hospital and from city to city.[26]

García[27] suggests the primary measures for preventing ESBL-producing bacteria are frequent handwashing, disinfecting surfaces that may serve as vectors, and reducing inappropriate antibiotic use—especially that of cephalosporins and quinolones. According to García, nitrofurantoin and fosfomycin have good activity in ESBLs, so they appear the best treatment options for uncomplicated UTIs caused by ESBL-producing microorganisms.[27] The results of this study reaffirm this proposal and coincide with reports by other authors who say that most community-acquired UTI-causing ESBL-producing *E. coli* are susceptible to these two antibiotics.[4,26,28] This could be due to the infrequent use of nitrofurantoin and fosfomycin trometamol in recent years, considering that the latter is not available in Cuba. However, due to bacterial capacity for mutation and continued adaptation to the environment, resistance surveillance and rational antibiotic use should be maintained.[15]

This research is limited by the fact that we did not know the patients' clinical characteristics, which kept us from classifying the type of urinary infection, and because we did not study the

susceptibility pattern of all bacterial uropathogens that caused UTIs; our study was limited to *E. coli*. However, guidelines for urinary tract infection treatment are based on the susceptibility of *E. coli*, the most commonly-isolated bacteria.

Another limitation of the study is that colistin susceptibility was only evaluated as a preliminary step in screening for the mcr-1 gene; we did not perform molecular studies to confirm whether any of the studied isolates carried this gene or its variants.

Considering the high resistance to ciprofloxacin and known circulation of the high-risk clone ST-131 in community isolates in Cuba, we recommend performing molecular epidemiology studies of isolates to help determine whether they belong to this clone.

CONCLUSIONS

There is a high frequency of resistance of uropathogenic *E. coli* to antibiotics most commonly used in medical practice, with extended-spectrum beta-lactamase–producing bacteria as the mechanism for resistance. Multidrug-resistance patterns include three or more of the antibiotic classes most commonly used for community infections. Fosfomycin and nitrofurantoin are the most active antibiotics in extended-spectrum beta-lactamase–producing bacteria. All isolates were susceptible to colistin.

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Original Research

Lymphocyte Subsets in Defense Against New Pathogens in Patients With Cancer

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ABSTRACT

INTRODUCTION Immunity in cancer patients is modified both by the cancer itself and by oncospecific treatments. Whether a patient's adaptive immunity is impaired depends on their levels of naive lymphocytes and other cell populations. During the COVID-19 pandemic, cancer patients are at greater risk of progressing to severe forms of the disease and have higher mortality rates than individuals without cancer, particularly while they are receiving cancer-specific therapies. An individual's protection against infection, their response to vaccines, and even the tests that determine the humoral immune response to SARS-CoV-2, depend on lymphocyte populations, meriting their study.

OBJECTIVE Estimate blood concentrations of lymphocytes involved in the immune response to new pathogens in cancer patients.

METHODS We carried out an analytical study of 218 cancer patients; 124 women and 94 men, 26–93 years of age, who were treated at the National Oncology and Radiobiology Institute in Havana, Cuba, March–June, 2020. Patients were divided into five groups: (1) those with controlled disease who were not undergoing cancer-specific treatment; (2) those undergoing debulking surgery; (3) patients undergoing chemotherapy; (4) patients undergoing radiation therapy and (5) patients currently battling infection. We evaluated the following peripheral blood lymphocyte subsets via flow cytometry: B lymphocytes (total, naive, transitional, memory, plasmablasts and plasma cells); T lymphocytes (total, helper, cytotoxic and their respective naive, activated, central memory and effector memory subsets); and total, secretory and cytotoxic natural killer cells and T natural killer cells. We also estimated neutrophil/lymphocyte ratios. Lymphocyte concentrations were associated with controlled disease and standard cancer therapy. For variables that did not fall within a normal distribution, ranges were set by medians and 2.5–97.5 percentiles. The two-tailed Mann–Whitney U test was used to measure the effect of sex and to compare lymphocyte populations. We calculated odds ratios to estimate lymphopenia risk.

RESULTS All cancer patients had lower values of naive helper and cytotoxic T lymphocyte populations, naive B lymphocytes, and natural killer cells than normal reference medians. Naive helper T cells were the most affected subpopulation. Memory B cells, plasmablasts, plasma cells, activated T helper cells, and cytotoxic central memory T cells were increased. Patients undergoing treatment had lower levels of naive lymphocytes than untreated patients, particularly during radiation therapy. The risk of B lymphopenia was higher in patients in treatment. The odds ratio for B lymphopenia was 8.0 in patients who underwent surgery, 12.9 in those undergoing chemotherapy, and 13.9 in patients in radiotherapy.

CONCLUSIONS Cancer and conventional cancer therapies significantly affect peripheral blood B lymphocyte levels, particularly transitional T helper lymphocytes, reducing the immune system's ability to trigger primary immune responses against new antigens.

KEYWORDS Cancer, lymphocyte subsets, flow cytometry, immunity, virus diseases, Cuba

INTRODUCTION

In cancer patients, infections are a risk factor for morbidity and mortality, since these tend to be more severe due to secondary immunodeficiency that can develop during the course of the disease and its treatment. The adjusted death rate for infections in individuals with cancer may be three times higher than the general population.[1,2] Patients with hematologic malignancies are at increased risk of infection, compared with patients who have solid tumors, especially when undergoing hematopoietic cell transplantation.[2] Neutropenia, lymphopenia, alterations of anatomical barrier systems (rupture of epithelial surfaces and basement membranes, either due to tumor invasion or induced by therapies), splenic and humoral defects and therapeutic immunosuppression all play a role in immunopathogenesis and affect infection incidence and severity.[3]

IMPORTANCE Knowing lymphoid cell concentrations in cancer patients allows us to design better vaccination strategies for new pathogens like SARS-CoV-2 and avoid false negatives in antibody tests.

Cancer patients have shown greater susceptibility to COVID-19.[4,5] Those with active malignancy experience more severe disease, with COVID-19 mortality rates at 5%–61%, and mean estimated mortality at 25.6%.[2] Risk of dying from COVID-19 is increased (OR = 9.31) when patients have metastatic disease.[6] Compared to persons without cancer, these patients have a higher probability of SARS-CoV-2 infection, of severe manifestations of the disease and of fatal outcomes. These effects are mediated by tumor location, disease stage and treatment type.[3,5,7] Of all these factors, only treatment type can be modified.

The immune response (IR) to infection is complex and requires a functioning immune system (IS) to achieve effective antimicrobial response. Viral infections require special attention, since antiviral treatments are not as effective for them as antibiotics are for bacterial infections.[8,9] Viral infection control depends largely on balancing the innate and adaptive IS, which influence infection and recovery.[8]

The main cells in innate immunity for viral infection control are natural killer (NK) cells, plasmacytoid dendritic cells and neutrophils, which act immediately through extracellular traps.[10,11]

Specific adaptive immunity requires more time to develop than innate immunity, especially in the case of new pathogens like SARS-CoV-2, requiring establishment of a new primary IR. The mature cells involved in adaptive immunity are: helper T lymphocytes (Th), cytotoxic T lymphocytes (Tc) and B lymphocytes, in their varying stages of differentiation (naive, activated, memory, effector and terminally differentiated cell forms). B lymphocytes differentiate into plasma cells that produce antibodies or specific immunoglobulins.[12] Naive lymphocytes are mature T or B cells that reside in peripheral lymphatic organs and in circulating blood, which have never encountered their cognate antigen and are therefore charged with recognizing new pathogens. Their concentration in blood can be measured, as they recirculate in their role in immunosurveillance against new antigens.

IS alterations in cancer patients create challenges in diagnosing and treating emerging infections, as has been the case for COVID-19.[13] Quantitative and qualitative alterations have been noted in both innate and adaptive IS cells in these patients, thus considering cancer a secondary immunodeficiency. Naive T and B lymphocyte populations may be affected, which would compromise the primary immune response of Tc lymphocytes and immunoglobulin production in response to new pathogens such as SARS-CoV-2.[14–16] Patients with malignant hemopathies treated with stem cell therapy require special attention, due to the time required for IS reconstitution and the quality of IR after stem cell transplantation.[17]

Infection as a comorbidity in cancer patients is well-documented,[2,5,18] but few studies have investigated patient susceptibility to infection during epidemics based on alterations to immunopathogenic mechanisms. Changes to IS cells in cancer patients limit the use of treatment and screening strategies designed for the general population. Two of these limitations are of major concern: 1) serological screening tests for diagnosing infections that, due to their high sensitivity and feasibility of application, can result in false negatives due to the decreased function of B lymphocytes, resulting in fewer circulating antibodies produced in response to infection[15,16] and 2) prophylactic vaccination schedules that are not always as effective as in healthy people, due to IS alterations. This could necessitate modifications to the number and interval of vaccine doses, as well as deferring their application, in accordance with cancer stage and type.[19,20] Cancer patients are excluded from clinical trials testing new vaccines, but arguments can be made for their inclusion, because of their increased vulnerability to infection.[21]

Knowing the effect of standard cancer-specific treatments on IS cell proportions in cancer patients could help in designing strategies for controlling epidemics such as COVID-19, including vaccination schedules and detection strategies—and help adjust them to the needs of cancer patients. We carried out this study to evaluate the composition of lymphocytes in blood necessary to trigger a primary immune response against new antigens in Cuban cancer patients.

METHODS

Design, participants and sampling We conducted a cross-sectional analytical study in 218 cancer patients with various tumor stages and locations, from March through June 2020. We included 124 women and 94 men 26–93 years of age who were treated in the National Oncology and Radiobiology Institute (INOR) in Havana, Cuba. Patients with malignant hemopathies were not included.

Patients were divided into five groups:

- Patients with controlled cancer without oncospecific treatment (n = 39): followup patients with no evidence of active disease were considered ‘controlled’ after at least 12 weeks since the end of their primary/adjuvant treatment. Breast cancer patients who fulfilled this criteria and were on hormonal adjuvant therapy for 5 years or prophylactic treatment with zoledronic acid were also included.
- Patients who had not started chemotherapy or radiotherapy, and underwent cytoreductive surgery as a therapeutic standard, 1–7 days post-surgery (n = 54).
- Patients undergoing chemotherapy, regardless of the cycle and type of chemotherapy used, who did not undergo surgery or radiotherapy (n = 67).
- Patients undergoing radiotherapy who had not received prior or concomitant chemotherapy or surgery (n = 44).
- Patients diagnosed with acute infections confirmed via testing (related or unrelated to oncospecific therapies) (n = 14).

Peripheral blood samples were obtained by antecubital venipuncture, 4 mL of which were deposited in Vacutainer tubes (Becton Dickinson, USA) with ethylene aminotetraacetic acid added as an anticoagulant. Samples were processed within the first six hours after extraction.

Flow cytometry We designed a cytometry panel that allowed immunophenotyping of the following lymphocyte subsets (or subpopulations) in peripheral blood based on a $CD45^{+++}/SS^{low}$ window: Total B lymphocytes (CD19 + / CD20 +), naive (CD19 + / CD20 + / CD38 +/−), early activation (CD19 + / CD20 + / CD22 + / CD25 + / HLA-DR +), late activation (CD19 + / CD20 + / CD25 + / CD22 ++ / HLA-DR ++), transitional (CD19 + / CD20 + / CD22 +/− / CD38 ++), memory (CD19 + / CD20 + CD22 + / CD38 +/−); plasmablasts and plasma cells (CD19 + / CD20 − / CD22 − / CD38 +++); Total T (CD3 +), T helper (Th; CD3 + / CD4 +) and T-cytotoxic (Tc; CD3 + / CD8 +) lymphocytes; Th naive cells, activated, with central memory and effector memory; Naive Tc (CCR7 + / CD45RO −), activated (CD25 + / HLA-DR +), central memory (CCR7 + / CD45RO +) and effector memory (CCR7 − / CD45RO +); Total natural killer cells (NK; CD3 − / CD56 +) (secretory CD56 ++ and cytotoxic CD56 +); Natural killer T cells (NKT; CD3 + / CD56 +). We also estimated the neutrophil/lymphocyte ratio (NLR).

Samples were prepared according to manufacturer specifications for cell surface immunophenotyping, with an unwashed reed blood cell (RBC) lysis protocol (VeraLyse; Beckman-Coulter RBC

Lysis Buffer, France). We used a 10-color cytometer (Beckman-Coulter, France). 100 µL of blood was dispensed for staining with fluorochrome-conjugated monoclonal antibodies (Beckman-Coulter, France): anti-CD45 AA750 (Clone J33), anti-CD19 PC7 (Clone J3-119), anti-CD3 FITC (Clone UCHT1), anti-CD4 PB (Clone 13B8.2), anti-CD8 AA700 (Clone B9.11), anti-CD56 PE (Clone N901) (NKH-1), anti-HLA-DR PE (Clone Immu-357), anti-CD45RO PC5 (Clone UCHL1), anti-CCR7 PC7 (Clone G043H7), anti-CD20 FITC (Clone B9E9), anti-CD38 PE (Clone LS198-4-3), anti-CD25 PC5 (Clone B1.49.9) and anti-CD22 PC7 (Clone SJ10.1H11).

We performed daily quality controls of the Flow-Check fluorosphere cytometer, aligning the lasers and checking the water system. Fluorescence intensity was monitored with Flow-Set fluorospheres from the same company.

Data was processed with Kaluza Analysis V1.5a software (Beckman-Coulter, France), with a minimum of 50,000 events acquired. We used a manual and logical-sequential window, and the guidelines recommended by the Human Immunology Project's immunophenotype standardization.[22] We used published reference values.[23–25]

Statistical analysis To define value ranges, we evaluated normal distribution of variables via the Shapiro-Wilk test. Most variables did not follow a Gaussian distribution. Ranges were set through medians and 2.5–97.5 percentiles. We analyzed the effect of age on lymphocyte populations with a simple linear regression model, and applied the two-tailed Mann-Whitney U test to evaluate the effect of sex as well as the comparison between lymphocyte subpopulations. To evaluate association between variables, we calculated odds ratios. All tests were performed with an associated significance level of $p < 0.05$.

Ethics The study was approved by the INOR ethics committee. Participants provided written informed consent and we followed the principles for human subject research established by the Declaration of Helsinki.[26] Identifying information was kept confidential. Diagnostic means were selected under the guiding principle of maximum beneficence, the ethical norm of 'do no harm', and material accessibility.

RESULTS

Age and sex did not significantly influence patient lymphocyte ratios.

Most naive T and B lymphocyte and NK cell populations were significantly lower in cancer patients than the normal reference median. However, memory lymphocytes and activated Tc were elevated. The neutrophil-to-lymphocyte ratio (NLR) was within normal range (Table 1).

Significant differences were observed in the estimated medians for all lymphocyte populations' relative concentrations in the five groups (Table 2). We found a wide range inside the 2.5–97.5 percentiles for all lymphocyte subpopulations in the different groups, most notably in patients with untreated controlled disease and B lymphocyte subpopulations.

Patients with controlled disease had low total values of T lymphocytes and effector memory T lymphocytes (Th and Tc), but their naive T and B cells were not affected. Cytotoxic-type NK cells were lower than reference values.

Table 1: Lymphocyte subpopulation percentage values and Neutrophil-to-Lymphocyte Ratios in cancer patient's peripheral blood, as compared with normal reference values (N = 218)

Subpopulation	Normal values [54–56]	Cancer patient values	p*
	Median % (Range)	Median % (Range)	
Total B lymphocytes	10.7 (4.7–19.1)	6.8 (0.3–24.8)	<0.0001
Naive	65.1 (58.0–72.1)	62.2 (10.4–88.7)	0.0092
Transitional	6.2 (1.7–13.8)	1.4 (0.0–40.3)	<0.0001
Memory	10.9 (1.9–13.4)	14.5 (0.0–73.3)	<0.0001
Plasmablasts and plasma cells	1.3 (0.2–5.0)	2.0 (0.0–57.1)	<0.0001
Total T lymphocytes	73.0 (56.5–84.7)	67.4 (1.1–85.3)	<0.0001
Th	43.8 (30.3–55.7)	35.7 (4.0–59.0)	0.1243
Tc	26.0 (13.2–42.9)	22.5 (0.6–44.3)	<0.0001
Naive Th	31.3 (5.7–63.5)	18.7 (0.5–81.8)	0.0027
Naive Tc	43.1 (17.8–66.3)	23.3 (0.7–73.6)	<0.0001
Active Th	1.7 (0.8–4.4)	0.9 (0.0–14.6)	0.3997
Active Tc	1.0 (0.3–6.4)	2.1 (0.0–39.3)	<0.0001
Central memory Th	32.8 (19.4–51.9)	62.5 (0.0–93.9)	<0.0001
Central memory Tc	9.6 (3.4–22.4)	30.9 (0.0–84.6)	<0.0001
Effector memory Th	16.7 (7.4–31.9)	4.2 (0.0–69.3)	<0.0001
Effector memory Tc	18.9 (6.0–38.9)	0.6 (0.0–70.3)	<0.0001
Total NK cells	12.7 (3.7–28.0)	10.3 (0.0–49.1)	<0.0001
Secretory	6.4 (1.1–17.7)	1.5 (0.0–18.6)	<0.0001
Cytotoxic	≈ 90.7	67.8 (14.4–94.6)	<0.0001
NKT	5.5 (1.1–14.9)	5.8 (0.5–75.4)	0.0014
NLR	<2.7	2.5 (0.6–11.4)	0.7604

*: p value associated with a Wilcoxon signed rank test; NK: Natural Killer cells; NKT Natural Killer T cells; NLR: Neutrophil-to-Lymphocyte Ratio; Tc: cytolytic T lymphocytes; Th: helper T lymphocytes

Activated T lymphocytes, central memory, effector memory (Th and Tc) and transitional B lymphocytes were lower in patients who underwent cytoreductive surgeries. Cytotoxic-type NK cells were significantly lower. Neutrophil values were higher in patients with infection, with a mean of 10,025 cells/µL, increasing the NLR. However, neutrophil values were normal in other groups and the NLR did not rise (Table 2).

Patients undergoing chemotherapy had lower levels of naive Th and Tc cells, total and transitional B lymphocytes, and cytotoxic NK cells.

Patients with infections had lower levels of total T lymphocytes—at the expense of naive Tc—of total and naive B lymphocytes, and of cytotoxic-type NK cells. The NLR was higher, with increased neutrophils.

In the standard treatment and infection groups, proportions of patients with low total B lymphocyte values were higher than those in the untreated group (Table 3). Only the radiotherapy group showed a significant increase in patients with low levels of naive B lymphocytes compared to the untreated group. The proportion of patients with low naive Th and cytotoxic NK values was significantly higher in the treated and infection groups. In the treated groups, the proportion of patients with low levels of total T lymphocytes was lower compared to untreated patients (Table 3).

Table 2: Percentage values medians of lymphocyte subpopulations in cancer patients, by study group

Subpopulation	Untreated n = 39	Surgery n = 54	Q n = 67	R n = 44	P/Infec n = 14	Total n = 218	KW
	% (2.5–97.5)	% (2.5–97.5)	% (2.5–97.5)	% (2.5–97.5)	% (2.5–97.5)	% (2.5–97.5)	
Total B lymphocytes	11.2 (0.5–46.1)	9.2 (1.9–34.4)	4.3 (0.2–12.3)	3.9 (0.1–12.1)	5.2 (0.2–12.2)	6.8 (0.3–24.8)	<0.001
Naive[a]	57.6 (27.0–86.3)	67.1 (13.7–91.4)	69.7 (15.9–84.4)	56.9 (0.3–98.6)	35.5 (12.2–100)	62.2 (10.4–88.7)	<0.001
Early activation[a]	8.7 (0.7–47.5)	7.9 (0.0–47.5)	12.7 (0.9–68.3)	16.6 (0.0–69.6)	9.5 (0.0–35.5)	10.2 (0.0–58.5)	<0.001
Late activation[a]	0.7 (0.0–5.0)	1.9 (0.0–22.5)	3.1 (0.0–28.6)	4.3 (0.0–55.3)	6.5 (0.0–36.5)	2.3 (0.0–37.2)	<0.001
Transitional[a]	22.7 (0.0–50.3)	0.3 (0.0–48.3)	0.2 (0.0–3.6)	0.4 (0.0–4.5)	3.2 (0.0–07.2)	1.4 (0.0–40.3)	<0.001
Memory[a]	3.3 (0.0–71.8)	19.4 (0.0–71.5)	8.3 (0.0–63.6)	7.3 (0.0–88.2)	22.6 (0.0–41.7)	14.5 (0.0–73.3)	<0.001
Plasmablasts and plasma cells[a]	37.7 (0.1–79.7)	0.3 (0.0–55.5)	4.3 (0.1–50.0)	0.7 (0.0–19.8)	12.2 (0.0–21.6)	2.0 (0.0–57.1)	<0.001
Total B lymphocytes[b]	49.4 (0.7–84.8)	68.8 (0.6–84.9)	68.9 (42.6–86.8)	69.8 (38.0–89.2)	33.7 (19.1–53.0)	67.4 (1.1–85.3)	<0.001
Th[b]	11.3 (0.9–59.3)	41.6 (6.3–57.5)	33.6 (19.1–68.5)	38.6 (18.3–66.9)	7.8 (0.3–23.6)	35.7 (4.0–59.0)	<0.001
Tc[b]	4.8 (0.1–43.7)	24.3 (0.4–49.6)	24.7 (7.9–44.2)	24.0 (9.9–53.1)	15.9 (7.1–44.8)	22.5 (0.6–44.3)	<0.001
Naive (Th/Tc)[b]	41.4 (1.9–83.1) 29.2 (13.0–84.4)	46.5 (2.3–93.8) 25.5 (5.7–72.0)	6.1 (0.0–323.1) 8.3 (1.1–30.5)	12.9 (1.5–99.0) 25.1 (3.4–76.9)	20.4 (0.0–26.6) 13.7 (4.0–30.2)	18.7 (0.5–81.8) 23.3 (0.7–73.6.)	<0.001
Activated (Th/Tc)[b]	2.4 (0.0–17.0) 2.1 (0.0–16.2)	0.4 (0.0–17.2) 1.8 (0.0–11.9)	0.9 (0.0–8.0) 9.8 (2.8–24.9)	1.3 (0.0–15.8) 3.5 (0.0–49.7)	1.6 (0.0–5.1) 7.5 (3.2–14.9)	0.9 (0.0–14.6) 2.1 (0.0–39.3)	<0.001
Central memory (Th/Tc)[b]	62.4 (0.0–100) 11.6 (0.0–79.1)	24.5 (0.0–86.0) 1.5 (0.0–24.8)	59.6 (23.4–84.8) 40.8 (16.3–68.2)	69.3 (26.1–93.4) 35.8 (10.6–92.5)	20.4 (10.5–42.2) 15.5 (13.7 –23.8)	62.5 (0.0–93.9) 30.9 (0.0–84.6)	<0.001
Effector memory (Th/Tc)[b]	2.84 (0.0–69.5) 0.0 (0.0–41.3)	4.7 (0.0–32.3) 0.1 (0.0–2.2)	27.6 (4.1–69.5) 35.4 (0.0–70.0)	10.1 (0.2–58.9) 10.6 (0.0–73.6)	58.7 (31.3–82.1) 59.7 (41.8–75.5)	4.2 (0.0–69.3) 0.6 (0.0–70.3)	<0.001
Total NK cells	16.9 (0.0–66.8)	9.5 (0.5–31.8)	9.9 (2.3–18.4)	9.6 (1.6–26.6)	8.8 (6.1–13.4)	10.3 (0.0–49.1)	<0.001
Secretory[c]	0.3 (0.0–14.3)	2.0 (0.0–15.6)	5.6 (1.3–14.8)	1.1 (0.0–15.1)	6.2 (2.2–10.0)	1.5 (0.0–18.6)	<0.001
Cytotoxic[c]	64.7 (11.8–87.5)	69.5 (12.6–87.1)	66.9 (6.8–88.5)	80.5 (18.3–96.6)	65.1 (55.8–81.2)	67.8 (14.4–94.6)	<0.001
Cytotoxic state[c]	25.9 (2.4–54.4)	31.9 (0.0–64.3)	20.4 (4.6–38.5)	23.1 (0.0–585.3)	9.9 (3.3–23.6)	19.7 (0.0–53.5)	<0.001
Total NKT cells	31.9 (1.3–76.5)	5.6 (0.5–30.9)	2.3 (0.8–16.3)	6.4 (0.7–81.9)	6.5 (4.3–8.9)	5.8 (0.5–75.4)	<0.001
NLR	2.4 (0.6–9.7)	2.9 (1.0–6.5)	2.6 (1.1–11.5)	2.7 (0.2–7.5)	3.3 (0.2–7.6)	2.5 (0.6–14.4)	0.405

(2.5–97.5): Range between percentiles;
a: Percentage of total B lymphocytes
b: Percentage of total T lymphocytes
c: Percentage of total NK cells

KW: p-value associated with Kruskal-Wallis test for comparison of groups; NK: Natural Killer cells; NKT Natural Killer T cells; NLR: Neutrophil/lymphocyte ratio, median of each group; P/Infec: Patients with infection; Q: Chemotheraphy; R: Radiotherapy; Tc: cytolytic T lymphocytes; Th: helper T lymphocytes

Table 3: Proportion of patients with low lymphocyte subset levels, by study group

Subset with low values	Without treatment (n = 39)	Surgery (n = 54)	p*	Q (n = 67)	p*	R (n = 44)	p*	P/Infec (n = 14)	p*	Total (N = 218)
	%	%		%		%		%		%
Total B lymphocytes	1.9	20.4	0.008	59.7	<0.001	65.9	<0.001	25.1	0.006	39.3
Naive B cells[a]	17.9	20.4	0.764	14.9	0.686	38.6	0.039	40.2	0.097	29.4
Plasmablasts and plasma cells[a]	28.2	48.1	0.054	13.4	0.061	40.9	0.228	30.8	0.855	31.8
Total T lymphocytes	69.2	20.4	<0.001	40.1	0.004	15.9	<0.001	68.4	0.956	70.1
Naive Th[b]	7.7	16.7	0.204	65.7	<0.001	29.5	0.012	12.5	0.593	32.7
Total NK cells	23.1	16.7	0.443	16.4	0.397	9.1	0.081	10.3	0.306	24.2
Cytotoxic[c]	12.8	96.3	<0.001	92.5	<0.001	59.1	<0.001	88.2	<0.001	85.3
Total NKT	5.1	9.3	0.451	16.4	0.088	4.5	0.898	9.2	0.588	9.1

*: p value associated with a Wilcoxon signed rank test; a: Percentage of total B lymphocytes b: Percentage of total T lymphocytes
c: Percentage of total NK cells. All comparisons are made with respect to the cancer patient group, without treatment
NK: Natural Killer cells; NKT Natural Killer T cells; P/Infec: Patients with infection; Q: Chemotherapy; R: Radiotherapy;Th: helper T lymphocytes

In all treatment groups, transitional B lymphocytes were low, but were high in patients with infections and in untreated patients (variability was high in the latter group). In all groups, naive B lymphocyte medians were normal but significantly lower in patients who had not undergone treatment and in patients with infections compared to treated patients (p = 0.001, Mann-Whitney U) (Figure 1A).

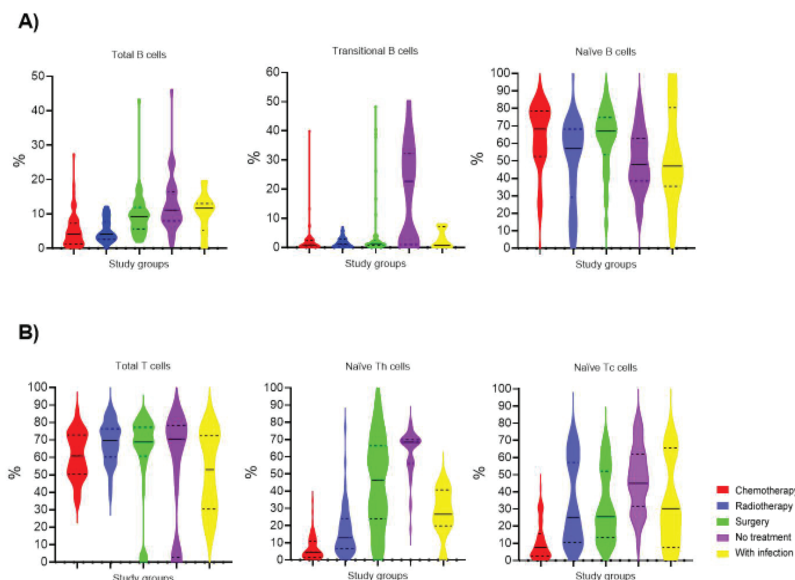
Patients with infections had the lowest total lymphocyte medians and heterogenous values with bimodal distributions around the first and third quartiles. More than half of chemotherapy patients had decreased naive Th lymphocytes, and those who had undergone surgery had a high dispersion of percentage values for these lymphocytes with a bimodal distribution similar to that described for total T lymphocytes. Naive Th lymphocytes were higher than established reference values in the untreated group. Patients in chemotherapy had low Tc lymphocyte values.

Cancer patients undergoing surgery had an eight-fold higher risk of low transitional B lymphocytes than those who did not receive oncospecific treatments. Cancer patients who underwent surgery had a lower risk for low concentrations of memory B lymphocytes, Th and Tc, with risk reductions of 7.8, 5.5 and 8.3 times, respectively (Table 4).

Chemotherapy patients had a 12.9-fold greater risk than untreated patients for total B-cell lymphopenia and were 32.5 times more likely to have decreased naive Th lymphocyte levels. Risk of transitional B-cell lymphopenia was 2.5 times higher in chemotherapy patients. However, chemotherapy was associated with protection against total and memory T lymphocyte depletion. The risk of lymphopenia was reduced 3.8 times for Th, 9.1 times for Tc, and 33.3 and 11.1 times for central memory Tc and effector memory Th lymphocytes, respectively.

Radiation therapy was associated with a 13.9-fold increased risk of total B-cell lymphopenia. Additionally, it was associated with 10.1- and 7.5-times higher risks of transitional B-cell lymphopenias and naive Th lymphopenias respectively (Table 4), but was a protective factor against central memory and effector memory Th and Tc lymphocyte lymphopenias.

Figure 1: Main lymphocyte subpopulations involved in primary immune responses. A) Distribution of main B lymphocyte subpopulations according to the five study groups of cancer patients. B) Distribution of main T lymphocyte subpopulations according to the five study groups of cancer patients



Tc: cytotoxic T lymphocytes; Th: T helper lymphocytes; (N = 218 patients)

DISCUSSION

Cancer patients suffer from IS dysfunction due to a failure in immune surveillance of malignant tumors. As the disease progresses, IS deficiency worsens, which explains immune tolerance of increasing tumor burdens. For this reason, cancer is considered a cause of secondary immunodeficiency.[27] When standard therapies are applied—chemotherapy,[28,29] radiotherapy[30] and cytoreductive surgery[31,32]—either alone or in combination, they lead to major

Table 4: Estimates and 95% confidence intervals of odds ratios for the subpopulation presence, by lymphocyte population and cancer therapy type

Lymphocyte subpopulation	Odds Ratio	95% CI
Surgery		
Transitional B lymphocytes	8.00	3.1–20.9
Memory B lymphocytes	0.13	0.02–0.64
Helper T lymphocytes (Th)	0.18	0.07–0.43
Cytotoxic T lymphocytes (Tc)	0.12	0.05–0.32
Naive Th lymphocytes	5.60	1.1–27.7
Central memory Tc lymphocytes	12.20	4.1–36.3
Chemotherapy		
Total B lymphocytes	12.90	4.1–40.7
Transitional B lymphocytes	2.50	1.1–5.9
Helper T lymphocytes (Th)	0.26	0.11–0.60
Cytotoxic T lymphocytes (Tc)	0.11	0.04–0.28
Naive Th lymphocytes	32.50	7.2–147.6
Central memory Tc lymphocytes	0.03	0.004–0.26
Effector memory Th lymphocytes	0.09	0.03–0.22
Radiotherapy		
Total B lymphocytes	13.90	4.1–46.1
Transitional B lymphocytes	10.10	3.4–29.7
Helper T lymphocytes (Th)	0.13	0.04–0.34
Cytotoxic T lymphocytes (Tc)	0.05	0.01–0.17
Naive Th lymphocytes	7.50	1.1–36.1
Central memory Tc lymphocytes	0.28	0.1–0.9
Effector memory Th lymphocytes	0.20	0.07–0.55

immunodeficiency.[29] Chemo- and radiotherapy, which mainly affect proliferating cells, can destroy IS cells, especially those of developing leucocyte populations.[27,33] The selective cytotoxic effect on these cells could influence the fact that no significant decreases were found between men and women, or were associated with age.

In the case of lymphocytes, naive subpopulations require cycles of proliferation and differentiation to give rise to effector and memory cells, and are thus more affected than memory cells by chemotherapy and radiation.[3] Memory cells are mostly quiescent and are more abundant, as they have already undergone clonal expansion.[27,34] In our study, the decrease in naive lymphocytes was related to the effects of cancer and cancer-related therapies. These cells were affected by a double depletion mechanism, that of their precursors during maturation and that of naive clones when they are activated by cognate antigen recognition, as they undergo proliferation cycles in both conditions. This is why cancer patients have compromised defenses against infectious agents that are coming into contact with the body for the first time.[6,29,34]

The increase in memory B and T lymphocytes could be relative since their naive counterparts decreased in percentage. Memory Tc cells may increase, mainly in cancers that involve regional lymph nodes depending on disease stage, such as in breast cancer.[35,36] In these cases, tumor antigens arriving from IRs are systematically introduced to secondary lymphoid structures that generate memory clones that circulate in blood.

There is evidence that increases in memory B lymphocytes and Th lymphocytes are interrelated in some cancers, especially when there is a high density of B cells in tertiary lymphoid structures developed in the microenvironment. Increase of these populations is due mainly to clonal expansion, stemming from a great diversity of specificities, particularly in patients younger than 68 years.[37] Montfort concludes that the increase in memory B lymphocytes and antibody-producing plasma cells is due to antigenic diversity generated by the tumor, favored by chemotherapy.[38]

The risk cancer patients will suffer IR alterations is increased when they undergo oncospecific therapies, manifested by a decrease in leukocytes and an increased risk of infection. Our finding of a normal NLR median for all cancer patients was related to a decrease in neutrophils, which could be due to the influence of cytotoxic therapies, as they have a rapid turnover in circulation, with continuous replacement of new cells produced in bone marrow.[39] However, patients with infections had neutrophilia with high indices, contributing to the wide range we observed—up to 11.4.

NLR is an important biomarker for prognosis of these patients, since its increase is associated with poor outcomes.[40] This correlates with the literature, since patients undergoing treatment with chemo- and radiotherapy generally have lymphopenia and a low NLR, sometimes requiring treatment with granulocyte colony stimulating factor. In patients with infections, this index increased, which suggests poorer prognoses. In patients that recover, cell restoration kinetics are different for leukocytes and lymphocytes, as lymphocytes require more time to recover. Adaptive immunity takes anywhere from six months to a year to restore itself following chemotherapy. This delay can negatively impact patient prognosis after completion of cytotoxic therapies, as neutrophils normalize much faster than lymphocytes and thus NLR rises at the expense of persistent lymphopenia.[30,41]

Other research evaluating the impact of cancer and its treatments on IS cell ratios is based on lineage analysis, but few studies examine distribution of naive or memory populations. Our study shows that the cell population most affected by cancer treatment is B lymphocytes, which helps explain why the humoral response is also affected. This coincides with publications by other authors;[31,42–44] however, the main lymphocyte subpopulations involved in primary IR were also decreased in some study groups.

Recent research indicates that in chemotherapy patients, not only are naive cells lost, but memory cells (both B and T) are also diminished, manifested by a decrease of antibody titers against previously administered vaccines. This has been the case in antigen-dependent responses.[41,43] However, protection is preserved against latent viruses like cytomegalovirus, suggesting memory cytotoxic T cell preservation. This could be due to the fact that memory cells are mostly quiescent, and this type of therapy acts especially well on proliferating cells.[33,44] We observed the opposite in the defense against new infections produced by viruses like Zika, SARS-CoV-2 or West Nile, in which the naive cell repertoire is compromised and they are the ones generating the primary immune response.[30,43]

Our results coincide with these findings, as there was a significant decrease in naive populations, which resulted in a compromised primary response. In the case of total T lymphocytes, no risk of

lymphopenia was found in the chemotherapy group, possibly due to the relative estimation of their subpopulations, and the decrease in naive cells did not cause a percentage decrease in T cells. These results are especially important during the COVID-19 pandemic, when cancer patients are more susceptible to severe forms of the disease and death.[2,5]

The tissues most affected by radiation are bone marrow and intestinal mucosa. Both are essential in the IS, since the first produces naive lymphocytes and the second is one of the largest secondary lymphoid organs. Radiation's cytotoxic effects increase with dose and treatment time. The main mechanisms of radiation-induced death in lymphocyte populations are primary necrosis for T lymphocytes, secondary necrosis for B lymphocytes, and apoptosis for NK cells. NK lymphocytes are the most radiosensitive immune cells, followed by B lymphocytes and lastly, T cells.[38]

Within T cell and B cell subpopulations, naive cells are the most radiosensitive, because they proliferate when activated. Although radiation therapy is localized, its effects on lymphocytes are systemic.[44] Dovšak showed radiotherapy's immunosuppressive effects in oral cancer patients and its negative impact on naive cells, even when irradiation was local. NK cells were also affected. This depression can persist longer than a year following treatment cessation.[31]

Transitional B lymphocytes come from bone marrow, are precursors of naive cells, and complete their maturation in other organs. Cells with the CD38^{hi} subphenotype are known to be tolerant to circulating autoantigens, like tumor antigens, and the mechanism behind this tolerance is anergy, which often ends in apoptosis.[45] This could explain the depletion found in this lymphocyte subphenotype. In all groups studied, lymphopenia has multiple repercussions for patients (Figure 1A). On one hand, the repertoire of naive cells derived from transitional cells is reduced and the generation of humoral responses to new antigens is compromised—as is the case for new pathogens like SARS-CoV-2—but on the other hand this could lead to better cancer prognoses, as the cells that produce interleukin-10 are reduced at a systemic level, decreasing antitumor responses.

Cancer patients who underwent surgery had less risk of lower B lymphocyte, Th and total Tc lymphocyte values, which suggests tumor removal modifies inter-department lymphocyte distribution, although it does not necessarily favor production of naive lymphocytes. Cytoreductive cancer surgery improves patient immune status as it eliminates the tumor microenvironment that produces both local and systemic immunosuppressive effects, but it does so by reducing suppressive lymphocytes.[46,47] However, these surgeries are a stressor and they produce a decrease in certain lymphocyte populations, although this decrease is temporary.[47]

The decrease in transitional B lymphocytes after debulking surgeries could be related to inflammatory response and surgical healing processes. After the operation, bone marrow increases production of red blood cells, neutrophils and T lymphocytes related to homeostasis. B lymphocytes are perhaps less necessary to this process and transitional cells belong to the final stage of this lymphocytic lineage's maturation. Naive Th cells had widely dispersed percentage values and exhibited a bimodal distribution similar to that described for total T lymphocytes. This could be due to heterogenicity in the tumor microenvironment's cellular composition, which has a systemic impact and depends on tumor type and disease stage.[48]

The prognosis for an unfavorable NLR evolution is greater when dependent on a decrease in lymphocytes. NLR increase denotes dysfunctional and sometimes suppressive inflammation, which translates clinically into reduced patient survival and poor tumor cell response to therapies inhibiting PD1 receptors.[49] It is also associated with poor prognoses in infections with uncontrolled inflammatory reactions like those seen in COVID-19,[50] so this indicator should be measured in cancer patients, especially during oncospecific therapies. Absence of a high NLR in all study participants is because although patients with infections had neutrophilia and increased NLR, patients who received chemo- and radiotherapy had neutropenia and decreased NLR.

Antibody levels against pretreatment infections, including COVID-19, are lowered in cancer patients treated with chemotherapy.[15,16] In hematologic malignancies like leukemia, the antibody response to vaccines is also affected before starting chemotherapy, so the deficit caused by the disease is added to that caused by the therapy, implying that patients should be reimmunized three to six months after finishing treatment.[51–53] Antibody production is affected in cancer patients, so it would be advisable during new epidemics to carry out diagnostic tests that detect the causal agent—rather than estimating antibody presence in response to the causal agent—as this could be decreased or absent and result in false negatives, even if the patient has the infection.

Vaccination strategies in cancer patients should be based on sound scientific evidence, to not deprive them of vaccines or subject them to unnecessary risks. Indication for patient vaccination depends on vaccine design, cancer type, the state of the patient's immune system, and treatment timing and type. In our experience with cancer patients, protective effects of immunization are obtained one month after immunosuppressive treatment cessation. However, in the case of malignant blood diseases like leukemia, vaccination is not always recommended, as treatments are usually prolonged. Malignant B cell neoplasms such as lymphoma, chronic lymphocyte leukemia and multiple myeloma will likely have different antibody responses to other cancer types in which the cells responsible for producing antibodies in response to infection are not affected by treatment. This is important when deciding whether to administer vaccines, where protection is related to antibody production, so indication for vaccination depends on vaccine design and expected response.

In addressing pandemics like COVID-19, vaccination strategies for cancer patients must be considered since high percentages of the population require vaccination and the decision whether to vaccinate patients or their cohabiting relatives should be prioritized. This decision must take into account cancer type, stage, the type of oncospecific treatment, the possible response of IS cells to these conditions and other preexisting comorbidities, as well as the type of vaccine to be administered.

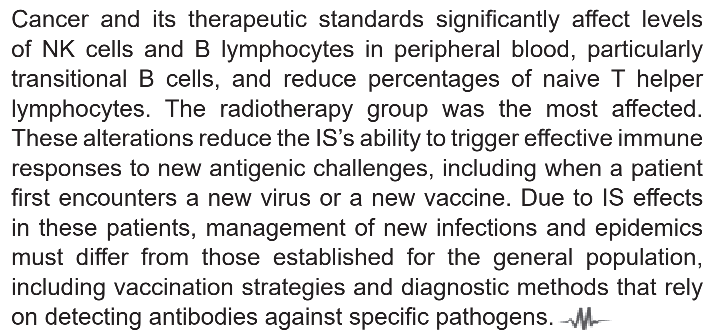
Considering our results, it is recommended that vaccines—particularly those with attenuated live agents—not be administered during convalescence from major surgeries, in the first cycles of chemotherapy, or during full-dose total body irradiation. The

proposal would be to vaccinate with a first dose two weeks before treatment or two weeks after treatment, at minimum, and finish the immunization schedule before starting another treatment cycle.[54,55]

Although this study was aimed at obtaining an overview of cancer as a group of diseases and establishing the effects of modifiable factors like therapeutic standards and infections, a stratified study examining age, sex, cancer type, disease stage and particularities within treatment groups is needed. Non-inclusion of these variables and stratification criteria constitute the main limitations of the present study.

CONCLUSIONS

Cancer and its therapeutic standards significantly affect levels of NK cells and B lymphocytes in peripheral blood, particularly transitional B cells, and reduce percentages of naive T helper lymphocytes. The radiotherapy group was the most affected. These alterations reduce the IS's ability to trigger effective immune responses to new antigenic challenges, including when a patient first encounters a new virus or a new vaccine. Due to IS effects in these patients, management of new infections and epidemics must differ from those established for the general population, including vaccination strategies and diagnostic methods that rely on detecting antibodies against specific pathogens.



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Genome-Wide mRNA Expression Analysis of Acute Psychological Stress Responses

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ABSTRACT

INTRODUCTION Most previous studies have examined the effects of acute psychological stress in humans based on select gene panels. The genomic approach may help identify novel genes that underline biological mechanisms of acute psychological stress responses.

OBJECTIVE This exploratory study aimed to investigate genome-wide transcriptional activity changes in response to acute psychological stress.

METHODS The sample included 40 healthy women (mean age 31.4 ± 11.6 years). Twenty-two participants had a stress experience induced by the Trier Social Stress Test (experimental group) and 18 did not (control group). Psychological stress levels and hemodynamic changes were assessed before and after the Trier Social Stress Test. Peripheral blood samples obtained before and after the Trier Social Stress Test were processed for mRNA sequencing.

RESULTS Psychological and hemodynamic stress parameters indicated that the Trier Social Stress Test induced moderate levels of stress in the experimental group. Six genes (HCG26, HCP5, HLA-F, HLA-F-AS1, LOC1019287, and SLC22A16) were up-regulated, and five genes (CA1, FBXO9, SNCA, STRADB, and TRMT12) were down-regulated among those who experienced stress induction, compared with the control group. Nine genes of eleven were linked to endocrine system disorders, neurological disease, and organismal injury and abnormalities.

CONCLUSION Of the genes identified in this study, HCP5, SLC22A16, and SNCA genes have previously been proposed as therapeutic targets for cancer and Parkinson disease. Further studies are needed to examine pathological mechanisms through which these genes mediate effects of psychological stress on adverse health outcomes. Such studies may ultimately identify therapeutic targets that enhance biological resilience to adverse effects of psychological stress.

KEYWORDS Stress, psychological; sequence analysis, RNA; psychological tests; US

INTRODUCTION

Psychological stress is associated with adverse health outcomes, particularly with depression, cardiovascular disease and cancer.[1] While many studies have demonstrated a potential link between chronic stress and altered inflammatory cytokine levels, immune dysfunctions and delayed neuronal recovery,[2,3] it is still unclear how acute stress is processed at transcriptional levels. Recently, a study conducted in mice demonstrated that a single stress event left long-lasting changes in microRNA, messenger RNA and protein expressions in the amygdala.[4] Although many animal studies have attempted to determine the mechanisms underlying stress pathophysiology, human studies investigating subcellular responses to acute psychological stress are limited. In addition, it is recognized that gene expression in animal models is not readily translated to humans.[5]

Available data collected in humans demonstrate that acute psychological stress is a potent trigger of inflammatory, neuroendocrine and metabolic responses, resulting in predisposition to disease. For example, acute stress increases proinflammatory gene expression, including interleukin 6 (IL-6), interleukin 1 beta (IL-1β), type 1 T-helper/type 2 T-helper (TH1/TH2) cytokines, catecholamine receptors, nuclear factor kappa beta (NF-kB), I kappa beta kinase (IκB), or tumor necrosis factor alpha (TNF-α);[6-10] the hypothalamic–pituitary–adrenal (HPA) axis-related genes;[11] immune activity;[12,13] and glucose metabolism.[14] However, most studies have investigated expression of only a few select genes, and only a limited number of studies have examined genome-wide transcriptional activity in response to acute psychological stress using microarrays.[15,16] The RNA-sequencing (RNA-seq) genomic approach to identifying differentially-expressed genes may contribute to a better understanding of the biological mechanisms underlying acute psychological stress responses. It may also identify novel genes that influence previously known and unknown stress response pathways. We investigated the effect of acute psychological stress on genome-wide transcriptome profiles in whole-blood samples via RNA-seq and explored which genes may be differentially expressed after stress induction using a randomized controlled design. We used the Trier Social Stress Test (TSST), a standard laboratory procedure used to reliably induce stress in human research participants.[17,18] This exploratory approach may identify genes that can then be studied in future confirmatory work.

IMPORTANCE This study highlights the need to understand the role of HCP5, SLC22A16, and SNCA genes in linking psychological stress to cancer and Parkinson disease.

METHODS

Participants All measurements and blood samples were obtained during a previous study, which evaluated changes in arterial stiff-

ness after stress induction.[19] After institutional review board approval for the previous study, a convenience sample of 85 women was recruited from Charlottesville, Virginia, USA, communities, using flyers, emails and word of mouth. The study included female adults aged 18–55 years. This selected sex and age range reduced sample heterogeneity and obviated the need to control for potential confounding effects of older age and menopause on cardiovascular function. To minimize potential confounding effects, women were also excluded who were taking medications for psychological issues (e.g., anxiolytics and antipsychotics), had any diagnosed cardiovascular disease (e.g., hypertension, diabetes mellitus or hyperlipidemia), or had gone through menopause. In the previous study, participants were randomized by coin flips into either the intervention (stress induction) or control (no stress induction) group. Financial considerations prohibited genetic sequencing on all 85 participants. Because the previous study aimed to test changes in transcriptome profiles in relation to arterial stiffness before and after stress induction, only the 40 participants who had exhibited changes in arterial stiffness were selected for genetic sequencing; 22 received the experiment (experimental group), and 18 did not (control group). This sample was used in the current study to analyze transcriptome changes related to acute psychological stress induction.

Ethics All procedures were conducted after the approval from the Institutional Review Board at University of Virginia, USA. Written informed consent was obtained from all patients included in the study.

Background Age, race, per capita income and education level were self-reported by participants.

Psychological factors Subjective acute psychological stress levels were measured by the Subjective Units of Distress Scale (SUDS) [20] and the state anxiety subscale of the Spielberger State-Trait Anxiety Inventory (STAI).[21]

Subjective Units of Distress Scale: Current and peak distress was measured by SUDS, widely-used one-item scale.[20] The question asked before the experiment (for the experimental group) or the sitting period (for the control group) was: "On a scale of 0 to 10, with 0 'not distressed at all' and 10 'the most distressed', what is your distress level now?" The question asked after the experiment (or after the sitting period) was: "On a scale of 0 to 10, with 0 'not distressed at all' and 10 'the most distressed', what was your highest level of distress during the experiment or the sitting period?".

State anxiety subscale of the Spielberger State-Trait Anxiety Inventory: State anxiety was also measured before and after the experiment (or after the sitting period for the control group). The 20-item state anxiety subscale uses a 4-point Likert-like scale to assess anxiety intensity, tied to "how one feels right now, that is, at this moment" (e.g., feelings of worry, tension). The total score is derived from the sum of the items with higher scores indicating greater anxiety.[21] This scale has been used extensively and has strong construct and divergent validity and internal consistency in large samples.[22] In the current study, baseline state anxiety scale internal consistency was excellent (Cronbach's α = 0.90).

Physiological factors

Mean arterial pressure and heart rate Blood pressure (BP) and heart rate (HR) are commonly used to assess physiological responses to stress. In this study, BP and HR were included as indicators of acute psychological stress, along with SUDS and STAI's state anxiety subscale. BP and HR were measured using the Welch Allyn Vital Signs Monitor 300 Series (Welch Allyn, USA). After measuring mid-section circumference of the nondominant upper arm, the proper-sized cuff was applied snuggly with the artery marker on the cuff placed over the brachial artery.[23] BP was measured on the arm kept still, at the level of the heart. Given that concurrent use of systolic BP and diastolic BP may cause multicollinearity issues, mean arterial blood pressure (MAP) was used by calculating diastolic BP +1/3 (systolic BP–diastolic BP).

Body mass index (BMI) This is an anthropometric measurement that may affect physiological response to stress;[24] thus, BMI was included as a covariate in transcriptome analysis. Height (m) and weight (kg) were measured to determine BMI (kg/m^2). Height was measured using a wall stadimeter (Accu-Hite, USA), and weight was measured with an electronic scale (Penn Scale, USA).

Procedures Participants' physiological conditions were standardized according to the European Society of Cardiology's recommendations for measuring cardiovascular function.[25,26] To minimize variance caused by circadian patterns, study procedures were conducted in a quiet room between 1:00 PM and 3:00 PM. All participants refrained from vigorous exercise and from consuming coffee, tea, bananas, chocolate, cocoa, citrus fruits and vanilla for one day before data collection, because vigorous exercise and consumption of such foods may change cardiovascular hemodynamics. Participants ate the same breakfast of cereal (35 g), milk (250 mL) and orange juice (250 mL) at 8:00 AM, followed by a fasting period until data collection was completed in the afternoon.

Pre-test data collection For baseline stress measurements, participants completed the SUDS and the STAI subscale. Next, height and weight were measured. After 10 minutes resting in the supine position, BP was measured. Blood was drawn by an experienced nurse–phlebotomist. Participants were then randomized by coin flip to either experimental or control group.

Trier Social Stress Test (TSST) For the experimental group, acute psychological stress was induced by using the TSST, which is a valid and reliable tool used to induce acute psychological stress and to study biological responses to stress in laboratory settings.[17,18] The intention of the TSST is to create perceived uncontrollability and fears of negative social evaluation. These two components are considered central in biological stress reactivity activation, such as activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic adrenal–medullary axis.[27] The TSST requires speech performance and verbal arithmetic performance in front of an audience, and is known to induce considerable changes in corticotropin,[28] cortisol,[29,30] blood pressure [29] and heart rate.[31]

The TSST was conducted in an intervention room located across the hall from the room where baseline measures were collected. Individuals assigned to the experimental group were given instructions for the upcoming TSST task. They had 10 minutes to prepare a 5-minute speech in which they were asked to convince two interviewers that they were a strong candidate for their dream job. Participants were told that their performances would be videotaped and evaluated by interviewers. Participants stood in front

of a video camera and two interviewers (research staff dressed in lab coats). During the presentation, the interviewers maintained neutral expressions and periodically took notes. If participants stopped early, they were encouraged to continue. If a participant repeatedly looked at the interviewers or stopped talking for more than 10 seconds, they were told that "you have X minutes left but you can let me know if you wish to stop". Each presentation was immediately followed by a mental arithmetic test, which involved sequentially subtracting the number 13 from 1022. If a mistake was made, they were asked to start again from the beginning. This continued until the participant had completed the task for the full five minutes.[17] Participants in the control group spent about 20 minutes quietly sitting in the same intervention room without undergoing the TSST.

Post-test data collection Immediately after the TSST period or the sitting period, participants in both groups completed the SUDS a second time. Next, in a supine position, BP was measured, and peripheral blood was drawn again from peripheral catheters, which had been placed earlier. Finally, participants were asked to complete the STAI subscale again before being fully debriefed and compensated for their participation.

RNA extraction, library preparation and sequencing Peripheral blood (2.5 mL) was collected in a PAXgene RNA tube (QIAGEN, USA) and stored at –80 ˚C until ready for RNA extraction. RNA was extracted from whole blood using the PAXgene Blood RNA system (QIAGEN, USA). RNA sample quality was evaluated using an Agilent 4200 TapeStation (Agilent Technologies, USA) by the RNA Integrity Number (RIN), and the quantity of RNA was measured using a Qubit (Life Technologies, USA). All samples used for this study had excellent purity (A260/A280 ≥1.9; A260/A230 ≥2) and showed no visible signs of degradation (RIN ≥9). We used the TruSeq Stranded mRNA library prep kit (Illumina, USA) to generate mRNA-sequencing libraries. These kits generated high-quality libraries for sequencing by fragmentizing RNA, performing reverse transcription and ligating the indexed adapters. This allowed individual libraries to be pooled in an equimolar fashion, minimizing the potential technical bias of run variation. Pooled libraries were then sequenced with an Illumina NextSeq 500 instrument (Illumina, USA).

Analysis Study participant characteristics are described by means and standard deviation (SD) for continuous variables, and by frequency and percent for categorical variables. Condition differences in participants' characteristics at baseline were examined using independent sample t-tests. To compare psychological and physiological responses between the experimental and control groups, repeated measures of covariance analyses (RM-ANCOVAs) were conducted; controlling for factors which may affect physiological responses to stress (age, MAP and BMI). The within-subjects factor was 'time' (pre- and post-stressor), and the between-subjects factor was 'stressor' (TSST and control). Interactions of within-subjects factor and between-subjects factor were tested. All statistical analyses were performed with SPSS Statistics 25 for Windows (SPSS, USA).

Bioinformatic analysis of RNA-sequencing data We performed bioinformatics quality control using FastQC, version 0.11.7 (Babraham Bioinformatics, UK). Poor quality reads and adapter sequences were filtered out by running CutAdapt, version 2.5.[32] To confirm the quality of the library and sequencing, we used RNA-SeQC[33] for quality control specific to RNA-sequencing, and assessed total number of reads, depth of reads, average read length, average coverage across the gene, number of identified genes, PCR duplication rate, ribosomal content and exon/intron representation. We aligned the raw reads to the GRCh38 reference genome using STAR version 2.6.1a.[34] We counted number of reads mapped to genes using HTSeq, version 0.11.0.[35] We performed differential gene expression analyses between experimental and control groups using DESeq2, version 1.30.1 [36] while controlling for age, MAP and BMI as potential covariates. The gene count table was imported to DESeq2. Read distribution was modeled as a negative binomial distribution with mean and variance estimated from data. P values were calculated by using the Wald test. Multiple testing correction was performed with Benjamini-Hochberg's False Discovery Rate (FDR) adjusted by the Independent Hypothesis Weighting method with a cutoff of 0.05 (on FDR),[37] accounting for age, BMI and MAP as covariate variables. R version 4.0.3 (2020-10-10) and BiocManager version 3.12 (Bioconductor, USA) were used.

Construction of pathway–gene–process network Biological pathways and networks related to stress induction were identified using Ingenuity Pathway Analysis (Ingenuity Systems, USA). Genes were selected as inputs for pathway testing when FDR was ≤0.05. Right-tailed Fisher's exact test was conducted to calculate significance values of pathway fittings.

RESULTS

Characteristics of participants Average participant age was 31.4 years (SD 11.6). Most study participants were Asian (90.0%), and ethnicity was not assessed. Average BMI was in the normal range with a mean of 23.16 (SD 4.69) kg/m^2. While the sample tended to be well educated, average per capita income was low with a mean of $19,298.39 per year (SD 12,921.45), because many participants

Table 1: Study participant characteristics

	All N (%) or Mean (SD)	Experimental Group n (%) or Mean (SD)	Control Group n (%) or Mean (SD)
Number of participants	**40 (100.0)**	**22 (55.0)**	**18 (45.0)**
Age (years)	31.4 (11.6)	29.6 (10.0)	33.6 (13.2)
Race			
Asian	36 (90.0)	20 (90.9)	16 (88.9)
Black	4 (10.0)	2 (9.1)	2 (11.1)
Body mass index (kg/m^2)	23.16 (4.69)	22.62 (4.25)	23.81 (5.25)
Per Capita Income ($)	19,298.39 (12,921.45)	20,089.74 (15,037.39)	18,726.85 (11,584.51)
Education			
High school graduate	7 (17.5)	2 (9.0)	5 (27.8)
Associate degree	10 (25.0)	8 (36.4)	2 (11.1)
College/university degree	13 (32.5)	6 (27.3)	7 (38.9)
Graduate degree	10 (25.0)	6 (27.3)	4 (22.2)

SD: Standard deviation

were graduate/undergraduate students. Both groups were quite similar for all demographic characteristics (Table 1).

Effects of TSST on psychological stress and physiological measures RM-ANCOVA results were as follows: interactions between the within-subjects factor (time) and the between-subjects factor (TSST) were significant, showing that participants who completed the TSST had significantly higher scores on SUDS ($F_{1,32}$ = 59.89, p = 0.000, and η^2 = 0.65) and state anxiety ($F_{1,35}$ = 10.62, p = 0.002, and η^2 = 0.23) after stress induction compared to the control group, after controlling for age, MAP and BMI. Participants in the experimental group also showed significantly higher systolic BP ($F_{1,35}$ = 26.53, p = 0.000, and η^2 = 0.43), diastolic BP ($F_{1,35}$ = 18.76, p = 0.000, and η^2 = 0.34) and HR ($F_{1,35}$ = 7.49, p = 0.010, and η^2 = 0.18) after stress induction, compared with the control group, after controlling for age and BMI (Table 2).

Table 2: Trier Social Stress Test effects on psychological stress and physiological measures

	Mean (SD)				P Value for ANCOVA (condition per time interaction)
	Pre-test		Post-test		
	Exp.	Control	Exp.	Control	
SUDS	2.05 (1.84)	2.73 (1.98)	5.46 (1.89)	1.67 (1.54)	<0.001
SA	31.64 (7.29)	31.89 (7.91)	35.50 (10.34)	29.06 (8.78)	0.002*
Systolic BP	101.18 (6.56)	104.11 (10.15)	110.27 (9.62)	104.94 (8.91)	<0.001†
Diastolic BP	60.50 (5.99)	62.83 (8.35)	65.36 (6.91)	61.78 (8.33)	<0.001†
Heart Rate	58.41 (7.84)	60.61 (7.06)	61.86 (8.41)	59.78 (6.71)	0.01*

ANCOVA: analysis of covariance; BP blood pressure; Exp.: Experimental group; SA: Spielberger Sate-Trait Anxiety Inventory–state anxiety subscale; SD: standard deviation; SUDS: Subjective Units of Distress Scale
* Age, body mass index, and mean arterial pressure were controlled.
† Age and body mass index were controlled.

Differentially-expressed genes after stress induction in experimental group compared with control group Illumina's NextSeq 500 sequencer generated around 30 million paired-end reads with read lengths of 75 bp (2 x 75 bp) per sample/library. A total of 22,021 genes were expressed. Tables 3 and 4 present the significantly up- and down-regulated genes associated with stress induction. Figure 1 shows normalized mRNA expression in terms of read counts for the differentially regulated genes under the acute stress condition (TSST). The Ingenuity Pathway Analysis (IPA) (QIAGEN, Germany) identified one related network; "Endocrine System Disorders, Neurological Disease, Organismal Injury and Abnormalities" with a score of 27 (Figure 2). Nine molecules of 11 were found in this network.

DISCUSSION

This exploratory study investigated genes that were differently-expressed between two conditions that varied as to whether or not participants were exposed to the TSST, an acute psychological stressor. The psychological and physiological stress parameters (SUDS, state anxiety/STAI, BP and HR) indicated that the TSST successfully induced moderate—but statistically significant—levels of acute psychological stress in experimental group participants. Results showed significant up-regulation of six genes (HCG26, HCP5, HLA-F, HLA-F-AS1, LOC1019287, and SLC22A16) and down-regulation of five genes (CA1, FBXO9, SNCA, STRADB, and TRMT12) in the stress-induced group, compared with the control group.

Table 3: Up-regulated genes following stress induction in experimental group compared with control group

Gene symbol	Gene name	log2 (Fold-change)	Adjusted p Value (FDR)
HCG26	Human leukocyte antigen complex group 26	0.36	0.001
HCP5	HLA complex P5	0.31	0.043
HLA-F	Major histocompatibility complex class I, F	0.29	0.049
HLA-F-AS1	HLA-F antisense RNA 1	0.33	0.006
LOC1019287	Undefined	0.82	0.001
SLC22A16	Solute carrier family 22 member 16	0.78	0.041

FDR: false discovery rate; HLA: Human Leukocyte Antigen

Table 4: Down-regulated genes after stress induction in experimental group compared with control group

Gene symbol	Gene name	log2 (Fold-change)	Adjusted p Value (FDR)
CA1	Carbonic anhydrase1	–1.08	0.001
FBXO9	F-box protein 9	–0.27	0.004
SNCA	Synuclein alpha	–0.86	0.048
STRADB	STE20 related adaptor beta	–0.71	0.043
TRMT12	tRNA methyltransferase 12 homolog	–0.28	0.030

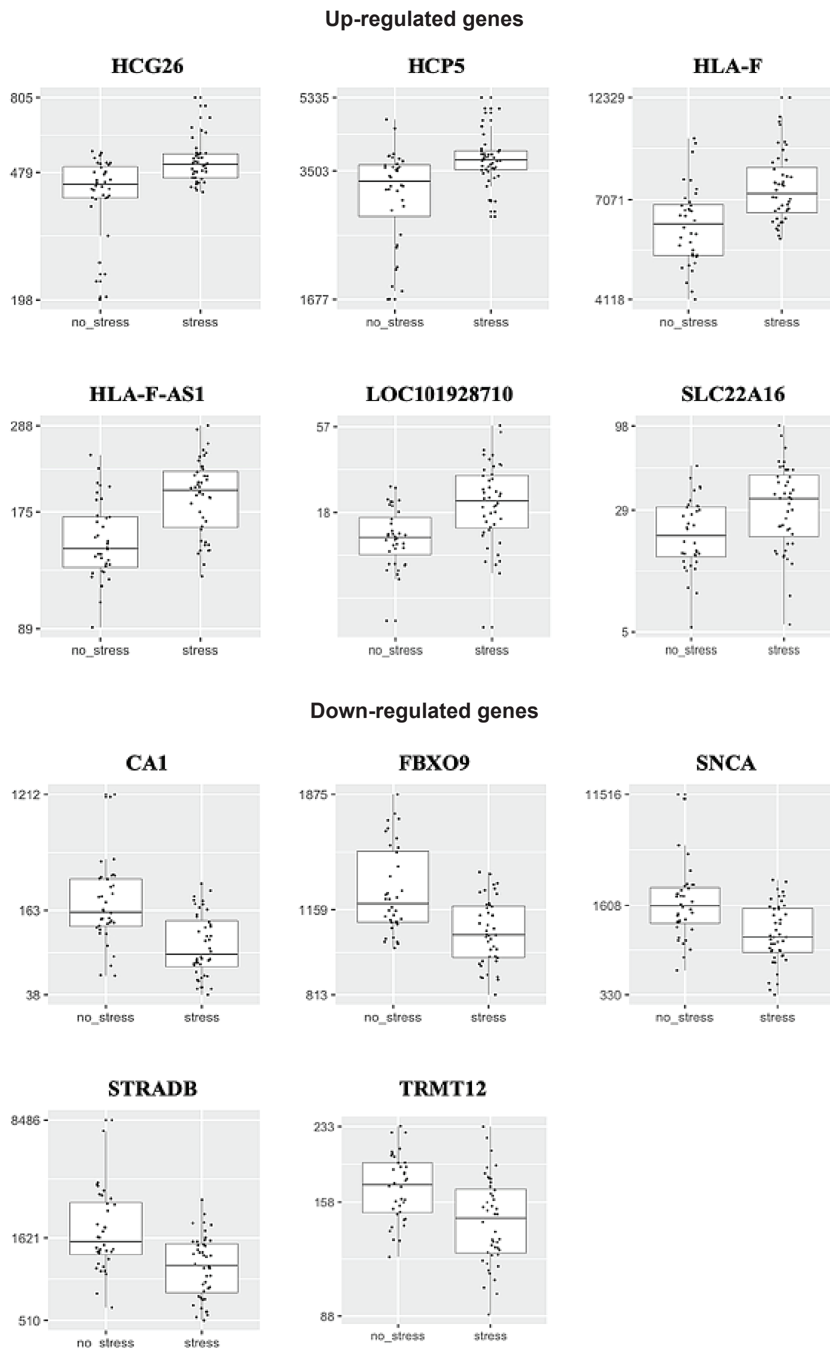
FDR: False discovery rate

IPA analysis demonstrated that 9 genes of 11 are implicated in endocrine system disorders, neurological disease, and organismal injury and abnormalities. This study revealed novel genes that have not been previously reported in relation to psychological stress. While this research was exploratory and requires confirmatory studies, it indicates that ultimately these genes may help elucidate pathophysiological mechanisms through which psychological stress is linked to disease conditions.

Up-regulation of genes linked to psychological stress induction Among six up-regulated genes, four genes (HCG26, HCP5, HLA-F, and HLA-F-AS1) are affiliated with the Human Leukocyte Antigen (HLA) complex, which is also referred to as the Major Histocompatibility Complex (MHC) in humans. The HLA complex is a group of proteins on the cell surface known to play a critical role in the immune system.[38] All four genes are also long non-coding RNAs (lncRNA), defined as RNA with more than 200 nucleotides that have no protein-coding capacity. The crucial function of lncRNA and its regulatory role in tumor occurrence and progression has been recognized in multiple studies.[39]

Histocompatibility leukocyte antigen complex p5 (HCP5) is known to play important roles in cancer cell proliferation, migration and invasion in many cancers.[40,41] With the down-regulation of HCP5's anti-tumorigenic effect,[42] HCP5 is considered a promising biomarker and therapeutic target. On the other hand, only a few studies have been conducted on HCG26, HLA-F, and HLA-F-AS1, and the molecular mechanisms by which they are involved in biological processes remain largely unknown. HCG26 is in HLA complex group 26.

Figure 1: Normalized read counts (Y-axes) for differentially-expressed genes in experimental group (stress) compared with control group (no stress)



One study exploring the roles of lncRNAs in follicular development demonstrated that HCG26 expression was up-regulated in patients with polycystic ovary syndrome and was associated with follicle count and cell proliferation.[43] HLA-F encodes HLA Class I Histocompatibility Antigen, Alpha Chain F.[44] A previous study on patients with breast cancer showed that HLA-F expression was positively associated with tumor size and poor clinical outcomes.[45] HLA-F antisense RNA 1 (HLA-F-AS1) has been reported as up-regulated in colorectal cancer cell tissues and could promote colorectal cancer cell proliferation.[46] Acute stress induces leukocyte redistribution and increases cellular adhesion, molecule expression and chemotaxis, all of which are critical in immune cell recruitment and migration.[12] As per acute psychological stressor's effects on leukocytosis, our findings suggest that acute psychological stress may dysregulate these four lncRNAs in the HLA system, which are known to play an important role in immune responses.

While we still do not know LOC1019287's function, the function of SLC22A16 has been well-documented.[47] Membrane transporters are proteins that carry molecules across the cell membrane. Solute carrier (SLC) transporters are one of two large groups of membrane transporters. The SLC22 family contains cation and carnitine transporters, which include SLC22A16.[47] Previous studies have suggested that SLC22A16 may be a novel target for cancer treatment. For example, a study on acute myeloid leukemia demonstrated that SLC22A16 showed the greatest differential expression in acute myeloid leukemia cells among different carnitine transporters, compared with normal cells.[48]

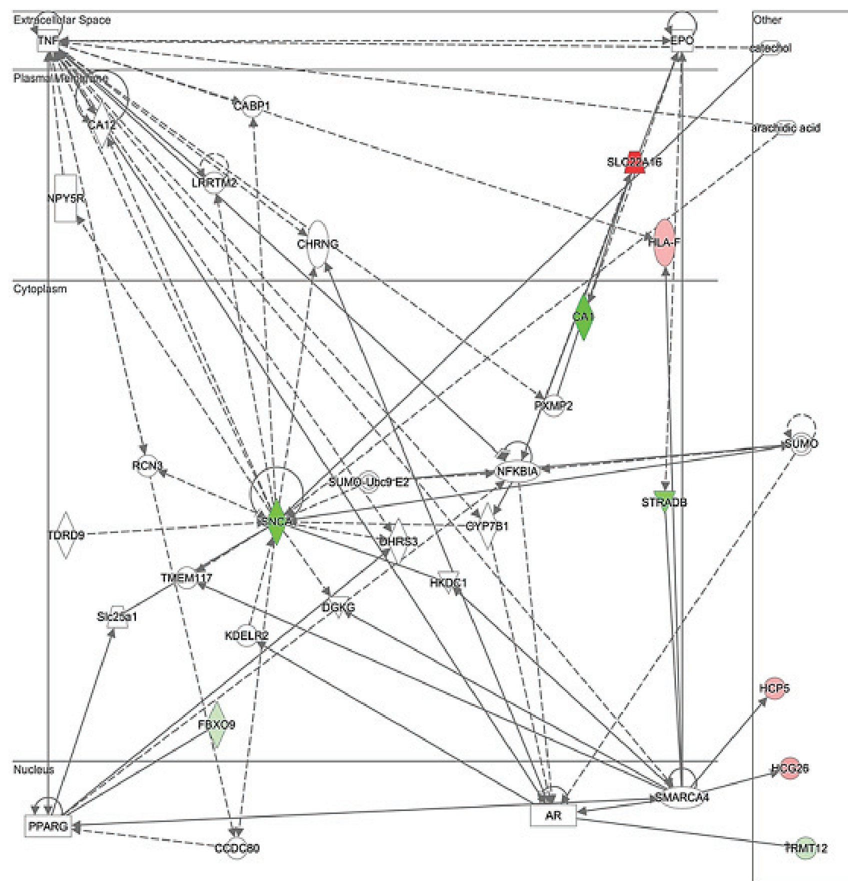
Another study identified SLC22A16 as one of 13 hub genes involved in nasopharyngeal carcinoma carcinogenesis or progression, and could be useful as a diagnostic biomarker for nasopharyngeal carcinoma.[49] It has also been reported that SLC22A16 up-regulation is an independent unfavorable prognostic indicator in gastric cancer.[50] While the results from our exploratory analyses need to be interpreted cautiously, coupled with previous reports on the high correlation between SLC22A16 and different types of cancer, they can inform future studies that may elucidate the causal relationship between stress and cancer.

Down-regulation of genes linked to psychological stress induction SNCA has previously been associated with psychological stress in animal models.[51] SNCA encodes α-synuclein, one of three families of synuclein that are soluble proteins found in nervous system tissue. Variants within SNCA have been studied extensively due to SNCA's link to Parkinson disease.[52] α-synuclein is known to have a role in synaptic vesicle cycling,[53] and overexpression of SNCA has a detrimental effect on neuron function.[52] Plasma and serum α-synuclein are suggested potential diagnostic biomarkers in Parkinson disease patients.[54]

There is interest in the association between psychological stress and Parkinson disease, and one study showed that chronic mild stress accelerates aggregation of α-synuclein in male mice,[51] suggesting a different directional effect than observed in this study. Given our result suggesting that acute stress down-regulates SNCA, it appears that acute and chronic psychological stress may exert different effects on SNCA expression, but clearly both the animal models and our study procedures need to be replicated and extended.

Down-regulated genes after stress induction also included CA1, F-box only protein 9 (FBXO9), STRADB, and TRMT12. CA1

Figure 2: The network identified by Ingenuity Pathway Analysis



Green-colored genes are up-regulated genes and red-colored genes are down-regulated genes. The darker the color, the more up or down-regulated the genes.

encodes carbonic anhydrase 1 which belongs to a family of zinc metalloenzymes. CA1 is known to catalyze the reversible hydration of carbon dioxide and be involved in the regulation of hemoglobin's affinity for oxygen.[55] A previous study on patients with colon cancer showed that higher CA1 expression levels were linked to higher survival probability than lower CA1 expression levels, and identified CA1 as a potential biomarker due to its predictive role in colon cancer status and survival time.[56] A recent study showed that CA1 is up-regulated in septic patients, suggesting the protective response of white cells in hostile environments like sepsis.[57] CA1 down-regulation in our study suggests that the gene may respond differently to severe stress conditions. FBXO9 is a member of the F-box protein family, which constitutes one of the four subunits of the ubiquitin protein ligase. A study showed that primary tumors with FBXO9 loss expressed high levels of proteins associated with metastasis and invasion.[58] STRADB encodes STE20-related kinase adapter protein beta enzyme, which is involved in cell cycles and apoptosis.[59] Reduced STRADB expression is associated with increased cell cycle length and consequent slowing down of the cell cycle.[60] TRMT12 is one of the tRNA methyltransferases that catalyze RNA methylation. TRMT12 is highly expressed in a large cohort of primary tumors,[61] and different cancer cell lines.[62]

Although not extensively studied, previous research suggests that CA1, FBXO9, and TRMT12 are implicated in cancer development. Future studies are needed to explore how various types of stress (e.g., acute vs. chronic psychological stress, or psychological vs. physiological stress) may differently regulate expression of these genes.

The strengths of this study include using a genomic approach to explore stress responses in healthy human subjects, use of RNA-sequencing, which is superior to the use of microarrays in gene expression profiling,[63] and successful induction of psychological stress in a laboratory setting, using the TSST. Nevertheless, this study has several limitations. First, relatively few genes were differentially expressed in the two groups.

Furthermore, inflammatory genes like cytokines, that have shown differential expression in prior studies, have not been expressed differently in the two groups of our study. This may be due to our study's convenience sampling. Selecting participants that exhibited changes in arterial stiffness after stress induction may generate the results that reduce generalizability of the study findings, given that the participants may be more likely to demonstrate hemodynamic changes compared to others. Second, because this sample is small, and includes only females, the majority of whom were of Asian descent, sex, race and other confounders should also be considered. Third, while use of gene expression patterns in whole blood cells is convenient and may have advantages for translational research, RNA-seq studies of homogeneous cell populations or specific tissues can be more informative.[64] Fourth, people with high levels of trait anxiety are more likely to respond in stressful situations with increased anxiety. Therefore, future studies should explore whether higher scores on trait anxiety play a role in transcriptional activity related to acute stress situations. Fifth, the findings from this exploratory study should be replicated in a study conducted with a larger sample that includes quantitative reverse transcription PCR (RT-qPCR). Last, the potential confounding effects of lymphocyte subset redistribution were not controlled for in analyses. Lymphocyte redistribution in response to acute stress and sympathetic nervous system activation has been well-described;[65] thus, isolated leukocyte subpopulation analysis should be considered in future studies.

CONCLUSION

This study highlights previously unreported associations of 11 genes with acute psychological stress and provides further evidence of stress-induced alterations of the gene expression profile. At present, we cannot explain the molecular mechanisms of these genes in stress responses. Future followup studies should validate the identified genes and explore the underlying mechanisms linking psychological stress, the identified genes, and their associated diseases, including cancer and neurodegenerative diseases. Such studies will clarify pathological mechanisms by which vulnerabilities to the diseases may be initiated or aggravated by psychological stress and may ultimately identify therapeutic targets that will enhance biological resilience to adverse effects of psychological stress.

FINANCING

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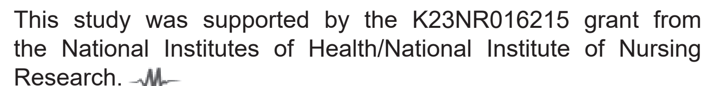

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Methanol Toxicity Outbreaks in the Americas: Strengthening National Prevention and Response Measures

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Unintentional poisonings remain a substantial global health challenge, resulting in an estimated 106,683 deaths and 6.3 million disability-adjusted life years in 2016.[1] Of these poisonings, methanol toxicity results from the metabolic breakdown of ingested methanol found in cleaning products, antifreeze, paints, and harmful and potentially lethal acidic compounds, among others. Consumption of tainted alcoholic beverages has recently been documented as one source of toxic exposure whose clinical diagnosis and management is too often delayed.[2]

At the 1992 UN Conference on Environment and Development (the 'Earth Summit') and the 1994 Intergovernmental Forum on Chemical Safety, global leaders highlighted the need to strengthen capacities for diagnosis, surveillance and response concerning exposure to harmful chemicals by setting up national poison control centers. These centers offer key clinical guidance for diagnosis and management of poisonings, conduct data monitoring and include a clinical treatment area or toxicology laboratory. Over the past two decades, however, only 47% of WHO member states have confirmed the existence of poison control centers, leaving significant gaps across Africa and Asia.[1] Moreover, recent outbreaks of methanol toxicity reported worldwide—including in the Czech Republic and Honduras in 2012, Libya in 2013, Kenya in 2014 and Nicaragua in 2015—highlight the need for greater attention to reducing harmful chemical exposure to promote population health.[3,4]

On the patient care side, clinicians are challenged to take a comprehensive medical history and thoroughly evaluate symptoms such as decreased consciousness and visual acuity, nausea, vomiting and abdominal pain, in order to exclude differential diagnoses of metabolic acidosis or other conditions. They must be prepared to administer correct care promptly to avoid complications like kidney failure, blindness or death.[3] Low-resource settings with weak health system infrastructures, however, may not have antidotes, medical services or intensive care support readily available, further jeopardizing low-income and other vulnerable populations.

In the Dominican Republic (DR), we analyze data collected from the surveillance system of the Ministry of Health's General Division of Epidemiology. The Ministry recently reported five significant outbreaks of methanol poisoning, as nationally notifiable events (i.e. individuals requiring medical attention), due to ingesting contaminated or counterfeit alcohol from clandestine distilleries. Specifically, outbreaks occurred in December 2017 (41 intoxications/29% mortality), December 2019 (4 intoxications/50% mortality), April 2020 (369 intoxications/62% mortality), November 2020 (9 intoxications/56% mortality) and April 2021 (25 intoxications/44% mortality).[4] Yet to date, no formal poison control center exists in the country.

To address this concerning health problem, the Administration of president Luis Abinader published Executive Order 275-21 (*Decreto Presidencial*) in April 2021, which expanded the scope of Executive Order 288-96 to apply Law 50-88 on Drugs and Controlled Substances in the Dominican Republic; the Order is designed to strengthen oversight of controlled substances in the DR,[5] and recognizes the legal production, distribution and importation of methanol, isopropanol and propanol, but strictly for industrial use. Federal agencies were mandated to evaluate drinks sold in markets and dismantle clandestine factories producing tainted alcohol. The Ministry of Health also developed public educational campaigns to raise awareness on the hazards of consuming illicit alcoholic beverages.

> **We recommend national governments implement a four-pronged approach that can reduce exposure to harmful substances and protect population health**

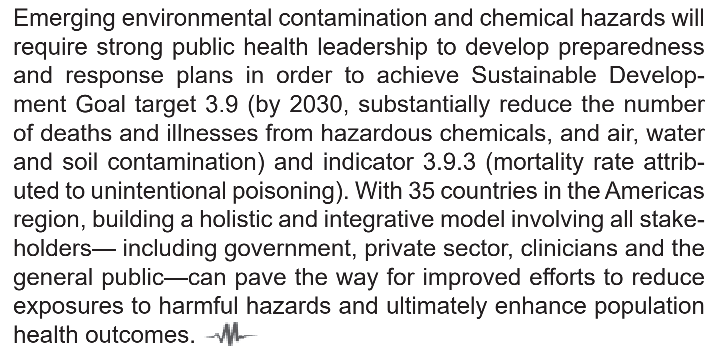
Recommendations for the Americas Region In the Americas, limitations in clinical diagnostic tools, recommended medical treatment, emergency or intensive care unit capacity and insufficient health worker training hinder quality health care delivery across communities. In order to close this practice gap, we recommend national governments implement a four-pronged approach that can reduce exposure to harmful substances and protect population health. These strategies include continuing national government oversight of the industrial use of methanol, monitoring commercial sales of alcoholic beverages, strengthening public health infrastructure and implementing capacity-building activities.

First, national oversight should prioritize laws to regulate industrial use of methanol as a primary material or adjuvant in product manufacturing; these laws should support quality control measures that allow for tracking product purchases, commercial distribution and customs declarations. With such legal mechanisms in place, production can be monitored and the clandestine use of methanol in liquor production limited and finally discontinued.

Second, strict guidelines are needed to help ensure that liquor distributors and other points of commercial sales do not acquire, distribute or store unlicensed products whose consumption can be dangerous to health. Additional public policies enforcing standardized processes prohibiting inclusion of 'home brew' manufacturing of unauthorized alcohol products would also aid in controlling such poisonings.

Third, clinical guidelines and surveillance must streamline emergency management for methanol toxicity across health institutions. National epidemiological surveillance in hospitals and primary health care centers can help leaders promptly identify outbreaks and at-risk communities. Moreover, investment in poison control centers can support surveillance programs, offer appropriate treatment and management with clinical expertise, and reinforce connections between health workers, community leaders and local residents.

Fourth, continuing education seminars for health workers can hone clinical suspicion and offer periodic updates on data trends. Furthermore, health professionals' training and graduate studies should incorporate scientific content and toxicology case studies into curricula. Community engagement led by health workers can also improve health literacy through local educational campaigns and public service announcements prepared especially for public transportation, newspapers and social media. Finally, with support from regional health agencies like PAHO, knowledge transfer across the Americas can be guided by countries with established poison control centers, advanced clinical protocols and robust surveillance programs.



Emerging environmental contamination and chemical hazards will require strong public health leadership to develop preparedness and response plans in order to achieve Sustainable Development Goal target 3.9 (by 2030, substantially reduce the number of deaths and illnesses from hazardous chemicals, and air, water and soil contamination) and indicator 3.9.3 (mortality rate attributed to unintentional poisoning). With 35 countries in the Americas region, building a holistic and integrative model involving all stakeholders— including government, private sector, clinicians and the general public—can pave the way for improved efforts to reduce exposures to harmful hazards and ultimately enhance population health outcomes.

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