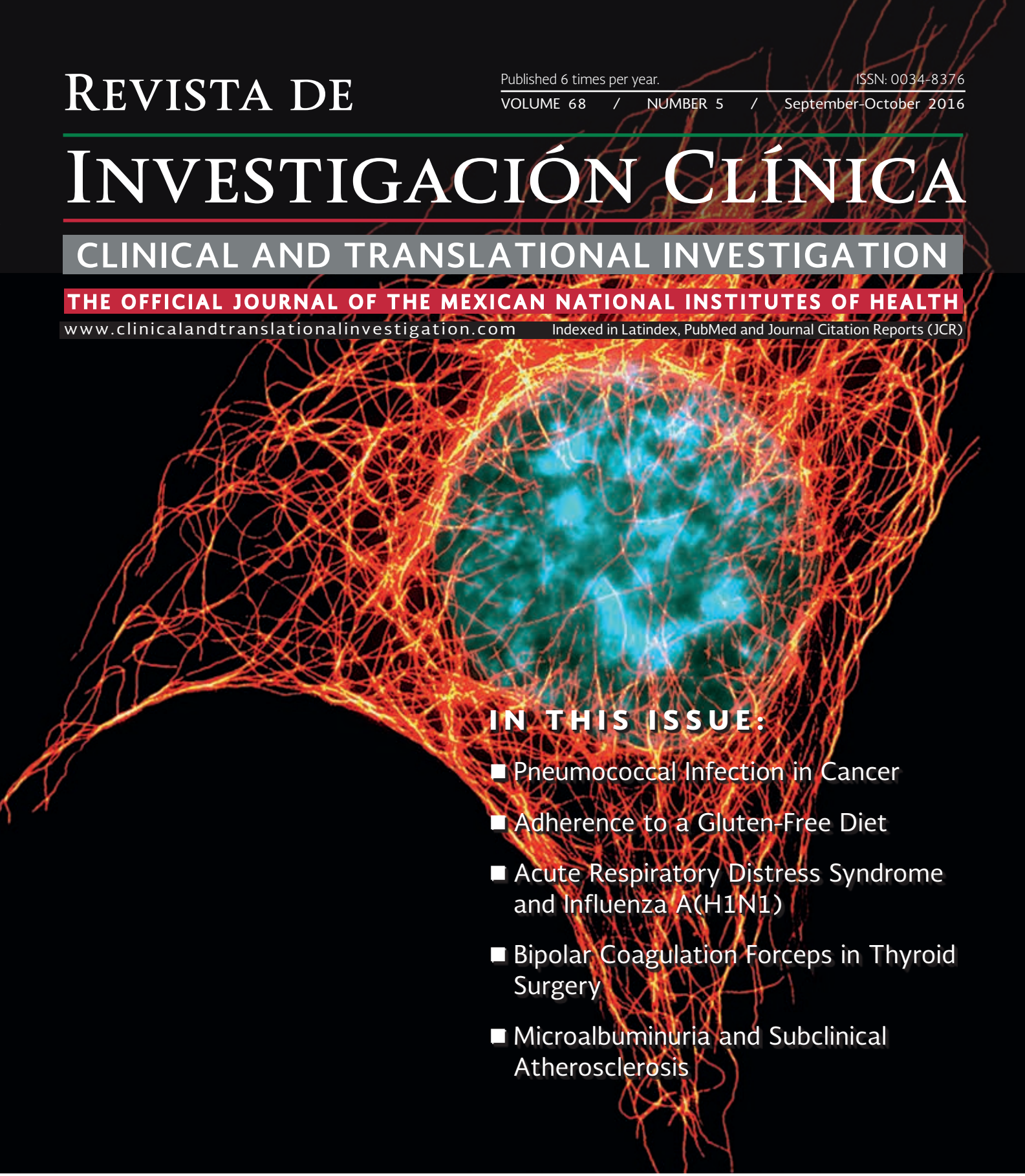
Published 6 times per year. ISSN: 0034-8376
VOLUME 68 / NUMBER 5 / September-October 2016

Revista de Investigación Clínica

Clinical and Translational Investigation

THE OFFICIAL JOURNAL OF THE MEXICAN NATIONAL INSTITUTES OF HEALTH
www.clinicalandtranslationalinvestigation.com Indexed in Latindex, PubMed and Journal Citation Reports (JCR)



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SECRETARÍA DE SALUD

Published 6 times per year. ISSN: 0034-8376
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CLINICAL AND TRANSLATIONAL INVESTIGATION

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Revista de Investigación Clínica is the Journal of the Secretaría de Salud

Revista de Investigación Clínica is the Journal of the Secretaría de Salud and Official Journal of the National Institutes of Health of Mexico.

Administration: Comisión Coordinadora de Institutos Nacionales de Salud y Hospitales de Alta Especialidad. CCINSHAE.

Editorial: Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. INCMNSZ

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Impreso en papel totalmente libre de cloro

Este papel cumple los requisitos de ANSI/NISO Z39.48-1992 (R 1997) (Papel Permanente)

Edición impresa en México

ISSN: 0034-8376
Ref.: 2931AX165

Cover: HeLa cell with the microtubules labeled with anti-alpha-tubulin and Alexa-555 and the nucleus with DAPI. The imagen was captured by high resolution microscopy (SIM), Elyra System PS.1, Carl Zeiss employing the software ZEN 2012 and a 630x magnification. Courtesy of Dr. Christian Hellriegel, Carl Zeiss Microscopy GmbH, Halle-Neustadt Area, Germany and Microscopy Unit, Instituto Nacional de Cancerología-Instituto de Investigaciones Biomédicas-Red de Apoyo a la Investigación (RAI), UNAM, Mexico.

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Rev Inves Clin. 2016;68:221-8 ORIGINAL ARTICLE

Invasive and Complicated Pneumococcal Infection in Patients with Cancer

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ABSTRACT

Background: In susceptible patients, *Streptococcus pneumoniae* can cause complicated pneumonia and invasive pneumococcal disease. The aim of this study was to assess the clinical and antimicrobial features of complicated and invasive pneumococcal disease in patients with cancer. **Methods:** We conducted a retrospective study including all *S. pneumoniae* isolates between January 1, 2007 and December 31, 2015 in an oncology center. Capsular serotyping was done in isolates from sterile sites. **Results**: There were 103 episodes: 69 with invasive pneumococcal disease and 34 with complicated pneumonia. Sixty-two patients were male (60%); mean age was 50 years. Eighty-four isolates were susceptible to penicillin (81.6%), 11 (10%) were intermediate, and eight (8.3%) were resistant. Serotyping was performed in 64 isolates; the main serotypes identified were 3 (n = 13) and 19A (n = 11). No patient had a record of vaccination. Mortality at seven days attributed to pneumococcal infection was different in invasive pneumococcal disease (n = 18, 28.6%) vs. pneumonia (n = 3, 8.9%; p = 0.04). Thirty-day mortality related with the infectious process was statistically different between both groups: 21 patients with invasive pneumococcal disease (30.4%) and six with pneumonia (17.6%; p = 0.04). By logistic analysis, the risk factor associated with mortality was not having received appropriate antimicrobial treatment in the first 48 hours. **Conclusions:** *S. pneumoniae* is a pathogen related with high mortality in patients with cancer. Pneumococcal immunization needs to be reinforced in this population. (REV INVES CLIN. 2016;68:221-8)

Key words: Cancer. Invasive pneumococcal disease. Pneumonia. *Streptococcus pneumoniae*.

INTRODUCTION

Streptococcus pneumoniae is an important cause of community acquired pneumonia, bacteremia, and meningitis. In susceptible patients, pneumococcal pneumonia could lead to hospitalization related to respiratory distress and in some cases requiring mechanical ventilation. Invasive pneumococcal disease (IPD) is a serious, life-threatening infection that remains an important global cause of major illness despite the introduction of pneumococcal vaccines[1,2].

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Received for publication: 07-07-2016
Accepted for publication: 15-09-2016

The incidence of IPD and pneumonia is higher in immunocompromised individuals, even in the modern antibiotic era[1,2]. Patients with cancer are especially susceptible to severe pneumococcal infections, which carry significant morbidity and mortality[3-5]. Appropriate empirical antibiotic treatment initiated as soon as the infection is clinically suspected is mandatory to improve outcomes, especially in patients with neutropenia[3,4]. Certain cancer patients are more vulnerable, including those with Hodgkin's lymphoma or multiple myeloma[3].

Information on this group of patients is scarce. Therefore, we assessed the clinical features, antimicrobial susceptibility, serotypes, and outcomes of complicated and IPD in patients with cancer at a tertiary level oncology center in Mexico.

METHODS

Study design and subjects

We retrospectively analyzed all consecutive *S. pneumoniae* infections in patients who were seen at the National Cancer Institute (INCan) in Mexico City, Mexico, between January 1, 2007 and December 31, 2015.

INCan is a 135-bed referral teaching hospital for adult patients with cancer located in Mexico City, with an average of 170,000 medical visits, 35,000 chemotherapy sessions, and 7,500 hospital discharges per year. The study was undertaken with Institutional Review Board approval (INCAN/CI/388/15).

Data on demographic characteristics, underlying malignancies, concurrent diseases, main clinical symptoms, antimicrobial therapy, intensive care unit admission, type of pneumococcal disease, number of days of antimicrobial therapy, type of antimicrobials used (beta-lactams, third-generation cephalosporins, macrolides, fluoroquinolones, clindamycin, and vancomycin), and infection outcomes were retrieved from patients' medical records and computed institutional databases.

Laboratory procedures

Microbiologic evaluations were performed at the INCan Laboratory of Microbiology. *S. pneumoniae* was isolated by standard microbiological procedures. Antimicrobial susceptibility was tested by the oxacillin disk diffusion method for penicillin susceptibility, following the Clinical Laboratory Standards Institute (CLSI) methods and criteria[6]. For non-meningitis isolates, an oxacillin zone of ≥ 20 mm was considered susceptible to penicillin and predicted susceptibility for ampicillin and ceftriaxone. Capsular serotyping was conducted in isolates from sterile sites and was performed at the National Public Health Institute (INSP, Cuernavaca, Morelos, Mexico), by the Quellung reaction with type- and factor-specific antisera (Statens Serum Institut, Copenhagen, Denmark). Minimal inhibitory concentration was determined for penicillin, erythromycin, and chloramphenicol in these isolates.

Definitions

Pneumonia: Radiographic criteria on chest X-ray or computed tomography scan (CT) plus one or more of the following: fever (≥ 38°C) or hypothermia (< 35°C), new cough with or without sputum production, pleuritic chest pain, dyspnea, and altered breath sounds on auscultation.

Severe or complicated pneumonia: CURB-65 pneumonia severity score of ≥ 2 and the presence of pleural effusion, empyema or cavitation.

Invasive pneumococcal disease: Isolation of *S. pneumoniae* from a normally sterile body fluid such as blood, cerebrospinal fluid, pleural fluid, or ascites.

Appropriate antimicrobial treatment: When an antimicrobial agent had been initiated within the first 48 hours of the first symptoms and if the patient had received it for at least 72 hours, including an antibiotic to which *S. pneumoniae* was susceptible.

Clinical cure: Resolution of clinical symptoms and signs, and/or sterilization of blood or respiratory tract cultures after antimicrobial treatment, and/or radiographic resolution of pneumonia in patients who had presented with pulmonary infiltrates.

Death related with pneumococcal infection: Persistence of a clinical condition of sepsis at the time of death, or when death occurred during the first week after blood cultures were taken.

Overall case fatality rate: Death by any cause within 30 days of IPD onset.

Table 1. Clinical characteristics of 103 patients with cancer and *Streptococcus pneumoniae* infection classified as invasive disease or pneumonia episodes

Characteristic – n (%)	Total (n = 103)	Invasive disease (n = 69)	Pneumonia episodes (n = 34)	p
Age (years)*	52 (35-65)	52 (33-67)	55 (37-61)	0.248
Male	62 (60.2)	39 (56.5)	23 (67.6)	0.278
Body mass index*†	24.5 (22-28)	25.2 (22-28)	23 (21-26)	0.480
Hematologic neoplasia	45 (43.7)	33 (47.8)	12 (35.3)	0.227
Solid tumor	58 (56.3)	36 (52.2)	22 (64.7)	
Status of cancer				0.473
Recent diagnosis	41 (39.8)	24 (34.8)	17 (50.0)	
Progressive disease	36 (34.9)	26 (37.7)	10 (29.4)	
Relapse	12 (11.7)	9 (13.0)	3 (8.8)	
Clinical remission	11 (10.7)	10 (14.5)	1 (3.0)	
Stable disease	3 (2.9)	0	3 (8.8)	
Comorbidities				0.176
Diabetes mellitus	17 (16.5)	14 (20.3)	3 (8.8)	
Current active smokers	27 (26.2)	14 (20.3)	13 (38.2)	
Obesity	18 (17.5)	13 (18.8)	5 (14.7)	
HIV	3 (2.9)	1 (1.5)	2 (5.9)	
Other‡	12 (11.7)	7 (10.1)	5 (14.7)	
Previous hospitalization#	32 (31)	22 (32)	10 (29)	0.798
Lymphocytes (cells/mm^3)*	600 (300-1,050)	555 (265-850)	850 (350-1,450)	0.128
Neutrophils (cells/mm^3)*	5,250 (1,200-10,500)	4,650 (1,050-11,850)	5,850 (2,050-9,550)	0.708
Neutropenia (< 500 cells/mm^3)	21 (20.4)	16 (23.2)	5 (14.7)	0.436
Days of neutropenia*#	2 (1-9)	2 (1.0-10.5)	1 (1-3)	0.327
Recent chemotherapy§	49 (47.6)	37 (53.6)	12 (35.2)	0.08
Days from chemotherapy to infection*	13 (7-24)	13 (7-24)	13.5 (7-23)	0.883

*Interquartile range; †body mass Index = kg/m^2; ‡chronic pulmonary disease, coronary heart disease, liver failure, previous breast cancer, multiple sclerosis, chronic alcoholism; #during the last month; §chemotherapy applied during the previous three months.

Statistical analysis

Quantitative variables were calculated as median and interquartile range (IQR). Categorical data were analyzed using the chi-square or the Fisher exact test, as appropriate. For the analysis, the events were divided into two groups: IPD and severe or complicated pneumonia. A logistic regression model was employed to examine the effects of multiple risk factors on mortality. Variables included in the model were those found to reach a significance level of $p \leq 0.1$ in the univariate analysis. Overall survival (OS) rates were estimated by means of the Kaplan-Meier method and the log-rank test. Values for $p \leq 0.05$ were considered statistically significant. Data was analyzed using STATA v.12 (Stata Corp., College Station, TX, USA) statistical software.

RESULTS

There were 103 episodes of pneumococcal infections during the eight-year study period. Sixty-nine (67%) were classified as IPD and 34 (33%) solely as

pneumonia. Sixty-two patients were male (60.2%); median age was 52 (IQR, 35-65) years. Forty-five patients (43.7%) had a malignant hematological disease. Other clinical and demographic characteristics are depicted in table 1.

The most frequent pneumococcal manifestation was bacteremic pneumonia (n = 43; 41.7%), followed by pneumonia (n = 37; 36%). Ninety-four patients (91.3%) were hospitalized, with a median hospital stay length of eight days (IQR, 4-15 days).

Chest X-ray was performed in 94 patients (91.2%) and 10 patients had CT; 84 (79%) had findings related with lung infection. More than one lobe of the lung parenchyma was affected in 24 patients (23.3%), cavitation was seen in three (2.9%), and pleural effusions of different sizes in 19 (18.5%) patients. Other clinical findings are presented in table 2.

Twenty infections (19.4%) were classified as hospital acquired, with no differences between IPD and non-invasive disease (p = 0.982).

Eighty-four isolates were susceptible to penicillin (81.6%), 11 (10%) were intermediate, and eight (8.3%) were resistant. Serotyping was performed in 64 isolates (60.1%). The main serotypes identified were 3 (n = 13), 19A (n = 11), 19F (n = 5), 4 (n = 4), and 11A (n = 4) (Fig. 1). Forty-nine (76.5%) isolates belonged to a serotype contained in the 23-valent pneumococcal polysaccharide vaccine (PPV-23) (Pneumovax 23®; Merck, USA), and 43 (67.1%) to a serotype contained in the 13-valent pneumococcal conjugate vaccine (PCV-13) (Prevnar 13®; Pfizer, USA). No patient had a record of having received pneumococcal vaccination.

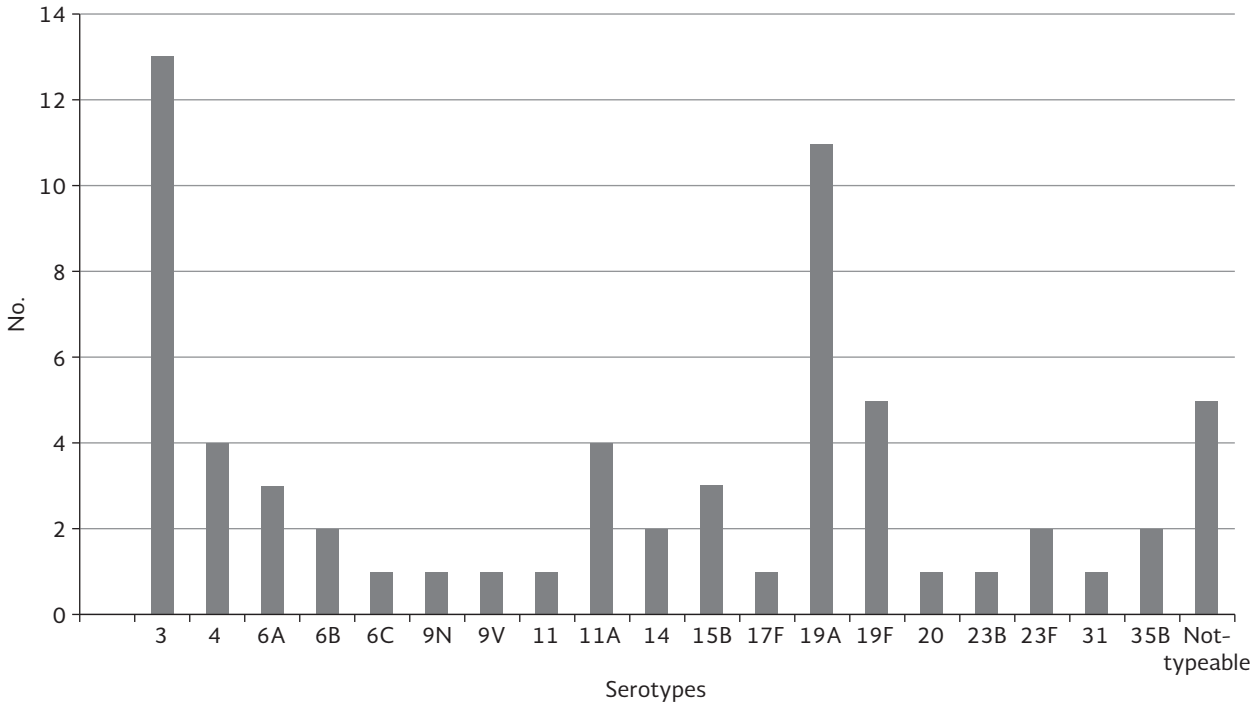
The majority of patients received ceftriaxone (n = 48; 57.1%), followed by vancomycin (n = 29; 34.5%), aminoglycosides (n = 28; 33.3%), β-lactams (n = 25; 29.8%), macrolides (n = 25; 29.8%), and ceftazidime (n = 20; 25%). Two or more effective drugs were simultaneously administered in 55 (65.4%) patients (Table 2). Combined antimicrobial treatment was associated as a protector variable in the univariate analysis for seven-day mortality (p = 0.008), but it was not confirmed in logistic regression analysis (p = 0.726). There were no differences in the analysis of 30-day mortality.

Table 2. Clinical characteristics related with *Streptococcus pneumoniae* infections in patients with cancer (n = 104)

Characteristic	No. of episodes (%)
Main clinical symptoms	
Fever	67 (65.0)
Cough	53 (51.5)
Dyspnea	41 (39.8)
Sputum	41 (39.8)
Malaise	19 (18.5)
Chest pain	15 (14.6)
Abdominal pain	14 (13.6)
Headache	12 (11.7)
Confusion/Disorientation	11 (10.7)
Infection type	
BSI*	7 (6.8)
Pneumonia	37 (35.9)
Pneumonia plus BSI	43 (41.7)
Empyema	3 (2.9)
Lung abscess	1 (1.0)
Meningitis plus BSI	3 (2.9%)
Peritonitis	6 (5.9)
Cholangitis	3 (2.9)
Hospitalization	94 (91.3)
Length of hospitalization (days)†	8 (4-15)
Intensive care unit (ICU) admission	8 (8.2)
Length of ICU (days)†	7 (6.0-9.5)
Deaths in the ICU	5 (5.1)
Patients who received antimicrobial treatment ≥ 48 h	84 (81.6)
Prescription of antimicrobials	
Ceftriaxone	48 (57.1)
Ceftazidime	21 (25.0)
Vancomycin	29 (34.5)
β-lactams	25 (29.7)
Macrolides	25 (29.7)
Clindamycin	14 (16.7)
Fluoroquinolones	13 (15.5)
Combined antimicrobials‡	55 (65.5)
Days with antimicrobials†	10 (7-13)
Outcome (30-day follow-up)	
Complete resolution of infection	61 (59.2)
Death related with pneumococcal infection	27 (26.2)
Death related with another cause	15 (14.6)

*Blood stream infection (BSI) without another documented source of infection; †median interquartile range; ‡combination of antimicrobials: ceftriaxone + clarithromycin (n = 18), ceftazidime + amikacin (n = 9), ceftriaxone + amikacin (n = 7), ceftriaxone + vancomycin (n = 6), ceftazidime + vancomycin (n = 6), piperacillin/tazobactam + clarithromycin (n = 5), and ceftazidime + clindamycin (n = 4).

Figure 1. Serotypes identified in 66 samples from patients with pneumococcal infection.



Fifteen patients (14.6%) died during the first 48 hours, all related with the infectious disease: 11 with IPD and four with pneumonia (p = 0.768). At seven days post-culture, 21 patients (20.4%) had died: 18 with IPD (28.6%) and three with pneumonia (8.9%; p = 0.04). At 30 days, 42 patients had died (40.1%): 32 with IPD (46.4%) and 10 with pneumonia (29.4%; p = 0.09). When deaths were directly attributed to the infectious process, there was a significantly higher mortality in patients with IPD (n = 21; 30.4%) than in those with pneumonia (n = 6; 17.6%; p = 0.04) (Fig. 2).

Logistic regression analysis showed that a risk factor for 30-day mortality was inappropriate antimicrobial treatment (Table 3).

DISCUSSION

This study assessed the disease burden, risk factors, microbiological features, and outcomes of pneumococcal infections in patients with cancer.

The mean age of these patients was younger than that reported in other series (52 vs. 64 years of age)[2,3]. This was probably related with the underlying neoplastic disease since patients with hematologic malignancies, who are usually younger, comprised 44% in our series.

A previous study reported that multiple myeloma presents a higher risk of pneumococcal infection related with defects in complement activation, neutrophil function, and functional hypogammaglobulinemia[2]. We found lymphoma as the most prevalent hematologic disease (17.5%), followed by leukemia (10.3%), and multiple myeloma (8.3%). We explain this finding because lymphoma is the most frequent hematologic disease seen at our hospital, followed by acute leukemia, and multiple myeloma as the third most frequent hematologic disorder.

About 20% of these cases were classified as hospital-acquired; other studies have reported that the prevalence of hospital-acquired pneumococcal pneumonia is 10-20%[7,8]. This finding is important since hospitalization is a risk factor for multidrug-resistant bacterial pneumonia; thus, it is important to consider penicillin-resistant pneumococcal strains.

Solid tumors comprised 56% of the whole group, with lung cancer being the most prevalent malignancy

Figure 2. Kaplan-Meier curve. Mortality in the first 30 days after *Streptococcus pneumoniae* isolation, divided into invasive disease (n = 69) and pneumonia episodes (n = 34).

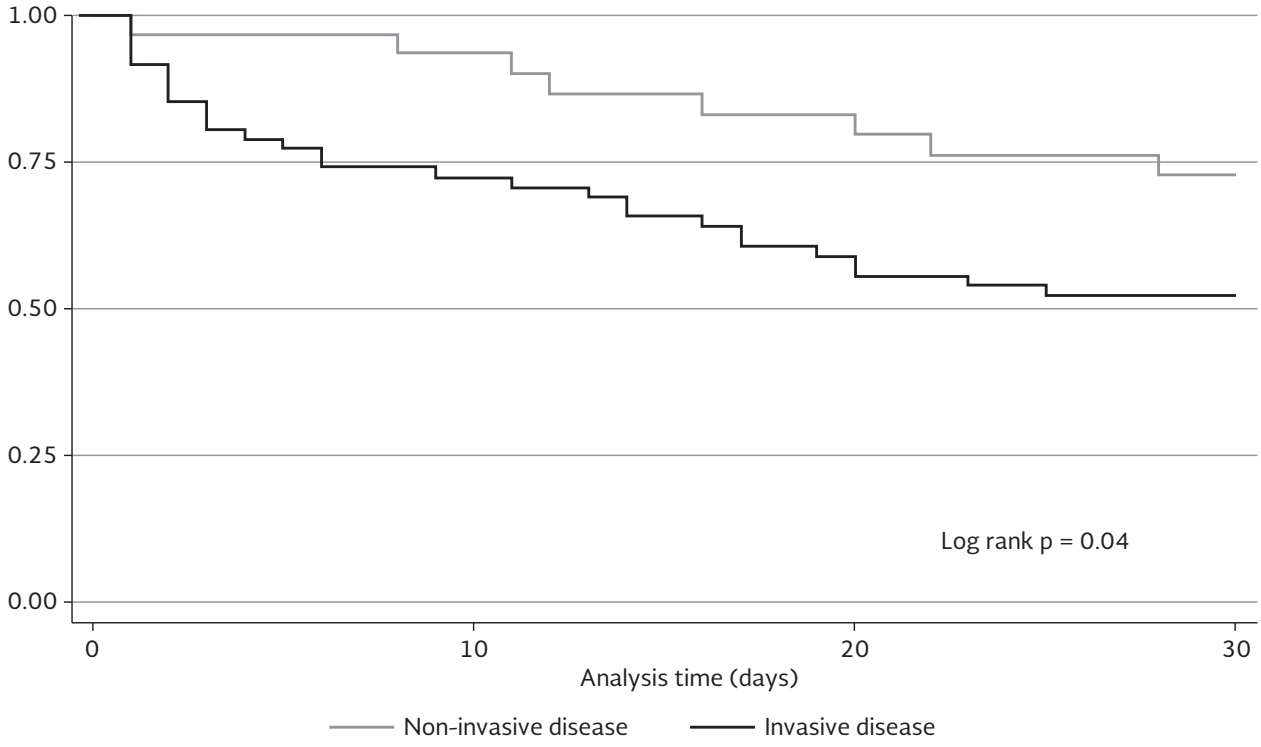


Table 3. Risk factors for 30-day mortality in patients with pneumococcal infection

Characteristic - n (%)	30-day mortality					
	Alive (n = 56)	Death (n = 41)	Univariate		Multivariate	
			OR (95% CI)	p	OR (95% CI)	p
Antimicrobial treatment			03.2 (4.8-1501.779)	< 0.0001	34.8 (4.24-286.12)	0.001
Appropriate	55	1				
Inappropriate	25	16				
Invasive disease			2.16 (0.82-5.9)	0.08	2.38 (0.828-6.86)	0.107
No	23	33				
Yes	10	31				
Cancer status			2.12 (0.86-5.25)	0.06	1.61 (0.62-4.15)	0.322
Remission/recent diagnosis	35	21				
Progression/relapse	18	23				

(n = 10; 9.7%) followed by gastrointestinal cancer (n = 9; 9.3%). Other studies in cancer patients have reported lung cancer as the most frequent solid tumor present in patients with IPD[2].

Bloodstream infection was the most frequent clinical presentation (52%); these were critically ill patients considering that 20% of them died in the first 48 hours and 47% died in the first month. One study conducted in patients with cancer and bloodstream infection by *S. pneumoniae* reported an early case mortality (< 48 hours) of 4.8% and an overall case fatality rate of 14.3% (< 30 days)[3], considerably lower than our findings. A higher mortality rate of pneumococcal

bloodstream infection (16.9%) has been reported in patients aged > 65 years and with underlying diseases or risk factors for immune suppression[9]. The elevated mortality reported herein could be explained by the fact that we included patients with invasive and complicated infections, such as meningitis, peritonitis, empyema, and lung abscess.

One question addressed in other studies is related to the use of single vs. combined anti-pneumococcal treatment, with other authors suggesting a benefit of employing two active antimicrobials[10]. In this study there was no benefit in survival by using combined antimicrobial treatment.

The prevalence of different pneumococcal serotypes varies by age group and geographic region, and differs with respect to virulence, invasiveness, and the ability to acquire resistance to different antibiotics[11]. Studies performed in patients with malignancy have found a higher prevalence of serotype 6A[2], different from what we found in this study where the main serotypes identified were 3 and 19A (20.3 and 17.1%, respectively). Serotype 3 is one of the most susceptible to beta-lactam antibiotics, while 19A is related with resistance to beta-lactams and is associated with the selection and dissemination of a clonal complex (ST 320)[11,12]. Since 2010, serotype 19A has shown a trend to increase in Mexico, related with PCV-7 used in the pediatric population[11]. Further studies are required to establish which are the major circulating serotypes in cancer patients.

Pneumococcal vaccines provide effective protection against severe and invasive disease in patients with a high risk of pneumococcal infections, such as cancer patients[13]. Nevertheless, it is important to consider that patients who have recently received antineoplastic treatment could present reduced vaccine immunogenicity[14]. In Mexico there are two different pneumococcal vaccines available. The pneumococcal polysaccharide vaccine (PPV-23) is currently recommended in patients with cancer; it is safe, inexpensive, and effective against invasive disease and improves the outcome and recurrences of patients with pneumococcal pneumonia[3]. The conjugated PCV-13 has a stronger and longer-lasting response because it contains a range of serotypes conjugated to a protein that allows for a T-cell-dependent response, thereby facilitating the formation of memory B-cells[3]. Both vaccines are recommended for administration to adults newly diagnosed with hematological or solid malignancies[14]. In data from the Regional System for Vaccines (SIREVA) Mexico network, 70% of the strains isolated were included in PPV-23 and PCV-13[15], similar to what we found in this series (72%). Pneumococcal vaccination policies at our hospital clearly have been insufficient and have impacted in complicated disease with high mortality in this group of patients. We need to increase our patients' coverage and make the rest of the medical team aware of the importance and need of expanding this coverage.

During the study period, 8.3% of *S. pneumoniae* isolates were penicillin-resistant and 10% were reported as penicillin-intermediate, similar to data from SIREVA Mexico network, which reported, in 2013, 12.5% penicillin-resistant and 8.3% intermediate strains. In Europe, penicillin resistance is reported in 9.3%, but in the Asia-Pacific Rim it is considerably higher (25%)[15,16].

This work has some limitations. It is a retrospective study carried out at a single center, although it is a tertiary level referral center, which receives patients from different geographic areas of the country. An important limitation is that we captured patients with positive cultures, so it is possible that some cases may have been missed if cultures were not done or if they were done after the administration of antibiotics.

Invasive pneumococcal disease is a severe life-threatening infection, with high morbidity and mortality in patients with cancer. Early initiation of antibiotics with activity against pneumococcus in patients with fever, respiratory symptoms and/or severe sepsis is essential to improve survival. It is imperative to reinforce pneumococcal vaccination programs.

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Rev Inves Clin. 2016;68:229-34 ORIGINAL ARTICLE

Adherence to a Gluten-Free Diet in Mexican Subjects with Gluten-Related Disorders: A High Prevalence of Inadvertent Gluten Intake

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ABSTRACT

Background: The rate of compliance with a gluten-free diet in patients with gluten-related disorders is unknown in most Latin American countries. **Objective:** To study the adherence to a gluten-free diet of Mexican individuals with celiac disease and non-celiac gluten sensitivity at the time of their first medical and nutritional consultation at a tertiary referral center. **Methods:** A cross-sectional study was performed. A specific questionnaire was used to gather information on demographics, clinical condition, and self-reported adherence to a gluten-free diet, and to determine strict compliance and intentional or inadvertent gluten consumption. All questionnaires were applied by a nutritionist with expertise in gluten-related disorders. **Results:** Fifty-six patients with celiac disease and 24 with non-celiac gluten sensitivity were included. Overall, 46 (57.5%) subjects perceived themselves as strictly adherent; however, inadvertent gluten intake was frequent in both celiac disease and non-celiac gluten sensitivity patients (39.2 vs. 33.3%; p = 0.2). Intentional consumption was more prevalent in subjects with celiac disease (48.8 vs. 29.1%; p = 0.048) and individuals with non-celiac gluten sensitivity showed better adherence (37.5 vs. 12.5%; p = 0.035). **Conclusions:** The importance of a gluten-free diet is underestimated by Mexican patients with celiac disease. The role of a team with expertise in gluten-related disorders is essential to identify inadvertent gluten intake. (REV INVES CLIN. 2016;68:229-34)

Key words: Celiac disease. Gluten-free diet. Gluten-related disorders. Non-celiac gluten sensitivity.

INTRODUCTION

A gluten-free diet (GFD) is only recommended for patients with gluten-related disorders (GRD); among them, the most studied condition is celiac disease (CD), an autoimmune enteropathy triggered by the ingestion of gluten that affects individuals with genetic susceptibility, in which the benefits of a GFD have been highly proven[1,2]. In non-celiac gluten sensitivity (NCGS), another GRD occurring in subjects who do not fulfill

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Received for publication: 21-08-2016
Accepted for publication: 27-09-2016

the criteria for CD but who present gastrointestinal and extra-intestinal symptoms related to the ingestion of wheat and similar cereals, the improvement of symptoms after a GFD is significant[3,4].

Adherence to a GFD has been shown to be a difficult task, attained by 44-90% of patients with CD[5]. Some of the factors related with the lack of compliance and inadvertent gluten intake are associated with a high prevalence of cross-contamination of foods and nutritional labeling difficulties[6-8]. In CD, a strict life-long GFD is aimed at avoiding metabolic, nutritional, and neoplastic complications, and although these have not been demonstrated in subjects with NCGS, their well-being improves, as in those patients with CD, when they follow a GFD[3,9,10]. Among the management strategies that seek to improve adherence to a GFD, nutritional assessment by supportive teams with expertise in GRD shows better outcomes[11]; however, there is a lack of information regarding these management strategies in most Latin American countries.

Celiac disease has been considered rare among native Mexicans, but a recent study indicates a prevalence of 0.7%, which is similar to that reported worldwide[12]. The prevalence of NCGS remains unknown in Mexico, although a self-reported questionnaire found that 7.8% of the studied population presents recurrent adverse reactions to gluten ingestion, which suggests that its prevalence could be as high as that published abroad[13]. Compliance with a GFD has not been studied in Mexican subjects with GRD; therefore, our aim was to investigate the adherence to a GFD in a group of patients with CD and NCGS seen at a tertiary referral center in Mexico City.

MATERIALS AND METHODS

A cross-sectional analysis was performed in 80 adult subjects with GRD who attended the Celiac Disease Unit at the Department of Gastroenterology at Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, a tertiary referral medical center in Mexico City.

Patients were divided in two groups: (i) subjects with CD, and (ii) subjects with NCGS. A diagnosis of CD was established in 56 symptomatic subjects based on the following criteria: (i) compatible clinical data; (ii) positive serological markers: anti-endomysium (EmA IgA/IgG), anti-transglutaminase (anti-tTG IgA), and/or anti-deamidated gliadin peptide (AGA-DGP IgA/IgG) antibodies; and (iii) villous atrophy typed according to Marsh-Oberhuber classification (3B-3C). Twenty-four individuals were considered as having NCGS due to: (i) gastrointestinal symptoms associated with wheat ingestion; (ii) negative serological markers; (iii) no/mild histological changes in duodenal biopsies; and (iv) symptom improvement while on a GFD.

Prior to this study, the patients with CD and NCGS were diagnosed by a gastroenterologist or a primary care physician and advised to follow a GFD during a similar estimated median time (72 vs. 43 months; p = 0.38). Before this evaluation, none of the patients had been followed up by a team with expertise in GRD.

During their first consultation at our unit, a nutritionist with expertise in GRD administered a specific questionnaire, which was divided into three sections. The first section collected the patients' demographic characteristics and evaluated their self-perception of current compliance with the GFD, classifying them into self-perceived as strictly adherent or non-adherent. The second section contained information regarding the time since diagnosis, time on a GFD, symptoms at onset, and symptomatic response to a GFD. In the third section, patients were given a list of 35 gluten-containing foods to select the products they considered as gluten-free and those ingested over the previous four weeks. If at least one of the products was consumed with the awareness of its gluten composition, it was classified as intentional non-adherence to the GFD. Otherwise, if the consumption was done unconsciously, it was considered inadvertent non-compliance. Strict compliance was defined when neither intentional nor inadvertent non-adherence occurred. The foods from the list were selected based on processed dairy products and gluten cross-contaminated grains popular in our country.

Statistical analysis

Categorical variables were presented as absolute and relative frequencies, whereas the continuous variables were summarized as means ± SD. The overall prevalence was reported as relative frequencies with the

Table 1. Subject characteristics

	CD (n = 56)		NCGS (n = 24)		p value
Gender					
Male	11	(19.6%)	7	(29.1%)	
Female	45	(80.4%)	17	(70.2%)	0.38
Age, years	59.4 ± 14.7		52.1 ± 11.9		0.03
Educational level, years	12.5 ± 3.9		14.04 ± 4.5		0.15
Duration of symptoms before diagnosis, months	12 (3-360)		30 (3-288)		0.12
	n	%	n	%	
Symptoms at onset					
Diarrhea	53	(96.4)	24	(100)	0.33
Steatorrhea	25	(44.6)	7	(29.2)	0.14
Abdominal distension	24	(42.9)	9	(37.5)	0.42
Lientery	22	(39.3)	10	(41.7)	0.51
Bloating	20	(35.7)	9	(37.5)	0.53
Abdominal pain	17	(30.4)	10	(41.7)	0.23
Flatulence	16	(28.5)	5	(20.8)	0.33
Tenesmus	6	(10.7)	7	(29.2)	0.04
Vomiting	13	(23.2)	1	(4.2)	0.03
Nausea	8	(14.3)	3	(12.5)	0.57
Time on GFD, months	72	(3-648)	43	(4-168)	0.38
Self-perceived as strictly adherent	29	(51.7%)	17	(70.8%)	0.00
Inadvertently non-adherent	22	(39.2%)	8	(33.3%)	0.29
Intentionally non-adherent	27	(48.2%)	7	(29.1%)	0.04
Strictly adherent	7	(12.5%)	9	(37.5%)	0.03
Mean gluten-containing foods consumed	2	(0-11)	1	(0-7)	0.00

CD: celiac disease; GFD: gluten-free diet; NCGS: non-celiac gluten sensitivity.

corresponding 95% confidence intervals (according to binomial distribution). Student's *t*-test and Mann-Whitney *U* test were used for evaluating normally and not normally distributed continuous data. Chi-square and Fisher's exact tests were used for the categorical data. Associations between demographics, symptoms at onset, time on a GFD and type of compliance were analyzed using binary logistic regression. Statistical significance was set up at p value < 0.05. The statistical package SPSS v20 software was used for data analysis.

The Ethics and Research Committees and the Institutional Review Board at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico, approved this study. All participants signed statements of informed consent as voluntary participants in the study. Patient's information was collected and saved according to the database standards of our public hospital and we followed the standards of practice and privacy from our institution.

RESULTS

From the 80 subjects included (mean age 58 ±14 years), 64 were female. Overall, 46 (57.5%) individuals perceived themselves as strictly adherent; however, when this cross-sectional study was concluded, it was found that 30 subjects (37.5%) had inadvertent gluten ingestion and 34 (42.5%) consumed gluten intentionally.

Demographic characteristics were similar between groups (Table 1). At the time of this study their clinical condition was stable; the most prevalent symptoms were diarrhea and abdominal distension, with frequencies of 75 and 36%, respectively. After following a GFD, almost all gastrointestinal symptoms disappeared in similar proportions in patients with CD and NCGS (67 vs. 75%; p = 0.7). Binary logistic regression model did not show a predictable association between groups' characteristics and adherence to a GFD. The specific questionnaire we used in this study is shown in table 2.

Table 2. Questionnaire

Section 1

a) Name _____ b) Age _____ c) Gender: ☐ Male ☐ Female d) Nationality _____
e) Occupation _____ f) Number of years of education completed _____
g) Marital status: ☐ single ☐ married ☐ divorced other _____

Questions	Answer	
1. Have you been diagnosed with a disorder related with gluten ingestion?	☐ Yes	☐ No
2. Which disorder have you been diagnosed with?	☐ CD	☐ NCGS
3. Do you follow a gluten-free diet (GFD)?	☐ Yes	☐ No
4. Do you consider that you follow a strict GFD?	☐ Yes	☐ No
5. Do you ingest gluten-containing foods intentionally?	☐ Yes	☐ No

Section 2

6. Who diagnosed your disorder?
☐ Gastroenterologist
☐ Primary care physician
☐ Other _____

7. How long have you been diagnosed with CD/NCGS? _____ Months
(Date of initial diagnosis _____)

8. How long have you followed a GFD? _____ Months

9. Please indicate which of the following symptoms you had at the time of diagnosis and which of them you still have at the present time.

Symptoms you presented at the time of diagnosis		Symptoms you present now	
☐ Diarrhea	☐ Steatorrhea	☐ Diarrhea	☐ Steatorrhea
☐ Abdominal Pain	☐ Lientery	☐ Abdominal Pain	☐ Lientery
☐ Bloating	☐ Incomplete emptying (tenesmus)	☐ Bloating	☐ Incomplete emptying (tenesmus)
☐ Abdominal distension	☐ Nausea	☐ Abdominal distension	☐ Nausea
☐ Flatulence	☐ Vomiting	☐ Flatulence	☐ Vomiting

Section 3 (Patients' marks)

10. Please mark (✓) the products that you consider are gluten-free.

☐ Achiote (Annato)	☐ Canned beans	☐ Gravy	☐ Processed rice
☐ Atole (cornflour beverage)	☐ Canned soup	☐ Ham	☐ Processed sauces
☐ Beer	☐ Champurrado	☐ Hamburger beef	☐ Sacramental bread
☐ Bread	☐ Chicken powder	☐ Ice cream	☐ Sausage
☐ Breaded food	☐ Chocolate candies	☐ Marshmallow	☐ Scotch
☐ Breakfast cereal	☐ Churros (fritters)	☐ Mexican traditional candies	☐ Snacks
☐ Bulk amaranth seeds	☐ Cookies	☐ Oat cookies	☐ Tamales
☐ Bulk rice	☐ Custards	☐ Pasta	☐ White tortillas
☐ Cake	☐ Flavored yoghurt	☐ Processed corn	

11. Please mark (✓) the products you have consumed, at least once, over the previous 4 weeks.

☐ Achiote	☐ Canned beans	☐ Gravy	☐ Processed rice
☐ Atole	☐ Canned soup	☐ Ham	☐ Processed sauces
☐ Beer	☐ Champurrado	☐ Hamburger beef	☐ Sacramental bread
☐ Bread	☐ Chicken powder	☐ Ice cream	☐ Sausage
☐ Breaded food	☐ Chocolate candies	☐ Marshmallow	☐ Scotch
☐ Breakfast cereal	☐ Churros	☐ Mexican traditional candies	☐ Snacks
☐ Bulk amaranth seeds	☐ Cookies	☐ Oat cookies	☐ Tamales
☐ Bulk rice	☐ Custards	☐ Pasta	☐ White tortillas
☐ Cake	☐ Flavored yoghurt	☐ Processed corn	

Table 3. Gluten-containing foods consumed (n = 80)

Inadvertently		Intentionally	
Product	**%**		**%**
Powdered chicken broth	27.2	Bread	57.6
Flavored yoghurt	21.7	Cookies	26.9
Sacramental bread	21.7	Processed sauce	26.9
Breakfast cereal	16.6	Powdered chicken broth	23.0
Traditional candies	16.6	Breaded food	23.0
Custards	14.8	Traditional candies	23.0
Processed sauces	11.1	Breakfast cereal	19.2

When compliance with the diet was compared between groups, subjects with NCGS were shown to be more adherent to the GFD than celiac individuals (37.5 vs. 12.5%; p = 0.035). Inadvertent gluten intake was not statistically different between groups (39.2 vs. 33.3%; p = 0.2); however, intentional disruptions were more frequent in subjects with CD (48.8 vs. 29.1%; p = 0.048). Individuals with inadvertent and intentional non-adherence consumed a median of two gluten-containing foods over the previous four weeks. The number of products inadvertently ingested by a single individual varied from one to seven. Foods consumed intentionally and by mistake are shown in table 3.

DISCUSSION

The recent Celiac Dietary Adherence Test (CDAT) seems to be a useful tool to assess GFD adherence, although it has not been validated in a Mexican population[14]. Biagi, et al. developed a score that verifies adherence to a GFD based on four questions that evaluate voluntary gluten intake, self-care when dining out, avoidance of gluten consumption by reading food labels, and eating products guaranteed by a celiac association. However, the reproducibility of this score in countries like ours is uncertain[15]. In Mexico, as in other Latin American countries, food labeling regulations do not require giving information on gluten content in all packaged products, so different strategies must be developed when evaluating adherence to a GFD. Until now, one of the most reliable strategies is patient monitoring by medical teams with expertise in GRD capable of identifying dietary habits and factors related with non-compliance[11]. This study shows, for the first time in our country, the rates of compliance with a GFD of Mexican patients with GRD who had not received proper nutritional advice. A specific questionnaire was performed by a nutritionist with expertise in CD, and the low rates of strict adherence that we found highlight the importance of improving the strategies to follow-up patients with CD and NCGS in Mexico.

In our study, a high proportion of individuals with CD and NCGS who attended their first medical and nutritional consultation at a CD unit, considered themselves to be adherent; however, a considerable amount of products prepared or contaminated with gluten were consumed accidentally. A recent systematic review demonstrated that the median of self-reported strict adherence to a GFD in subjects with CD is around 70% (range 42-91%)[5]; in our study, 57.5% (46) of the patients with a GRD believed themselves to be strictly adherent; however, inadvertent gluten intake was frequent (n = 30; 37.5%) and a low rate of strict compliance was found (20%). Gluten consumption was voluntary in 34 (42.5%) individuals of our group; bread and cookies were the products most frequently consumed intentionally, and the number of gluten-containing products eaten by a single individual varied from one to 11. Similar results were found by Hall, et al., who reported that 40.1% of 287 celiac subjects who responded to a self-completion questionnaire had intentional gluten consumption[8].

Compliance with dietary recommendations has been widely studied among subjects with CD and is poorly described in NCGS, so it is unclear whether this behavior is similar in both conditions. Interestingly, in

our study when groups were compared, subjects with NCGS showed to be more compliant with the GFD than celiac individuals (37.5 vs. 12.5%; p = 0.035). These surprising results suggest that NCGS patients could be more cautious while on a GFD, and that a high proportion of patients with CD ignore or underestimate the importance of this diet.

Interestingly, in our group the adherence to a GFD was not associated with a symptomatic response: gastrointestinal symptoms disappeared similarly in both CD and NCGS patients after a GFD (67 vs. 75%; p = 0.7). Although it is possible that the amounts of gluten ingested, or the time of exposure to gluten, could have been insufficient to cause symptoms in subjects who perceived themselves as strictly adherent, it supports the notion that compliance with the diet could be independent of the symptomatic condition, as has been previously described[16-18].

Our work was a cross-sectional evaluation that relied on patients' responses to study the adherence to a GFD. It was based on a simple questionnaire with a list of gluten-containing foods, whose results become of a high interest, not only for Mexicans, but also for other Latin American populations. It indicates that a high proportion of patients could be consuming gluten inadvertently. Furthermore, this is the first study performed in individuals with GRD in our country that suggests that the awareness of subjects with NCGS could be better than the celiac patients' when a GFD is followed. The low rates of strict adherence found in individuals without special nutritional advice underscores the importance of implementing new strategies to follow-up Mexican patients with GRD.

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PERMANYER

Rev Inves Clin. 2016;68:235-44 ORIGINAL ARTICLE

Acute Respiratory Distress Syndrome Secondary to Influenza A(H1N1)pdm09: Clinical Characteristics and Mortality Predictors

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ABSTRACT

Background: Acute respiratory distress syndrome secondary to influenza A(H1N1)pdm09 virus is the leading cause of death among this patient population. Expanding the knowledge of its course and predictors of mortality is relevant to decision making. We aimed to describe the clinical characteristics and identify factors associated with mortality in patients with acute respiratory distress syndrome secondary to influenza A(H1N1)pdm09 during the 2013-2014 influenza season. **Methods:** This is an observational study of a prospective cohort of 70 patients with acute respiratory distress syndrome and influenza A(H1N1) pdm09 seen in an academic medical center. Multivariate logistic regression was used to identify the independent mortality predictors. Bootstrap was used for internal model validation. **Results:** This cohort was represented by young adults (43 ± 11 years old). Obesity was present in 62.5% and was not associated with mortality. Mortality at 28 days and at discharge from the respiratory intensive care unit was 14 and 20%, respectively. All patients met the criteria for acute respiratory distress syndrome, 73% had vasodilatory shock, and 27.1% had acute kidney injury on respiratory intensive care unit admission. We observed a high incidence of intensive care unit-acquired weakness (81.4%). Ventilator-associated pneumonia developed in 47.1% and was not associated with mortality. In multivariate analysis, independent risk factors for intensive care unit mortality were age (odds ratio [OR] = 1.102), white blood cell count (OR = 1.22), and lactate dehydrogenase levels (OR = 1.004) on admission to the intensive care unit. **Conclusions:** We described the clinical characteristics and course of a cohort of patients with acute respiratory distress syndrome secondary to influenza A(H1N1)pdm09, and developed a predictive model of mortality based on the covariates age, levels of lactate dehydrogenase, and white cell count on admission to the respiratory intensive care unit. (REV INVES CLIN. 2016;68:235-44)

Key words: ARDS. Influenza A(H1N1)pdm09. Mortality. Prognostic factors. 2013-2014 influenza season.

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Received for publication: 02-07-2016
Accepted for publication: 05-10-2016

INTRODUCTION

In March 2009, the first cases of a novel respiratory infection caused by influenza virus A(H1N1)pdm09 were reported in Mexico. In contrast to the seasonal influenza virus, infection with the A(H1N1)pdm09 virus had a higher prevalence in young adults and obese and pregnant women and was associated with severe pneumonia, requiring frequent admission to the intensive care unit for mechanical ventilation and multisystem organ support. High mortality was reported in Mexico among patients who had acute respiratory failure and required mechanical ventilation[1,2]. This mortality contrasted strongly with that reported by other groups during the 2009 influenza pandemic[3,4].

Following the 2009 influenza pandemic, new outbreaks occurred in 2010-2011[5] and most recently from October 2013 through May 2014. During this last influenza season, a substantial increase was documented in the number of A(H1N1)pdm09-related hospitalizations and deaths, as was a proportionate shift of severe disease affecting middle-aged adults compared with the preceding A(H1N1)pdm09 2011-2012 epidemic in Mexico[6]. However, there are no reports on demographics, clinical characteristics, and outcome of those patients who developed severe pneumonitis and acute respiratory distress syndrome (ARDS) during the 2013-2014 influenza A(H1N1) pdm09 season. Increased knowledge on its course and predictors of mortality is relevant to decision making.

The objectives of this study were to describe the clinical characteristics and identify factors associated with mortality in patients admitted to our respiratory intensive care unit (RICU) presenting with ARDS secondary to viral pneumonitis by A(H1N1)pdm09 virus during the 2013-2014 influenza season.

MATERIAL AND METHODS

Study design and patient population

This is an observational study of a prospective cohort of patients admitted to the RICU from October 2013 through May 2014. We included all patients admitted to the RICU requiring mechanical ventilation and multisystem support for acute respiratory failure caused by primary viral pneumonia due to influenza A(H1N1) pdm09.

Nasopharyngeal swab specimens were collected on admission; lower respiratory secretions were also obtained in patients who had been intubated. Real-time polymerase chain reaction testing (RT-PCR)[7] was performed for A(H1N1)pdm09 and additional samples were obtained for testing with a multiplex PCR assay for coinfection with other respiratory pathogens (Allplex™, Respiratory Full Panel Assay, Seegene Technologies, Inc., USA.). A confirmed case was defined as a patient with an acute respiratory illness with laboratory-confirmed pandemic A(H1N1)pdm09 by strain-specific PCR. The study was approved by the institutional ethics committee, and the requirement of informed consent was waived due to the observational nature of the study.

Data collection and definition of variables and outcome

Data were obtained by two investigators and stored in an electronic database. The following variables were recorded: demographics, immunization status, comorbidities, time elapsed from the onset of symptoms to hospital admission, time to the first dose of oseltamivir, signs and symptoms, previous use of antibiotics, microbiological findings, and chest radiological findings. Cardiovascular and respiratory variables, parameters of mechanical ventilation, laboratory studies, and organ failure scores were evaluated on admission to the RICU. Organ failure was assessed using the Sequential Organ Failure Assessment (SOFA) scoring system[8]. Patients were followed throughout the course of their illness and development of complications was recorded.

Acute respiratory distress syndrome was defined according to the recent Berlin Definition[9]. Primary viral pneumonia was defined as the presence of pulmonary infiltrates in two or more lobules with negative cultures for bacteria in respiratory secretions and blood during the influenza season. Coinfection was defined as microbial growth in cultures of tracheal aspirates or bronchoalveolar lavage obtained within 48 hours of hospital admission. Ventilator-associated bacterial pneumonia was considered when there was recurrence of fever, leukocytosis, purulent sputum, and new infiltrates in a setting of greater respiratory impairment, plus positive cultures of respiratory secretions[10]. Acute kidney

injury (AKI) was defined according to the classification reported by Metha, et al.[11]. Oseltamivir was administered by a nasogastric tube at doses of 150-300 mg/24 hours as recommended by the World Health Organization (WHO)[12], with doses selected by the attending physician. Criteria for ICU admission and treatment decisions were not standardized for all patients and were decided by the attending physician. Outcomes were defined as 30- and 90-day all-cause mortality.

Statistical analysis

Data were described as counts or percentages for categorical variables, and as means and standard deviations (SD) or medians and interquartile ranges (IQR) for continuous variables. Differences between characteristics of the groups were analyzed using Student's *t*-test for independent samples or the Mann-Whitney rank-sum test for continuous variables, and the chi-square test or Fisher's exact test for categorical variables. Multivariate logistic regression was used to evaluate the association between clinical characteristics and mortality. All the factors associated with mortality in the univariate analysis were entered into the multivariate analysis with backward elimination ($p < 0.05$ to retain) to select the final set of predictors. The same model was selected using a forward stepwise procedure ($p < 0.05$ for admission). Results are presented as odds ratio (OR) and 95% confidence intervals (CI). Internal validation of the model was obtained by the statistical bootstrap procedure. In total, 1000 random samples with replacement were obtained from the original cohort. The model was developed again in each bootstrap sample yielding the new parameters' values to each bootstrap model. Finally, the mean, standard error, and confidence intervals of coefficients were obtained. Odd ratios were calculated as the exponential of coefficients. The distribution of the times of survival was analyzed by using the Kaplan-Meier method. A log rank test was run to determine if there were differences in the survival distribution for relevant covariates. The resulting model's predictive value was evaluated using a receiver operating characteristic (ROC) area under the curve (AUC). Confidence intervals and p values were considered significant for a two-tailed alpha level of 0.05. Data analysis was performed using SPSS for Windows 21.0 (SPSS, Inc, Chicago, IL, USA).

RESULTS

Characteristics of the study patients

Tables 1 and 2 show the demographic and clinical characteristics, comorbidities, physiological responses, and outcomes of patients. The average age was 47 ± 11 years; 61.4% were men and only 5% had been immunized against influenza. The median time to seek medical attention was three (IQR: 2-4) days, the median time from the onset of symptoms to admission to the emergency department was eight (IQR: 7-10) days, the median time to receive the first dose of oseltamivir was eight (IQR: 7-10) days, and 95.2% of patients received one or more antibiotics before admission. Obesity was the most common comorbidity, present in 62.5% of patients. The main symptoms were cough (100%), sore throat (59.4%), subjective feeling of fever (98.5%), shortness of breath (100%), headache (67.7%), intense fatigue (100%), and myalgia (84.6%). Hemoptysis was present in 36.9% of patients. On physical examination, the most common findings were crackles (96.9%), wheezing (15.4%), and cyanosis (52.1%).

Physiological response and severity of illness in the respiratory intensive care unit at admission

All patients required invasive mechanical ventilation. The partial pressure of arterial O_2 to the fraction of inspired O_2 (PaO_2/FIO_2) ratio was 95 ± 39, the median time on mechanical ventilation was 17 (IQR: 8-29) days, and 72.8% of patients required vasopressors. The median lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) levels on admission were 590 (IQR: 368-867) U/l and 764 (IQR: 226-1,894) U/l, respectively. The median SOFA score was 8 (IQR: 5-9). The median length of stay at the RICU was 18 (IQR: 1-32) days. Mortality at 28 days was 14.2% and at discharge from the RICU, 20%.

Comparison between survivors and non-survivors

Demographic data, clinical characteristics and comorbidities of patients who survived compared to those who did not survive are shown in table 2. Non-survivors were older than survivors (49 ±11 vs. 42 ± 10 years;

Table 1. Demographics, comorbidities, and clinical data of survivors and non-survivors

Patient characteristics on admission	All patients (n = 70)	Survivors (n = 56)	Non-survivors (n = 14)	*p* value
Age (years)	43 ± 11	42 ± 10	49 ± 11	0.02
Gender (M%)	61.4	61.4	61.5	0.80
BMI (kg/m^2)	31 ± 6.8	32.2 ± 6.9	30.7 ± 6.4	0.62
Influenza immunization (%)	5.7	7.0	0	0.57
Duration of symptoms to primary medical care, median (IQR) days	3 (2-4)	3 (2-4)	2 (1-3)	0.12
Previous medical care (%)	97.7	100	92.3	0.20
Duration of symptoms to hospital admission, median (IQR) days	8 (7-10)	8 (7-10)	7 (5-8)	0.35
Interval from symptom onset to swab, median (IQR) days	8 (7-10)	8 (7-10)	7 (5-8)	0.35
Pre-admission antibiotics (%)	95.2	94.7	90	> 0.99
Days on antibiotics	5 (4-6)	4.0 ± 1.5	5.3 ± 1.8	0.01
Duration of symptoms at first dose of Oseltamivir, median (IQR) (days)	8 (7-10)	8 (7-10)	7 (5-8)	0.35
Comorbidities, %				
Obesity (BMI > 30 kg/m^2)	62.5	57.8	61.5	0.77
Diabetes mellitus	8.7	7.0	15.3	0.09
Asthma	5.7	7.0	0	0.57
Hypertension	10.1	14.0	0	> 0.99
Cardiac failure	1.4	1.7	0	> 0.99
Cirrhosis	1.4	1.7	0	> 0.99
Chronic pulmonary disease	7.2	8.7	0	0.57
Chronic renal disease	1.4	0	0	> 0.99
HIV/AIDS	1.4	3.5	0	0.99
Smoker	17.4	24.5	30.7	0.74
Alcohol/Substance abuse	13.0	17.5	15.3	0.84
Symptoms and signs, %				
Cough	100	100	100	> 0.99
Subjective fever	98.5	98.2	100	> 0.99
Sore throat	59.4	50.8	38.4	0.34
Sputum	41.5	82.4	79.2	0.45
Rhinorrhea	78.5	46.4	21.4	0.13
Hemoptysis	36.9	38.5	30.7	0.90
Dyspnea	100	100	100	> 0.99
Headache	67.7	86.6	92.3	0.05
Fatigue	100	100	100	> 0.99
Myalgia	84.6	85.9	76.9	0.68
Diarrhea	4.6	3.5	7.6	0.49
Cyanosis	52.1	32.6	84.6	0.04
Wheezing	15.4	19.2	0	0.10
Crackles	96.9	96.4	100	> 0.99

BMI: body mass index; IQR: interquartile range.

Table 2. Laboratory values, physiological response, severity scores on admission to the respiratory intensive care unit, and outcome of survivors and non-survivors

Variable	Total (n = 70)	Survivors (n = 56)	Non-survivors (n = 14)	p value
MAP (mmHg)	73 ± 11	74 ± 12	70 ± 7	0.61
HR (beats/minute)	91 ± 21	92 ± 20	88 ± 24	0.71
Vasopressors %	72.8	68.4	92.3	0.01
Norepinephrine dose (mcg/kg/min)	0.096 ± 0.065	0.088 ± 0.036	0.137 ± 0.066	0.02
Ratio of PaO_2 to FIO_2	95 ± 39	76 ± 30	60 ± 25	0.06
PEEP, cm H_2O	12 ± 4	12 ± 3	13 ± 3	0.26
Tidal volume adjusted to ideal weight, ml/kg	9.6 ± 2.8	9.4 ± 2.9	10.3 ± 2.3	0.27
Dynamic compliance	24.4 ± 5.0	25.1 ± 5.7	21.4 ± 3.8	0.02
Prone positioning ventilation, %	7.1	5.3	14.2	0.26
Corticosteroid therapy, %	90	87.5	100	
White blood cell count	10.7 ± 5.1	9.9 ± 4.8	13.8 ± 5.2	0.01
Lymphocyte count	897 ± 548	873 ± 451	996 ± 848	0.45
Procalcitonin (ng/ml), median (IQR)	0.88 (0.25-4.7)	0.54 (0.25-4.25)	2.4 (1.37-6.95)	0.38
Platelet count	218 ± 100	214 ± 98	232 ± 114	0.51
BUN		19.4 ± 13.0	32.7 ± 19.0	< 0.01
Creatinine (mg/dl), mean (SD)	0.88 ± 0.75	1.21 ± 1.02	2.01 ± 1.54	0.02
Bilirubin (mg/dl), median (IQR)	0.69 (0.5-1.10)	0.69 (0.5-1.0)	0.80 (0.6-1.35)	0.21
AST (U/l), median (IQR)	76 (40-109)	74 (38-108)	99 (67-110)	0.80
ALT (U/l), median (IQR)	48 (35-68)	50 (37-69)	40 (34-54)	0.44
Albumin (g/dl), mean (SD)	2.1 ± 0.45	2.16 ± 0.45	1.88 ± 0.42	0.04
INR, mean (SD)	1.12 ± 0.16	1.11 ± 0.16	1.15 ± 0.15	0.50
TTP (sec), mean (SD)	28.7 ± 4.3	28.5 ± 4.4	29.6 ± 3.8	0.50
LDH (U/l), mean (SD)	590 ± 760	597 ± 328	987 ± 442	< 0.001
Creatine kinase (U/l), mean (SD)	1,150 ± 967	1,376 ± 1,787	1,201 ± 1,049	0.727
SOFA score, median (IQR)	8 (5-9)	7 (4-8)	10 (8-11)	< 0.001
RICU length of stay (days), median (IQR)	18 (11-32)	19 (10-32)	18 (15-26)	0.53
Duration of mechanical ventilation, median (IQR) days	17 (8-29)	17 (7-29)	18 (15-26)	0.47
Ventilator-associated pneumonia, %	47.1	42.8	64.2	0.09
ICU-acquired weakness, %	81.4	82.1	78.5	> 0.99
Delirium, %	72.8	69.6	85.7	0.32

ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; HR: heart rate; INR: international normalized ratio; IQR: interquartile range; LDH: lactic dehydrogenase; MAP: mean arterial pressure; PaO_2 to FIO_2: partial pressure of arterial oxygen to the fraction of inspired oxygen; PEEP: positive end-expiratory pressure; RICU: respiratory intensive care unit; SD: standard deviation; SOFA: Sequential Organ Failure Assessment; TTP: thrombotic thrombocytopenic purpura.

p = 0.02), although both groups represent a young population. There were no differences between survivors and non-survivors in distribution of comorbidities, time to seek primary medical attention, time from onset of symptoms to first dose of oseltamivir, time from onset of symptoms to hospital admission, or clinical manifestations and physical signs. However, the presence of cyanosis was more frequent among non-survivors (84.6 vs. 32.6%; p < 0.047). Obesity (body mass index, BMI > 30 kg/m^2) was the most frequent comorbidity and its prevalence was not different among survivors and non-survivors. There was no difference in the percentage of survivors and non-survivors who received antibiotics prior to hospital

admission (94.7 vs. 90%); however, non-survivors received antibiotics for a longer period of time (5.2 ± 1.9 vs. 4 ± 1.6 days; p = 0.022).

Cardiovascular features

Mean arterial pressure and heart rate were not different between survivors and non-survivors; however, a higher percentage of non-survivors required support with high doses of norepinephrine (92.3 vs. 68.4; p < 0.014). Non-survivors needed higher doses of norepinephrine during the first 24 hours to maintain mean arterial pressure (MAP) > 65 mmHg as compared to survivors (0.137 ± 0.066 vs. 0.088 ± 0.036 µg/kg/min; p = 0.022).

Respiratory function

Non-survivors had a lower PaO_2/FiO_2 ratio compared to survivors; however, this difference was not significant (60 ± 25 vs. 76 ± 30; p = 0.069). The required level of positive end-expiratory pressure (PEEP) and the tidal volume adjusted to ideal body weight were not different between the two groups. However, non-survivors showed lower dynamic compliance (non-survivors: 21.4 ± 3.8 vs. survivors: 25.1 ± 5.7; p = 0.027). A highly significant difference was observed in the levels of LDH between non-survivors and survivors (987 ± 442 vs. 597 ± 328; p = 0.000). Prone position ventilation was implemented in 5.3% of survivors and 14% of non-survivors (p = 0.260).

Renal function

The mean creatinine and blood urea nitrogen (BUN) values were significantly higher in non-survivors (Table 2). Acute kidney injury was present in 19 patients (27.1%), and of these, 15.7% were classified as AKI-1, 36.8% as AKI-2, and 47.3% as AKI-3. Hemodialysis was implemented in 77.7% of these patients. Mortality according to AKI classification was: no AKI or AKI-1: 0%; AKI-2: 28.5%; and AKI-3: 55.5%. Mortality in patients with AKI was 36.8% and for patients without AKI, 13.7%. AKI (2 or 3) was significantly associated with mortality in the univariate analysis (OR: 5.2; 95% CI: 1.4-18.5; p <0.01); however, it did not represent an independent risk factor in the multivariate analysis (OR: 1.7; 95% CI: 0.32-9.36; p = 0.512).

Infection features

Leukocyte count was higher in non-survivors and represented an independent risk factor for mortality. Procalcitonin levels were also higher in this group, although the difference was not statistically significant (0.54 [0.25-4.25] vs. 2.4 [1.37-6.95]; p = 0.386). However, in none of the cases was bacterial coinfection documented in the microbiological studies done within 48 hours of admission. Ventilator-associated bacterial pneumonia was present in 42.8% of survivors and 71.4% of non-survivors (p = 0.056). The most frequently isolated microorganisms were: *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, and *Staphylococcus aureus*. Other secondary infections were: invasive pulmonary aspergillosis, candidemia, *P. aeruginosa* and *S. aureus* bacteremia, and infected pressure sores.

Neurological manifestations

A high incidence of ICU-acquired weakness (ICU-AW, defined as a Medical Research Council [MRC] score[13] of < 48 points) was observed, and in a few cases, polyneuropathy and myopathy were documented by electromyography and nerve conduction studies. Delirium was observed in 87% of patients and was significantly associated with the length of stay in the RICU; however, there were no differences between the two groups.

Independent factors associated with mortality

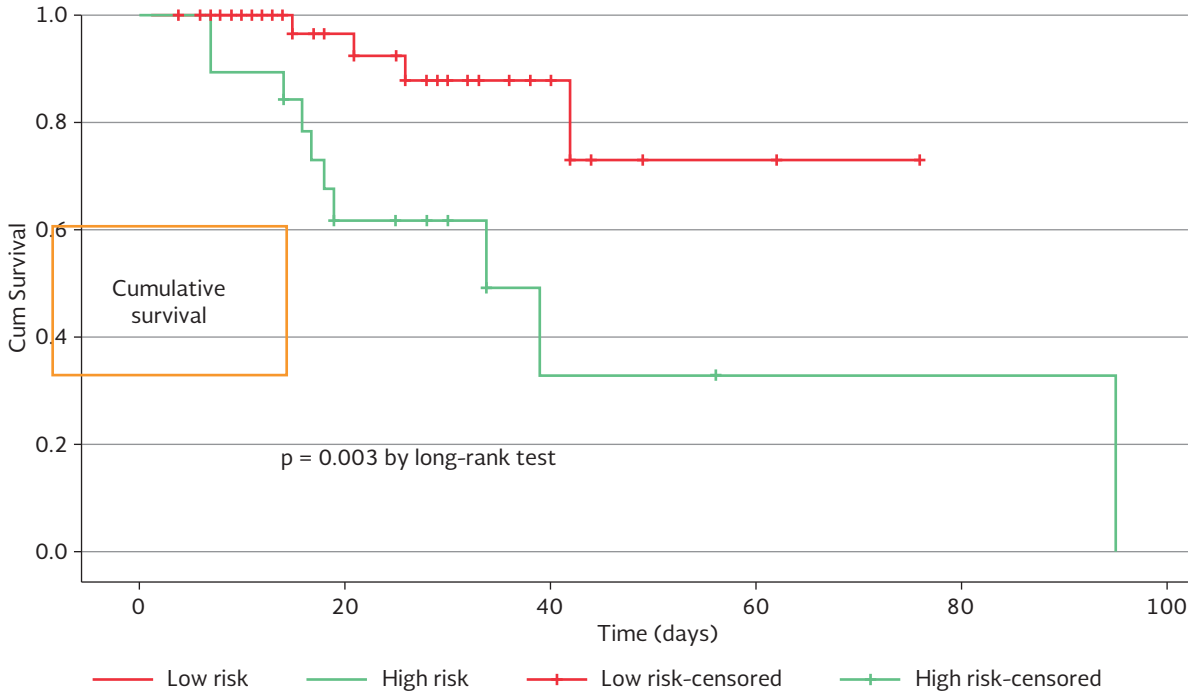
A logistic regression analysis was performed to ascertain the effects of age, white blood cell (WBC) count, norepinephrine dose in the first 24 hours, creatinine levels, and LDH levels on the likelihood of 90-day all-cause mortality. Of the five predictor variables, only three were statistically significant: age, WBC count, and LDH levels (Table 3). The logistic regression model was statistically significant, $\chi^2 = 27.402$, p < 0.0005. The model (Nagelkerke's R^2) explained 55% of the variance in the outcome variable and correctly classified 82.7% of cases. Sensitivity was 92.9%, specificity was 42.9%, positive predictive value was 60.0%, and negative predictive value was 86.6%. To test for a linear relationship between the continuous independent variables and the logit of the dependent variable,

Table 3. Results of multivariate logistic regression analysis and bootstrap

Variable	OR	95% CI	Bootstrap OR	95% CI
LDH	1.004	1.001-1.006	1.004	1.002-1.012
Age	1.102	1.019-1.191	1.102	1.032-1.321
WBC	1.222	1.046-1.428	1.222	1.051-1.900

LDH: lactic dehydrogenase; WBC: white blood cell count.

Figure 1. Survival curve in patients with acute respiratory distress syndrome secondary to influenza A(H1N1)pdm09 according to mortality score predictor. The cutoffs were: lactate dehydrogenase ≥ 700 U/l; age ≥ 55; white blood cell ≥ 12,000 cells/mm³. Patients with zero to 1 point were defined as having a low mortality risk, and patients with 2 to 3 points as high mortality risk. A highly significant difference between distributions of survival was observed (p < 0.001, by log rank test).



a Box-Tidwell approach was used, which adds an interaction term between an independent variable and its natural log to the equation. The interaction terms were not statistically significant. Increasing age, LDH levels, and WBC counts were associated with an increased likelihood of 90-day all-cause mortality. An un-weighted risk scale assigning one point to each risk factor was created using the independent risk factors. For purposes of the un-weighted risk scale, continuous variables were transformed into dichotomous variables by identifying the maximal sum of sensitivity and specificity. A score for each patient was then calculated. The cutoffs were: LDH ≥ 700 U/l; age ≥ 55; WBC ≥ 12,000 cells/mm³. Patients with zero to 1 point were defined as having a low mortality risk, and patients with 2 to 3 points as having a high mortality risk. A highly significant difference between distributions of survival was observed (p < 0.001, by log rank test) (Fig. 1).

DISCUSSION

In this study, we present the clinical characteristics and factors associated with mortality in 70 patients with influenza A(H1N1)pdm09 and ARDS admitted to our RICU during the 2013-2014 influenza season. This population was represented by young adults. Mortality at 28 days and at discharge from the RICU was 14% and 20%, respectively. This mortality compares

favorably with mortality reported during the influenza H1N1 pandemic of 2009 in Mexico[1,2].

Early administration of oseltamivir in patients with influenza has been reported to reduce the risk of complications and mortality. Rodriguez, et al. found that oseltamivir therapy initiated within two days of influenza symptoms was an independent variable associated with a significant reduction in mortality[14]. A recent meta-analysis reported that the risk of mortality increases 1.23 times for each day of delay in the initiation of treatment up to day 5[15]; furthermore, the interval between the onset of symptoms and initiation of antiviral therapy has been reported as an independent factor associated with increased severity of disease[14]. In our patients, the median time to first dose was eight days, reflecting a serious problem of logistics in primary health care services. Thus, the indiscriminate use of antibiotics as well as a failure in primary care attention probably contributed to the delay in hospital admission and the increase in severity of lung injury in our patients.

During the influenza pandemic of 1917-1918, most deaths were due to bacterial coinfection[16]. During the influenza pandemic of 2009, it was reported that bacterial coinfection had a significant prevalence that contributed to mortality[17]. However, Russell, et al. reported severe ARDS and multiple organ failure solely attributable to the A(H1N1)pdm09 virus[18]. Moreover, studies in animal models and in humans have demonstrated the ability of the influenza A(H1N1) pdm09 virus to trigger necrosis and apoptosis in the cells of the respiratory epithelium and alveolar pneumocytes, as well as an intense local and systemic cytokine response[19-21]. Thus, infection with the A(H1N1) pdm09 virus may be sufficient to cause severe lung injury, vasodilatory shock, and multiple organ failure in the absence of bacterial coinfection. In our patients, leukocytosis detected on admission was significantly associated with mortality, and procalcitonin levels were also higher in non-survivor patients, suggesting bacterial coinfection[22]. However, all cultures on admission were negative, suggesting unlikely bacterial coinfection, although it cannot be ruled out since almost all patients received one or more antibiotics before admission.

The prevalence of obesity in our patients was 65%, which is more than twice the prevalence of 28.1% reported for our country by the WHO[23]. This suggests an increased risk of infection with influenza A(H1N1) pdm09 virus in these patients, consistent with findings previously reported during the influenza pandemic of 2009[2,3]. Although an association between obesity and mortality in patients with influenza A(H1N1)pdm09 has been suggested[24,25], obesity was not associated with mortality in our cohort of patients. Our results agree with those reported by the Mexican and Canadian groups during the influenza pandemic of 2009[2,3] as well as with other studies showing that mortality in obese patients with ARDS is not increased[26].

We identified LDH as a strong independent mortality predictor. However, the source of this LDH cannot be determined with certainty because the LDH isoenzymes were not determined. Smith, et al. reported high levels of LDH in serum and bronchoalveolar lavage fluid in patients with pneumocystis pneumonia[27]. Their results suggest that the increase in serum levels of LDH is due to backflow of pulmonary derived LDH into the blood through a compromised alveolocapillary membrane. Thus, levels of LDH could reflect the severity of lung injury, and could prove to be a better predictor of mortality than the PaO_2/FiO_2 ratio in our cohort of patients. Our results are consistent with those reported by other researchers in patients with severe pulmonary viral infections[28,29].

A significant problem observed in our patients was a high incidence of early ICU-AW. The cause of this syndrome in our patients is probably multifactorial (obesity, steroids, neuromuscular blockage). However, myositis by the A(H1N1)pdm09 virus with necrosis of muscle fibers may have played a significant role. A history of severe myalgia and elevation of CPK is consistent with the diagnosis of viral myositis[30]. Cases of acute severe myositis by the A(H1N1)pdm09 virus have been reported[31], and the myotropism of the virus and its ability to produce muscle necrosis have been demonstrated in animal models and in humans[32,33]. Because the rapid course to ICU-AW was an important factor for prolonged mechanical ventilation, the early implementation of a physiotherapy program may be especially significant for limiting further muscle function impairment.

Acute kidney injury has been described as a complication in patients with influenza A(H1N1)pdm09 and an independent predictor for mortality. Bagshaw, et

al.[34] reported an incidence of 60.9% of AKI according to the RIFLE classification in 562 critically ill patients with H1N1 influenza; AKI was not an independent predictor for hospital mortality. In contrast, Martin-Loeches, et al.[35] reported an incidence of 17.6% in 660 critically ill patients, where AKI was an independent predictor for mortality. In our cohort, 27% of patients had AKI on admission, and mortality was significantly higher in those with AKI-3. However, in multivariate analysis, AKI did not represent an independent predictor for mortality. Early support with hemodialysis may have contributed to the reduction of mortality risk in this group.

We identified three factors independently associated with mortality. Based on these, we developed a score to classify the patients into strata of low and high risk of mortality. The internal model validation by the bootstrap method showed the stability of parameters. However, we were not able to conduct an external validation (gold standard). Although the results should be viewed with caution, they may be useful for stratifying the severity of a patient and may contribute to the decision-making process.

Our study had some limitations. First, results represent a single institution. Second, bias in patient selection cannot be ruled out since admission to the RICU depended on the judgment of the attending physician. Due to this selection bias, it is possible that patients with a more severe disease, and with an increased mortality risk, were not selected for admission to the RICU, which could have influenced the low mortality that we found compared with that reported in other institutions nationwide, although the severity of respiratory failure is similar to that reported in other centers. We think that the experiences gained in the pandemic and subsequent outbreaks and the care in a specialized unit are also important factors that influenced mortality, although a selection bias may be present. Third, although an internal validation of the predictive model was made, the gold standard is the external validation. Thus, the validity of the predictive factors for mortality derived from our cohort must be taken with caution; further validation with another data set is needed.

In conclusion, we described the clinical characteristics and course of a cohort of patients with ARDS secondary to influenza A(H1N1)pdm09, and developed a predictive model based on the covariates of age, levels of LDH, and WBC count on admission to the RICU. The model may be useful for stratifying the severity of the patient and may contribute to the decision-making process. However, external validation of this proposed model is still required.

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Rev Inves Clin. 2016;68:245-55 ORIGINAL ARTICLE

Gender Differences in Quantitative Electroencephalogram During a Simple Hand Movement Task in Young Adults

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ABSTRACT

Background: No consensus has been reached regarding the existence of gender differences during motor tasks in electroencephalography. This could lead to misinterpretation of electroencephalography clinical diagnosis and affect the calibration of brain-computer interfaces. **Objective:** To assess whether there are statistically significant gender differences in electroencephalography recorded during hand movements. **Methods:** Electroencephalography data were recorded from 18 women and 18 men while performing hand movements and rest. Electroencephalography power was computed for alpha (8-13 Hz), beta (14-30 Hz), and a broader band including alpha and beta (8-30 Hz) using wavelet transform. Statistical analysis was done using a General Linear Model for repeated measurements (α = 0.05). Additionally, topographic maps were computed for each gender. **Results:** Significant gender differences were found for the rest condition in all analyzed bands. For the hand movement tasks, gender differences were mainly found in the beta band and located in temporoparietal areas. Power decrease observed in topographic maps was located in the centro-parietal areas for females and the centro-frontal areas for males. Additionally, greater power decreases were observed for women in all analyzed frequency bands. **Conclusion**: Electroencephalography parameters used for the diagnosis of neuromotor diseases, as well as for brain-computer interface calibration, must take gender into account. (REV INVES CLIN. 2016;68:245-55)

Key words: Alpha rhythm. Beta rhythm. Electrophysiology. Sensorimotor cortex. Wavelet analysis.

INTRODUCTION

Several studies have intended to show the anatomical and physiological differences in the nervous system between men and women[1]. These differences may seem obvious, but they have not always been demonstrated, although the studies assessing them have been conducted under rigorous methodologies. In this regard, one of the issues that has been extensively studied is the difference in the performance of motor skills. There are some studies that suggest a male superiority in gross motor skills such as ball skills[2] and simple

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Received for publication: 05-08-2016
Accepted for publication: 05-10-2016

index finger tapping[3,4], while the performance of females was often found to be superior to males in fine motor skills such as hand writing tasks[5] and pegboard tasks[3]. However, other studies with similar tasks have found no significant gender differences[6-8]. Most of these studies have been conducted in school children using neuropsychological tests. The reason for these differences could be due to anatomical sex dimorphisms[9-12] or to dissimilarities in functional cerebral organization[13-15], which have been analyzed using imaging studies. Although there are several studies related to the matter, there is still no consensus on whether there is a real biological difference in the performance of motor skills between men and women.

Quantitative electroencephalography (EEG) is an important tool in the analysis and understanding of movement. Currently, EEG features in time and frequency domain related to movement have applications in the field of clinical diagnosis and in the design of devices known as brain-computer interfaces (BCI). In clinical diagnosis, markers for assessment of motor-related conditions have been described in EEG elicited during motor execution. Some of these conditions are amyotrophic lateral sclerosis[16], progressive myoclonic epilepsy[17], cerebral palsy[18], and spinal cord injury[19]. In the field of BCI, changes in EEG are primarily elicited by means of motor imagery, which has been demonstrated to activate similar cortical areas such as motor execution[20-22], and are used to control external devices. Although most BCI systems are tailored to the user's EEG features, some of them have been designed to be calibrated with EEG data from a group of subjects in order to reduce calibration time of the system, which are known as subject-independent BCIs[23].

Since both subject-independent BCIs and quantitative EEG markers for motor-related conditions rely on EEG data extracted from a population, gender differences could produce a source of high variability that could lead to misinterpretation and poor performance of the mentioned applications. To take this variability into account, an analysis of EEG features needs to be done during the performance of motor skills. Some research groups have reported gender differences in EEG power amplitude across frequency bands in resting conditions. These studies have found that females had higher power amplitudes in the alpha band during rest than males[24,25]. Other studies have shown gender differences in the EEG associated to the performance of cognitive tasks. Significant gender differences during a simple visual-evoked potential were found in the EEG delta, beta, and gamma frequency bands[26], with the females having higher power amplitudes; however, no gender differences were found in the alpha frequency band. During REM sleep, a higher alpha and delta amplitude for the female gender was also reported[27]. Studies related to mental rotation have also found significant gender differences in EEG power[28,29]. Few researchers have studied gender differences in EEG activity when performing voluntary movement execution; e.g. index finger movements from both hands after an acoustic stimulus showed a higher normalized activation for women in frontal electrodes, while for men a higher activation was shown in the temporoparietal regions[30].

Since several studies have found significant gender differences in EEG features during rest and cognitive tasks, it might also be possible to find gender differences during motor execution. The main goal of this paper is to determine whether significant gender differences are elicited in EEG spectral power features, while performing simple hand movements, and how these changes are influenced by frequency band, spatial, and time feature selection of the EEG. These differences could affect the outcomes of studies that aim to use time and frequency EEG data for the assessment of motor-related diseases or affect the performance of BCIs.

MATERIAL AND METHODS

Participants

We recruited 40 healthy young adults; four subjects were excluded prior to data analysis due to excessive blinking artifacts. Therefore, this study was performed with data from 18 females and 18 males. Age range for the total sample was 21-30 years with mean of 26.31 years and standard deviation (SD) of 2.99 years. The mean age for the female group was 26.33 years with a SD of 3.27 years, while for males the mean age was 26.28 years and SD of 2.78 years. A non-paired *t*-test revealed no statistical differences between the mean age of both groups ($t[34] = 0.055$; $p = 0.9516$). All participants were students with right hand dominance for writing, with normal or corrected-to-normal vision, and without a history of

psychological diseases or brain injuries. An expert in clinical electrophysiology discarded any abnormality in the EEG using a qualitative approach. An expert in neuropsychology evaluated the ability of participants to follow instructions and concentrate on repetitive tasks without being distracted by other stimuli. This was done using the subscales of digit detection and visual detection of the neuropsychological test NEUROPSI Attention and Memory[31]; this test was developed and standardized for Spanish-speaking populations. All the participants achieved a normal performance in the NEUROPSI test. The participants signed an informed consent approved by the Ethics and Research Committee of the National Institute of Rehabilitation in Mexico.

Experimental task

Subjects were seated in a comfortable armchair. Visual cues were presented in the screen of a personal computer. The instructions were explained before starting the EEG acquisition. The experimental paradigm is extensively described in a previous work[23]. Participants performed real movements and motor imagery of both hands (continuous opening and closing of the left or right hand). In the present work, only the trials comprising rest with eyes opened and real movement were analyzed.

Each trial lasted eight seconds (three seconds for rest and five for hand movement) and was repeated 20 times; the right and left cues appeared randomly 10 times each to avoid the participants' habituation. The average frequency for grasp movement of all participants was approximately 0.6 Hz. From the EEG signals, we extracted 10 time segments of right hand movement, 10 of left hand movement, and 20 of the resting task.

EEG signal acquisition

The EEG data were recorded with a Nicolet® amplifier model NicONE (California, USA) with 32 channels and 16-bits resolution at a sample rate of 256 Hz. Twenty-two gold electrodes were located over the scalp according to the international 10-20 system of electrode placement. Ground and reference electrodes were located in the central forehead line. Electrodes were also placed in the orbicularis oculi muscle on both eyes to record their movements. The skin surface was lightly abraded to reduce the impedance between the skin and the electrode. Electrode impedances were kept below 5 kΩ. Electromyography (EMG) electrodes were placed in both arms above the deep flexor and superficial muscles of the fingers to verify that the subjects correctly performed hand movements on the specified time intervals. We analyzed 11 electrodes (F3, F4, C3, C4, P3, P4, T3, T4, Fz, Cz, Pz) located over the sensorimotor cortex since this area is involved in the central nervous system's processing of movement execution. The brain processes related to movement have been correlated in quantitative EEG with cortical oscillations known as mu rhythm, within a frequency range of 8-13 Hz (the same frequency range as the alpha rhythm), and beta rhythm, within a frequency range of 14-30 Hz. More specifically, voluntary movement is identified in the EEG as a suppression of power called event-related desynchronization (ERD) or an increase of power called event-related synchronization (ERS)[32].

All the recordings were conducted under consistent conditions: constant sound noise, same illumination, and same experimenter. Control records with intervals of open eyes and closed eyes were recorded before the movement tasks to evaluate basal brain activity of the participants.

Signal pre-processing

The technique known as common average reference (CAR) was used to generate a more ideal electrode reference for the EEG recordings. In CAR, the average value of the entire electrode montage is subtracted from that of the channel of interest. Because it emphasizes components that are present in a large proportion of the electrode population, CAR reduces such components, including movements and blink artifacts. Uncorrelated random noise with a zero mean is minimized through the averaging process[33]. After that, the re-referenced EEG signals were band-pass filtered from 8 to 30 Hz and a band-stop filter of 59-61 Hz was used to reduce line artifacts.

Time-frequency analysis

The method used to compute the EEG power for each trial and channel is based on a time-frequency wavelet decomposition of the signal. The main advantage of this approach is that it provides a better compromise between time and frequency resolution than methods using Fourier transform[34].

The EEG signal was convoluted by complex Morlet wavelets[35], having a Gaussian shape in both the time domain and the frequency domain around its central frequency f_0, as explained by Tallon-Baudry, et al.[36]. In our analysis, the wavelet family used is defined by a ratio (parameter that determines the width of the wavelets in number of cycles) of six, with f_0 ranging from 8 to 30 Hz with a resolution of 0.5 Hz. The analysis was performed in the time interval of 1-7 seconds (s) of each trial.

For each task and EEG channel, we computed the average power in two time windows: for the rest condition (REST) power was averaged from 1 to 3 sec and, for the left hand movement (LEFTMOV) and right hand movement (RIGHTMOV) power was averaged from a time window ranging from 3.5 to 7.0 sec. The first 500 ms following the movement cue onset of each time-frequency representation were not used for data analysis, since it was observed that EEG changes elicited during motor execution are detected in the signal with 300-500 ms of delay. The frequency bands used to extract the above-mentioned windows are alpha (8-13 Hz), beta (14-30 Hz), and their combination (8-30 Hz), named in this study, alpha-beta. This last band was selected because, in many BCI experiments, a broad frequency band is selected in order to select features for the processing stage[37,38]. A database was built with the averaged power values from the 10 trials, for 11 EEG channels and the three performed tasks. Data from only 10 randomly selected REST windows was used for data analysis.

In order to visualize cortex activations during hand movement, relative changes of power with respect to a baseline interval (from 1.5 to 2.5 sec) were calculated. Grand-averaged topographic maps for each gender were obtained for alpha, beta, and alpha-beta frequency bands.

All EEG signals were read, preprocessed, and processed using the Matlab® R2014b software and the free license toolbox Fieldtrip[39].

Statistical analysis

Since we have repeated measurements of the same variable, and these were computed under different conditions on the same subjects, we used a general linear model (GLM) with repeated measures to analyze the averaged power data. For every frequency band, we used a mixed design with 10 × 3 × 11 (trials by tasks by channels) within-subjects factors (repeated-measures) and gender as between-subjects factor. The dependent variable is the EEG power (μV^2) in each condition. GLM were performed to analyze main effects, three-way interactions, and simple main effects to communicate these interactions. The EEG power in every trial was analyzed to determine if gender differences could be found along time, despite inter-trial variability. The significance values were calculated for 95% confidence ($\alpha = 0.05$). Simple main effects were computed using a Bonferroni correction. Statistical analysis was performed using the SPSS v.17 software.

RESULTS

Statistical analysis revealed significant main effects of 10 different trials, 11 electrodes located above the sensorimotor cortex and three tasks (right hand movement, left hand movement, and rest) in the three analyzed frequency bands. The effect of gender was statistically significant only in beta and alpha-beta bands. There was no statistically significant three-way interaction between the effect of trials, task, and gender, except in the alpha band (Table 1).

Since we did not have significant three-way interactions, we analyzed simple main effects to determine if there were gender differences at each level of task, trials, and channels. We plotted the estimated marginal means for simple main effects between: trials × task × gender, trials × gender × task, channel × tasks × gender, channel × gender × task. Statistically significant values were obtained from the pairwise comparisons tables and marked by an asterisk in the plots shown in figures 1 to 4.

Gender differences across trials

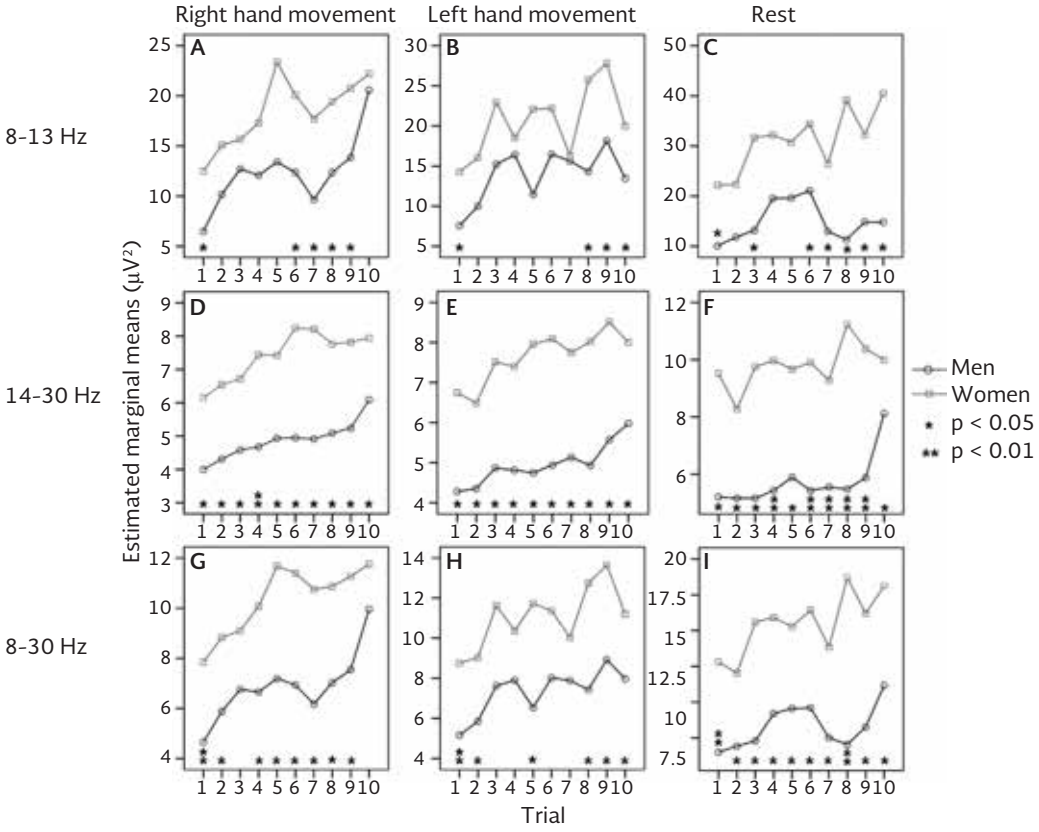
Figure 1 shows the estimated marginal means of EEG power for both genders in the RIGHTMOV, LEFTMOV, and REST tasks. Statistical differences were computed for each of the performed trials. In trial 1 and trials 6-10, significant gender differences ($p < 0.05$) were found in the alpha band for RIGHTMOV, LEFTMOV, and REST tasks (Fig. 1 A, 1 B, 1 C). Significant gender differences ($p < 0.05$) were found in beta in all trials

Table 1. Main effects and interactions for the general linear model for the three analyzed frequency bands

Independent variable	Alpha (8-13 Hz)				Beta (14-30 Hz)				Alpha-beta (8-30 Hz)			
	DF	DFe	F	p	DF	DFe	F	p	DF	DFe	F	p
Trials (A)	1	34	15.43	< 0.001*	1	34	18.61	< 0.001*	1	34	22.75	< 0.001*
Task (B)	1	34	9.18	0.005*	1	34	27.42	< 0.001*	1	34	15.18	< 0.001*
Channel (C)	1	34	4.76	0.036*	1	34	3.35	0.076	1	34	0.88	0.354
Gender (D)	1	34	2.51	0.122	1	34	6.49	0.015*	1	34	4.84	0.035*
A × B × D	1	34	4.99	0.032*	1	34	0.32	0.573	1	34	1.97	0.169
C × B × D	1	34	1.64	0.209	1	34	0.22	0.640	1	34	2.35	0.135

*Significant. DF: degree of freedom; DFe: degree of freedom for error; F: F-value.

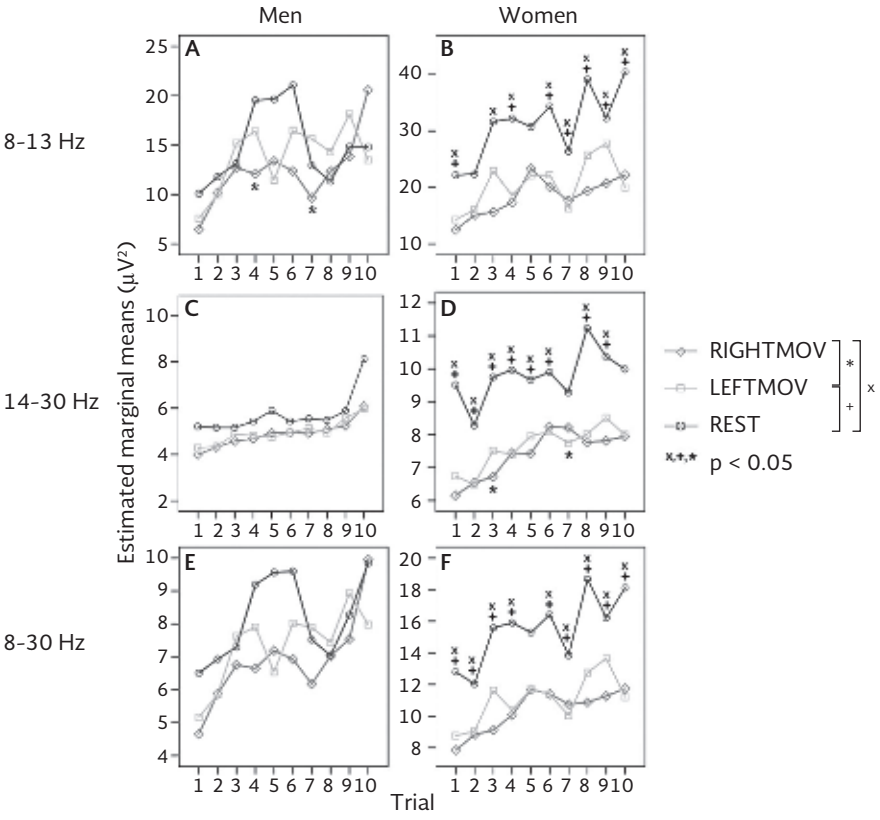
Figure 1. Estimated marginal means of EEG power in each trial for both genders. **A**, **B**, **C**: Gender differences in alpha band (8-13 Hz); **D**, **E**, **F**: in beta band (14-30 Hz); **G**, **H**, **I**; in alpha-beta band (8-30 Hz). One asterisk (*) indicates a significant difference with $p < 0.05$ and two asterisks (**) indicate significant difference with $p < 0.01$.



of the three tasks. It is important to observe that for the REST task, significant gender differences occurred with a 99% confidence level ($p < 0.01$) (Fig. 1 A, 1 B, 1 C). In the alpha-beta band, for most of the trials, significant gender differences ($p < 0.05$) were found in RIGHTMOV and REST, while for the LEFTMOV tasks less trials showed gender differences.

Figure 2 shows the estimated marginal means of power calculated for the 10 performed trials, separated by the RIGHTMOV, LEFTMOV, and REST tasks, observed for each gender. For men, significant differences ($p < 0.05$) between RIGHTMOV and LEFTMOV were only found in the trials 4 and 7 of the alpha frequency band (Fig. 2 A, 2 C, 2 D). For women, no

Figure 2. Estimated marginal means of EEG power in each trial for the three analyzed tasks. **A**, **B**: Statistically significant differences in alpha band (8-13 Hz); **C**, **D**: in beta band (14-30 Hz); **E**, **F**: in alpha-beta band (8-30 Hz). "x" indicates significant differences ($p < 0.05$) between RIGHTMOV and REST. "+" indicates significant differences ($p < 0.05$) between LEFTMOV and REST. "*" indicates significant differences between RIGHTMOV and LEFTMOV.



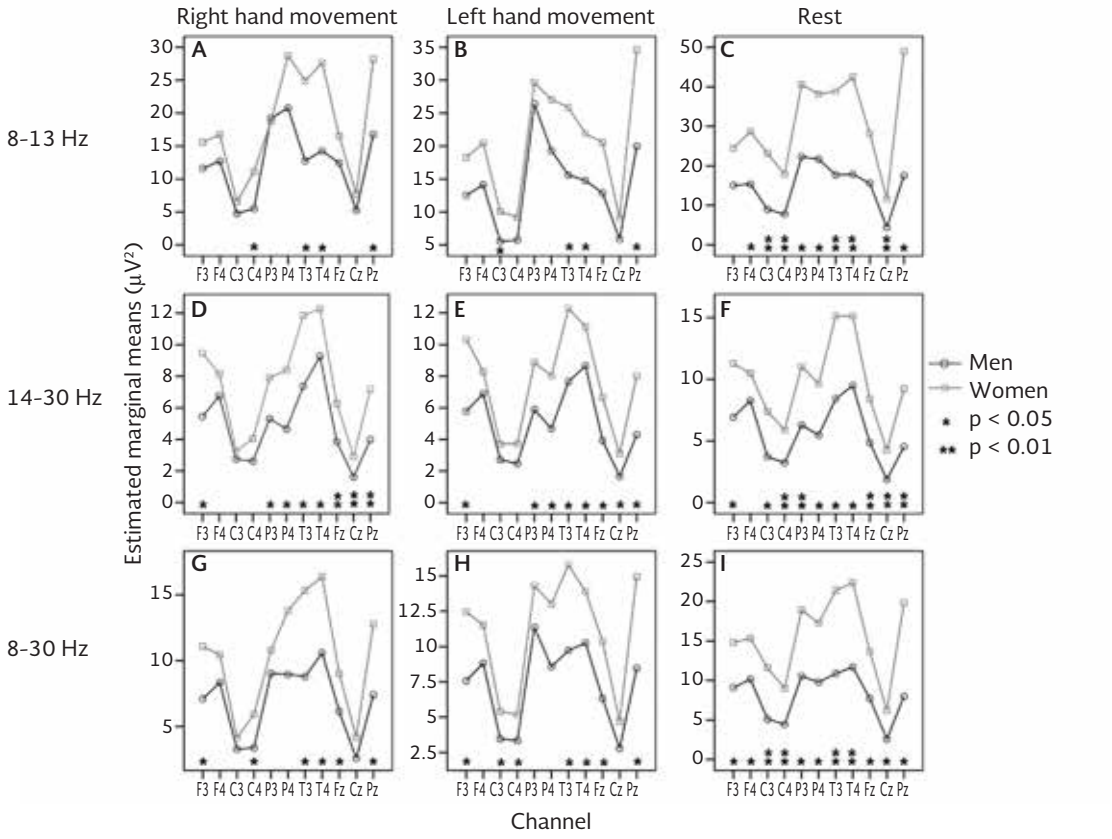
significant differences ($p < 0.05$) between RIGHTMOV and LEFTMOV were found in alpha or alpha-beta; however, these differences could be observed in the beta band in trials 3 and 7 (Fig. 2 D). For women, significant differences ($p < 0.05$) between RIGHTMOV and REST and between LEFTMOV and REST were found for all analyzed frequency bands and for most of the trials (Fig. 2 B, 2 D, 2 F).

Gender differences by EEG channel

For alpha, significant gender differences ($p < 0.05$) were found in RIGHTMOV in C4, T3, T4, and Pz (Fig. 3 A); for LEFTMOV these differences were found in C3, T3, T4, and Pz (Fig. 3 B); in REST these differences were found in most of the channels except for F3 and Fz (Fig. 3 C). For beta, significant gender differences ($p < 0.05$) while performing both RIGHTMOV and LEFTMOV were found for almost all channels except for F4, C3, and C4 (Fig. 3 D, 3 E), and for the REST, the only channel without significant gender difference was F4 (Fig. 3 F). For the alpha-beta band, significant gender differences ($p < 0.05$) were found in all channels of REST (Fig. 3 I), while for LEFTMOV no significant differences were found in F4, P3, P4, and Cz (Fig. 3 H), and for RIGHTMOV no significant gender differences were found in F4, C3, P3, and P4 (Fig. 3 G). In the three analyzed frequency bands, the recorded EEG channels with the lower mean power were C3, C4, and Cz, placed above the central area of the brain cortex. The recorded electrodes with the higher mean power were the ones placed above the parietal area in case of alpha, and in case of beta were the ones placed over the temporal area of the brain cortex.

Figure 4 shows the marginal means of EEG power for each one of the 11 acquired channels, calculated for the RIGHTMOV, LEFTMOV, and REST tasks. For men, no significant differences between different tasks were seen, except in beta for F3, F4 and C3 (Fig. 4 C). For women, significant differences ($p < 0.05$) between

Figure 3. Estimated marginal means of EEG power in each channel for both genders. **A**, **B**, **C**: Gender differences in alpha band (8-13 Hz); **D**, **E**, **F**: in beta band (14-30 Hz); **G**, **H**, **I**: in alpha-beta band (8-30 Hz). One asterisk (*) means a significant difference with $p < 0.05$ and two asterisks (**) means a significant difference with $p < 0.01$.



RIGHTMOV and LEFTMOV were observed in the alpha and alpha-beta bands in channels C3, P3 and Pz (Fig. 4 B, 4 F); for the beta frequency band significant differences ($p < 0.05$) between RIGHTMOV and LEFTMOV were only present in P3 (Fig. 4 D). Moreover, significant ($p < 0.05$) differences were observed, in the three analyzed frequency bands, between REST and the RIGHTMOV, LEFTMOV tasks in most of the channels (Fig. 4 B, 4 D, 4 F).

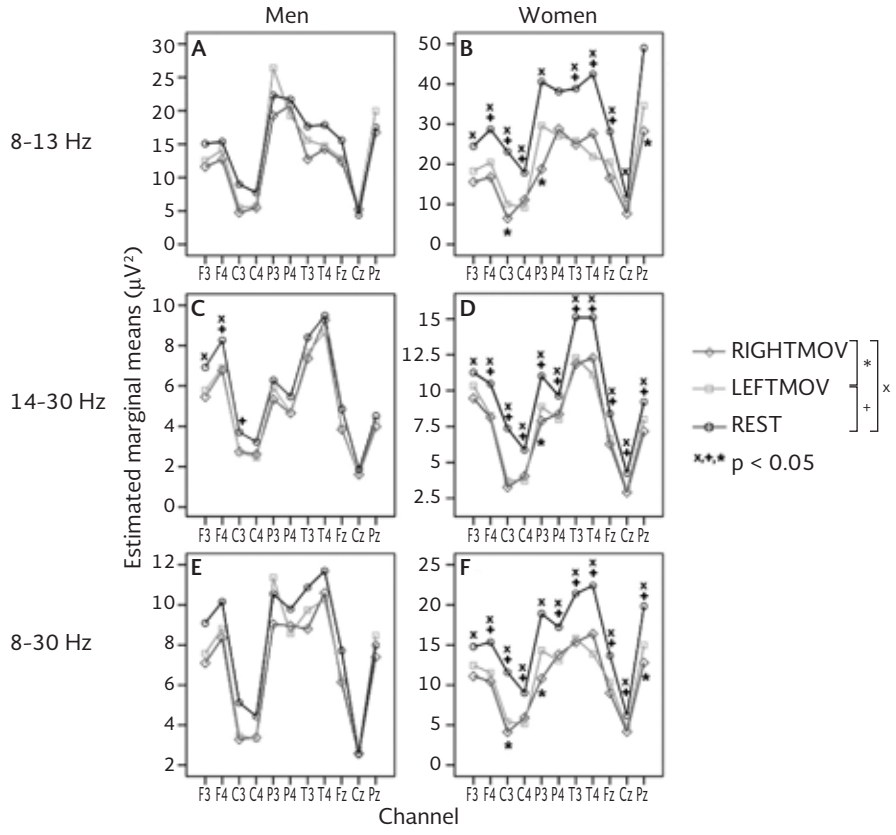
Brain topographic maps

Grand-averaged topographic maps of relative power of each gender are shown in figure 5. For both genders, a contralateral activation was elicited by RIGHTMOV (the dominant hand of the subjects) and an ipsilateral activation for LEFTMOV. For women, the more pronounced decreases in power were elicited in the alpha frequency band during RIGHTMOV and LEFTMOV. For men, the stronger decrease in power was elicited in alpha by RIGHTMOV, while for LEFTMOV the most significant decrease in power occurred in beta. Cortex activation in women was more pronounced and had a greater activation area than those from men for all of the analyzed frequency bands and for both RIGHTMOV and LEFTMOV tasks. For women, for both RIGHTMOV and LEFTMOV tasks, activations seem to concentrate in the centro-temporal regions (C3, T3, C4 and T4 electrodes), for all the analyzed frequency bands. For men, this dominance of centro-temporal activations is only seen in alpha, while predominant frontal (F3 and F4 electrodes) activations are elicited by LEFTMOV in beta and alpha-beta, and predominant centro-frontal and left centro-temporal activations (Fz, C3, T3) are elicited by RIGHTMOV in both beta and alpha-beta.

DISCUSSION

The results of this study showed significant gender differences in EEG spectral power calculated by means

Figure 4. Estimated marginal means of EEG power in each channel for the three analyzed tasks. **A**, **B**: Statistically significant differences in alpha band (8-13 Hz); **C**, **D**: in beta band (14-30 Hz); **E**, **F**: in alpha-beta band (8-30 Hz). "x" indicates significant differences ($p < 0.05$) between RIGHTMOV and REST. "+" indicates significant differences ($p < 0.05$) between LEFTMOV and REST. "*" indicates significant differences between RIGHTMOV and LEFTMOV.

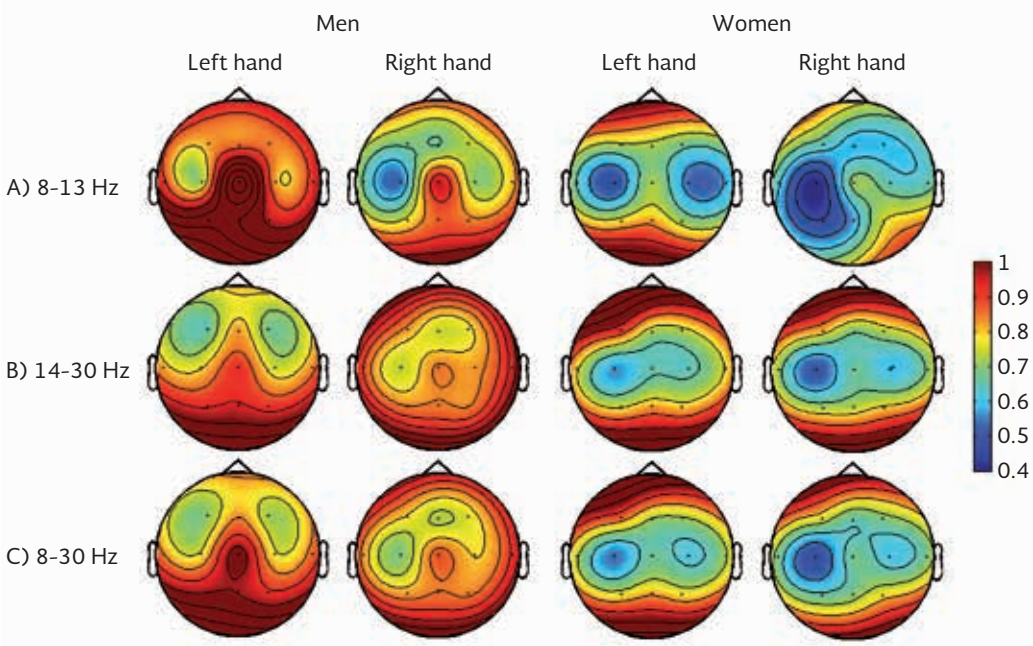


of time-frequency wavelets decomposition in young adults. These gender differences were found in EEG cortical activations during hand motor execution tasks and in a rest condition with open eyes.

Significant gender differences were found along time, despite inter-trial variability, for the RIGHTMOV, LEFTMOV, and REST tasks. These differences were found in the beta and alpha-beta frequency bands; however, for the alpha band, only the last trials showed significant gender differences. Power for the three analyzed tasks was higher in a 2:1 ratio for women in all the analyzed frequency bands. Gender differences for the beta band are similar to the ones reported by other studies that describe a higher power amplitude in the EEG for women in rest conditions[24,25] during the observation of a simple visual stimulus[26] and during mental rotation tasks[28,29]. These differences were observed along different cortical areas due to the distinct nature of each performed task. Hand movement tasks analyzed in this study seem to show a habituation process, observed as an increase in power as the performed trials progress, even though the order of the tasks was randomized, which is not observed in the REST task.

Temporal and parietal regions seem to have different activation or organization across genders since, for T3, T4 and Pz, significant differences were always found in RIGHTMOV, LEFTMOV, and REST tasks in all the analyzed frequency bands. For the alpha band, parietal (P3 and P4) and frontal (F3 and F4) areas do not seem to show significant gender differences in RIGHTMOV and LEFTMOV. While for beta band, central channels (C3 and C4) do not show significant gender differences for these motor tasks. These results suggest that motor cortex activation is similar for both genders, while somatosensory cortex activation is different for both genders, since temporoparietal brain activity is related to the sensory processing of hand movement[30].

Figure 5. Grand-averaged topographic maps for three frequency bands across each gender. **A**: Alpha band (8-13 Hz), **B**: beta band (14-30 Hz), **C**: alpha-beta (8-30 Hz). Relative power was computed with respect to a baseline interval from 1.5 to 2.5 seconds.



For all analyzed frequency bands, cortex activations for men were very similar when performing both hand movements and rest since there were no significant differences between tasks when analyzing power across trials or channels. Moreover, women showed significant differences between REST and either RIGHTMOV or LEFTMOV in all trials, channels, and frequency bands. Besides, for women, only in the central and parietal channels of the left hemisphere a significant difference between RIGHTMOV and LEFTMOV was found. Many BCI applications require discriminating between cortex activations during LEFTMOV and RIGHTMOV in order to translate them into control signals for external devices. However, the observations made in this study seem to indicate that many of the subjects in the sample generate very similar activation patterns for these two tasks. This highlights the importance of user training or neuromodulation to increase the ability of the subjects to generate different EEG patterns that can be recognized in their EEG signal. Some of the subjects could have a similar level of dexterity in both hands, which could explain the similarity in cortex activations while performing hand movements.

Topographic maps show that both genders elicit contralateral activations during RIGHTMOV, which is in accordance with the literature. However, women compared to men showed more pronounced power decrease with a larger activation zone, which involves the sensory association area. During LEFTMOV, gender differences were observed: for women, a bilateral activation with a small dominance of the central cortex area of the ipsilateral hemisphere is elicited, while for men, the activation is also bilateral but more focalized in the frontal area of the hemispheres, which is related to movement preparation. As well as for RIGHTMOV, women had more pronounced power decrease during LEFTMOV. The larger power decrease of the female gender was also reported by Cheng, et al. during the observation of hand movements by a group of subjects[40]. It is important to notice that the women's power decrease is constant across alpha, beta, and alpha-beta bands. A possible explanation for the observed bilateral activation during LEFTMOV could be that the non-dominant hand movement is a more complex task than the dominant hand movement (RIGHTMOV); therefore, both hemispheres are activated in order to correctly perform the movement. This activation is in concordance with two functional magnetic resonance studies that showed that left hand movement activates the ipsilateral hemisphere to a greater degree than right hand movement[41,42]. Additionally, Tomasi and Volkow[43] have

shown that women present a less functional lateralization, so they have a tendency to show bilateral hemispheric activation for verbal and non-verbal tasks, whereas men have a higher tendency to unilateral activation when performing such tasks.

The observed gender differences may be a product of physiological, anatomical, or psychological features among genders; for example, some neuroimaging-based morphological studies have suggested that women have a more complex and thicker brain cortex in the frontal and parietal lobes than men[44-46]. Other studies even suggest that women have an increased gray matter volume adjacent to the central sulcus, this being an area closely related to motor and somatosensory functions[47,48]. On the other hand, histological studies have shown an increased cortical neuron density in men with respect to women, with an increased number of neuronal processes (neuropil) in women[49]. All these anatomical and cellular differences can contribute to the gender differences in EEG power observed in the current study.

In this analysis, some of the features that could influence the results were controlled, including the subject's age, hand dominance for writing, and education level. Nevertheless, one limitation of this study is that a quantitative assessment of the handedness of subjects was not performed, so in order to further inspect this particular hypothesis the Edinburgh Inventory will be applied to the participants of future studies.

To the authors' knowledge, no study has analyzed gender differences, elicited in EEG power features during hand motor execution, in the recorded channels (spatial distribution), and the performed trials (time evolution). Therefore, this work contributes to improve the understanding of gender differences in the performance of gross motor skills in young adults. However, it is important to acknowledge that a larger sample of both subjects and trials per subject may be needed to achieve a larger perspective of the effect of time and the habituation effects that may be seen during motor execution tasks.

The observed results are a valuable contribution to the establishment of quantitative EEG markers for the diagnosis of neurological and motor disorders that take gender into account, and to BCI research.

ACKNOWLEDGEMENTS

The authors thank the Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico, for providing financial support with grant number SALUD-2015- 2-262061. The authors would also like to thank Saul R. Leon-Hernandez, Marlene A. Galicia-Alvarado and Blanca G. Flores-Avalos for their helpful comments.

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Rev Inves Clin. 2016;68:256-61 ORIGINAL ARTICLE

Therapeutic Effects of Bipolar Coagulation Forceps on Open Thyroid Surgery

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ABSTRACT

Background: The aim was to compare the therapeutic effects of bipolar coagulation forceps, harmonic scalpel, and conventional thyroidectomy on open thyroid surgery. **Methods:** A total of 527 patients who received open thyroid surgery in the Affiliated Drum Tower Hospital of Nanjing University Medical School between February 2013 and February 2016 were randomly divided into three groups: bipolar coagulation forceps, harmonic scalpel, and conventional thyroidectomy. There were no statistically significant differences in gender, age, disease constituents or mass diameter between the three groups. All surgeries were performed by the same surgeon. The surgical time, intraoperative blood loss, postoperative volume of drainage, postoperative hospital stay, and postoperative complications of the three surgical methods were compared. **Results:** The bipolar coagulation forceps and harmonic scalpel groups were significantly superior to the conventional thyroidectomy group ($p < 0.05$) in terms of surgical time, intraoperative blood loss, postoperative volume of drainage, and postoperative hospital stay, but the first two groups had similar outcomes ($p > 0.05$). There were significant differences between the three groups in temporary recurrent laryngeal nerve palsy and temporary hypoparathyroidism, and the results of the bipolar coagulation forceps group were significantly better than those of the other two groups ($p < 0.05$). No significant differences were found in airway depression due to postoperative bleeding or irritating cough induced by superior laryngeal nerve palsy between the three groups ($p > 0.05$). None of the patients in the three groups suffered from permanent recurrent laryngeal nerve palsy or permanent hypoparathyroidism. **Conclusions:** The effects of bipolar coagulation forceps on open thyroid surgery exceeded those of the harmonic scalpel and conventional thyroidectomy. This method is worthy of promotion in clinical practice. (Rev Inves Clin. 2016;68:256-61)

Key words: Bipolar coagulation forceps. Clinical application. Harmonic scalpel. Open thyroid surgery.

INTRODUCTION

Thyroid disease, which is currently one of the most common diseases worldwide, has been mainly treated by surgery. Albucasis, a Spanish doctor, successfully completed the first case of thyroid surgery in AD 952, but the postoperative mortality rate was extremely high[1]. Since Kocher and Billroth described the standardized method of thyroid surgery in detail for the first time in the late 19th century[2], thyroidectomy

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Received for publication: 25-08-2016
Accepted for publication: 14-10-2016

has been one of the most common head and neck surgeries. Subsequently, most thyroid diseases have been successfully treated by bilateral thyroidectomy. Due to the extremely rich blood supply in the thyroid, the mortality rate of thyroidectomy in early times was very high in the absence of effective hemostasis. Fifteen years later, the morality rate decreased from 12.8 to 0.5% by Kocher, who managed to ligate the upper and lower polar vessels of the thyroid[3]. Conventional thyroid surgery uses suture and ligation for hemostasis, which leads to considerable intraoperative blood loss, a blurred surgical field that interferes with accurate identification of the parathyroid glands, and hypoparathyroidism, thus affecting the quality of life of patients and even endangering their life safety.

With the development of medical science, the harmonic scalpel has been employed to allow endoscopic thyroidectomy and anterior cervical incision endoscopy-assisted thyroidectomy by performing excision and vascular closure simultaneously. Bipolar coagulation forceps (BCF), which was designed by Malis, induces satisfactory coagulation and hemostasis for small blood vessels. Using thin electrodes, it is suitable for treating small blood vessels near the recurrent laryngeal nerve and parathyroids on the back of the thyroid. Until now, BCF has seldom been applied in open thyroid surgery and its therapeutic effects have never been compared with those of the harmonic scalpel and conventional thyroidectomy.

MATERIALS AND METHODS

Baseline clinical data

A total of 527 patients who received open thyroid surgery in the Affiliated Drum Tower Hospital of Nanjing University Medical School from February 2013 to February 2016 were selected. Inclusion criteria: All patients had surgical indications for total thyroidectomy; written consent was obtained from all patients and their family members; this study was approved by the ethics committee of our hospital. The patients were randomly divided into three groups using a computer random number table, i.e. 180 cases in the BCF group, 167 cases in the harmonic scalpel group, and 180 cases in the conventional thyroidectomy (excision and ligation) group. The three groups had similar baseline clinical data such as gender, age and histological types ($p > 0.05$).

The patients consisted of 41 males and 486 females, with a male/female ratio of 1/11.85 and mean age of 43.2 ± 4.1 years. There were 98 cases of nodular goiter, 131 cases of thyroid adenoma, and 298 cases of papillary thyroid carcinoma. Surgical methods were: 124 cases treated with single thyroid lobectomy, 158 cases by total thyroidectomy, 176 cases received total thyroidectomy plus unilateral central lymph node dissection, and 69 cases received total thyroidectomy plus bilateral central lymph node dissection.

Surgical methods

Under general anesthesia, all patients received endotracheal intubation, with their shoulders boosted through hyperextension of the neck. A curved incision of about 3-5 cm was made along the dermatoglyph at a horizontal finger above the suprasternal notch. An electric scalpel was used to separate the space of loose connective tissues deep in the platysma muscle, up to the inferior margin of thyroid cartilage and down to the sternal notch. An incision was made along the *linea alba cervicalis* to find the thyroid capsule for surgery, sufficiently dissociating one or whole lobes in the space between true and false capsules. The harmonic scalpel group was treated by using an ultrasonic excision and hemostasis system (Johnson & Johnson, USA), with a 2 mm-wide Focus scalpel head. A ValleyBC BCF with a forceps tip diameter of 0.6 mm was used for the BCF group. The conventional thyroidectomy group was subjected to thyroid dissociation, forceps holding, excision, and ligation. According to thyroid lesions, the three groups underwent partial thyroidectomy, subtotal thyroidectomy, and thyroid cancer radical resection, respectively, performed by the same surgeon.

Observation indices

Surgical time (minutes)

The time of thyroidectomy was the time from thyroid exposure to resection. The time for thyroidectomy and central lymph node dissection was the time from thyroid exposure to resection and completion of central lymph node dissection.

Intraoperative blood loss (ml)

Intraoperative blood loss (unit: g, converted into ml according to the specific gravity) was the difference

between the weights of gauze before and after use. During surgery, an aspirator was not used to aspirate blood in the surgical field.

Postoperative volume of drainage (ml)

Postoperative volume of drainage was the volume drained from drainage tube placement until removal. Indication for drainage tube removal was if there was no obvious interstitial fluid under the skin flap, with the drainage volume in the tube of less than 15 ml/d.

Postoperative hospital stay

Postoperative hospital stay was from the first day after surgery to the day of discharge.

Surgical complications

Surgical complications included postoperative bleeding, recurrent laryngeal nerve palsy, hypoparathyroidism, and postoperative interstitial fluid under the skin flap.

There was active postoperative bleeding, and over 50 ml of blood was drained within one hour after surgery, which increased continuously. Space under the skin flap swelled clearly.

Recurrent laryngeal nerve palsy: All patients were subjected to fibro-laryngoscopy, and those with vocal cord paralysis were diagnosed. Temporary recurrent laryngeal nerve palsy was defined for cured, significantly or obviously relieved vocal cord paralysis after treatment, and permanent recurrent laryngeal nerve palsy was defined for unchanged paralysis. The recovery of patients with temporary recurrent laryngeal nerve palsy was examined by fibro-laryngoscopy and recorded.

Hypoparathyroidism: parathyroid hormone (PTH) and serum calcium levels were determined on the first postoperative day. Hypoparathyroidism was diagnosed when PTH levels were < 1.6 pmol/l and (or) serum calcium levels were < 1.9 mmol/l, or when patients had apparent hypocalcemia and numbness, with a serum calcium level of 1.9-2.1 mmol/l. If PTH and serum calcium levels were still below the above criteria three months after surgery, the patients were diagnosed as having permanent hypoparathyroidism. The patients were not routinely given calcium orally or intravenously before serum calcium examination.

Postoperative interstitial fluid under the skin flap: When there was obvious interstitial fluid and swelling after removal of drainage tube, which required fluid aspiration or reposition of the tube.

Statistical analysis

All data were collected from three independent replicate experiments, analyzed by SPSS22.0 and expressed as mean ± standard deviation. Inter-group comparisons were performed by Student's *t* test, and comparisons among multiple groups were conducted by one-way analysis of variance. A $p < 0.05$ was considered statistically significant.

RESULTS

Surgical outcomes

All excised samples were subjected to intraoperative frozen-section analysis. Total thyroidectomy and central lymph node dissection were performed for malignant tumors. The patients with lateral neck lymphatic metastasis who required lateral lymph node dissection were excluded. The surgical time, intraoperative blood loss, postoperative volume of drainage, and postoperative hospital stay of the three groups are listed in table 1. The BCF and harmonic scalpel groups had significantly better outcomes than the conventional thyroidectomy group ($p < 0.05$), whereas the former two groups had similar outcomes ($p > 0.05$).

Postoperative complications

The common complications after open thyroid surgery include postoperative bleeding, superior/recurrent laryngeal nerve injuries, and parathyroid dysfunction (Table 2). All three groups underwent airway depression due to postoperative bleeding resulting from uncontrolled high blood pressure. This symptom was effectively relieved by eliminating the hematoma using emergency treatment. Recurrent laryngeal nerve and parathyroid injuries were mainly manifested as temporary nerve palsy and temporary hypoparathyroidism, with the incidence rates of the

Table 1. Surgical outcomes (x ± s) in the three techniques of open thyroid surgery: bipolar coagulation forceps, harmonic scalpel, and conventional thyroidectomy

Observation index	Single thyroid lobectomy			Total thyroidectomy			Total thyroidectomy + unilateral central lymph node dissection			Total thyroidectomy + bilateral central lymph node dissection		
	BCF	Harmonic scalpel	Conventional thyroidectomy	BCF	Harmonic scalpel	Conventional thyroidectomy	BCF	Harmonic scalpel	Conventional thyroidectomy	BCF	Harmonic scalpel	Conventional thyroidectomy
Case number (n)	48	45	31	51	47	60	56	54	66	25	21	23
Surgical time (minutes)	39.5 ± 1.3	40.2 ± 1.5*	46.6 ± 2.3†‡	63.1 ± 3.1	59.8 ± 2.9*	71.6 ± 5.1†‡	61.2 ± 3.0	57.1 ± 2.1*	72.3 ± 4.3†‡	71.4 ± 6.1	62.3 ± 3.7*	75.4 ± 5.8†‡
Intraoperative blood loss (ml)	24.9 ± 0.6	21.1 ± 0.9*	30.2 ± 1.3†‡	39.8 ± 3.1	41.9 ± 3.8*	50.1 ± 3.8†‡	52.6 ± 4.7	45.9 ± 3.9*	55.3 ± 3.8†‡	57.9 ± 4.0	55.8 ± 4.1*	60.4 ± 5.1†‡
Postoperative volume of drainage (ml)	41.2 ± 4.2	49.5 ± 4.4*	55.1 ± 4.8†‡	88.4 ± 8.7	98.6 ± 10.1*	110.2 ± 11.5†‡	63.1 ± 5.4	78.6 ± 6.1*	82.8 ± 6.8†‡	126.2 ± 11.4	135.1 ± 12.1*	154.6 ± 12.4†‡
Postoperative hospital stay (days)	3.2 ± 0.4	3.6 ± 0.5*	4.1 ± 0.7†‡	4.6 ± 0.4	4.2 ± 0.6*	4.7 ± 0.6†‡	4.9 ± 0.7	4.7 ± 0.4*	5.1 ± 0.7†‡	5.2 ± 0.8	5.8 ± 0.6*	6.1 ± 0.7†‡

Comparison between harmonic scalpel and bipolar coagulation forceps groups, *$p > 0.05$; comparison between conventional thyroidectomy and BCF groups, †$p < 0.05$; comparison between conventional thyroidectomy and harmonic scalpel groups, ‡$p < 0.05$.
BCF: bipolar coagulation forceps.

Table 2. Postoperative complications (case [%]) in the three groups of open thyroid surgery: bipolar coagulation forceps, harmonic scalpel, and conventional thyroidectomy

Complication	BCF (n = 180)	Harmonic scalpel (n = 167)	p	BCF (n = 180)	Conventional thyroidectomy (n = 180)	p	Harmonic scalpel (n = 167)	Conventional thyroidectomy (n = 180)	p
Airway depression due to postoperative bleeding	1 (0.5)	1.0 (0.6)	> 0.05	1.0 (0.5)	3.0 (1.7)	> 0.05	1.0 (0.6)	3.0 (1.7)	> 0.05
Temporary recurrent laryngeal nerve palsy	2 (1.1 ± 0.12)	14.0 (8.4 ± 1.01)	< 0.05	2.0 (1.1 ± 0.12)	27.0 (15 ± 2.37)	< 0.05	14.0 (8.4 ± 1.01)	27.0 (15 ± 2.37)	< 0.05
Permanent recurrent laryngeal nerve palsy	0 (0)	0 (0)	> 0.05	0 (0)	0 (0)	> 0.05	0 (0)	0 (0)	> 0.05
Temporary hypoparathyroidism	4 (16)	3.0 (14.3)	< 0.05	4.0 (17.4)	7.0 (30.4)	< 0.05	3.0 (14.3)	7.0 (30.4)	< 0.05
Permanent hypoparathyroidism	0 (0)	0 (0)	> 0.05	0 (0)	0 (0)	> 0.05	0 (0)	0 (0)	> 0.05
Irritating coughing induced by superior laryngeal nerve palsy	2 (1.1)	6.0 (3.6)	> 0.05	2.0 (1.1)	10.0 (5.6)	> 0.05	6.0 (3.6)	10.0 (5.6)	> 0.05

BCF: bipolar coagulation forceps.

conventional thyroidectomy group significantly exceeding those of the other two groups ($p < 0.05$). All the affected patients suffered from hoarseness and numbness from the postoperative day 2-3, although without permanent injury. No significant differences were found in irritating cough induced by superior laryngeal nerve palsy between the three groups ($p > 0.05$).

DISCUSSION

After decades of development in open thyroid surgery, the requirements for specialist physicians have increased owing to continuously improved anesthetic techniques and updated surgical instruments[4]. Due to an increased incidence rate and a change in disease spectrum[5], single thyroid lobectomy and total thyroidectomy have been performed in most cases[6,7]. Therefore, protecting recurrent/superior laryngeal nerves and especially the parathyroid glands is the key to reducing surgical complications[8]. State-of-the-art surgical instruments should allow for an improvement in surgical efficiency and reduction in the incidence rates of complications as well.

This study compared the therapeutic effects of BCF, harmonic scalpel, and conventional thyroidectomy on open thyroid surgery, proving that BCF decreased the incidence rates of complications. Although traditional electric scalpel can effectively control bleeding, it causes considerable thermal damage, and the resulting smoke also easily affects the surgical field. On the other hand, the high-frequency electric scalpel inevitably stimulates nerves[9]. The harmonic scalpel system, which has been widely used in breast as well as head and neck surgeries[10], can stop bleeding by generating ultrasonic waves through the metal tip vibration to vaporize water in tissues and cells and to produce vascular closure through protein coagulation. Therefore, the harmonic scalpel is capable of performing tissue excision and hemostasis simultaneously so as to reduce vascular ligation[11] and to shorten surgical time[12] as advantages[13-15]. Nevertheless, in this study, harmonic scalpel produced intense heat between the tip and its surrounding tissues during firing, which induced significant thermal burns to the tender recurrent laryngeal nerve, superior laryngeal nerve, parathyroid, and even the skin of incision. Similar to a previous report[16], there were a large number of cases with postoperative temporary recurrent laryngeal nerve palsy and hypoparathyroidism. BCF, which has most commonly been used for ear nose and throat surgery and neurosurgery, causes hemostasis by using two tips to provide high-frequency electrical energy for tissues with lesions, aiming to dehydrate blood vessels between both tips[17,18]. Since BCF only works for the range between two tips, adjacent tissues are barely damaged or affected and remain safe[19]. We herein verified the advantages of BCF by comparing the treatment outcomes of three groups. First, similar to an ordinary forceps in shape, BCF can be flexibly used. Being capable of small vessel coagulation, it can reduce vascular ligation and contribute to surgeries with small incisions. Second, compared with harmonic scalpel, the tips of BCF are as tiny as 0.6 mm, so it is particularly suitable for delicate surgeries such as hemostasis of small blood vessels surrounding recurrent or superior laryngeal nerves. In addition, BCF can stop bleeding accurately and effectively without thermal burns to nerves[20]. Third, after a short time of firing, the electric current passes between the two tips of BCF so it only affects a few held tissues. Additionally, the temperature of the BCF tip is much lower than that of the harmonic scalpel tip so it does not cause thermal damage to surrounding tissues, which is a striking advantage. In this study, mitigation of temporary recurrent laryngeal nerve palsy and temporary hypoparathyroidism in the BCF group provided the best evidence. And fourth, BCF is readily applicable in primary hospitals due to its low cost and high cost-effectiveness ratio.

Regarding the surgical time, intraoperative blood loss, postoperative volume of drainage, and postoperative hospital stay, the BCF group and the harmonic scalpel group were superior to the conventional thyroidectomy group ($p < 0.05$), although without significant differences between the former two groups ($p > 0.05$). There were significant differences in temporary recurrent laryngeal nerve palsy and temporary hyperparathyroidism between the three groups, and the results of the BCF group significantly surpassed those of the other two groups ($p < 0.05$). The incidence rates of airway depression due to postoperative bleeding or irritating coughing induced by superior laryngeal nerve palsy were not significantly different among the three groups ($p > 0.05$). There were no cases of permanent recurrent laryngeal nerve palsy or permanent hyperparathyroidism in the three groups.

In summary, open thyroid surgery has become increasingly sophisticated, specialized, and minimally invasive, meeting with aesthetic requirements as well. Being both safe and feasible for open thyroid surgery, BCF allows performing thyroidectomy with small incisions. Well-trained specialist physicians can perform open thyroid surgery with ease after mastering the skills for using BCF. The low-cost, widely applicable BCF can decrease surgical expenses, have therapeutic effects similar to those of the harmonic scalpel, and also prevent complications, and thereby is worthy of promotion in the clinical practice.

ACKNOWLEDGMENTS

Lei Su and Jiayong Li contributed equally to this study.

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Rev Inves Clin. 2016;68:262-8 ORIGINAL ARTICLE

Microalbuminuria and its Association with Subclinical Atherosclerosis in the Mexican Mestizo population: the GEA study

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ABSTRACT

Background: Microalbuminuria is an early marker of atherosclerosis. Ethnic differences for both conditions have been reported. We studied microalbuminuria prevalence and its association with coronary artery calcification as an early atherosclerosis marker in a Mexican-Mestizo population free of diabetes and hypertension (healthy), as well as in hypertensive and diabetic subjects. **Methods:** In 1,472 adults (53.3 ± 9.4 years old, 50.3% women), anthropometric measurements, fasting blood glucose, and lipid profile were determined. A spot urine sample was used to quantify the albumin-to-creatinine ratio and to define microalbuminuria (20-200 mg/g in men, and 30-300 mg/g in women). A coronary artery calcification score was obtained by electron-beam computed tomography and subclinical atherosclerosis was defined as a score > 0. **Results:** Overall microalbuminuria prevalence was 9.3% (5.4% in healthy, 11.6% in obese, 12% in hypertensive, and 25% in diabetic subjects). Compared to "healthy" subjects without microalbuminuria, those with microalbuminuria had a ~3-fold higher prevalence of coronary artery calcification > 0, while normal-high albumin-to-creatinine ratio (OR: 1.8; $p < 0.05$) and microalbuminuria (OR: 2.6; $p < 0.001$) was independently associated with coronary artery calcification > 0 only among diabetic subjects. **Conclusions:** Microalbuminuria and high-normal albumin-to-creatinine ratio were independently associated with subclinical atherosclerosis, suggesting that they may confer a higher risk of future cardiovascular events. (REV INVES CLIN. 2016;68:262-8)

Key words: Coronary artery calcium. Microalbuminuria. Subclinical atherosclerosis.

INTRODUCTION

Kidney disease increases the risk of cardiovascular and all-cause mortality[1,2]. A moderate decrease in kidney function is also associated with subclinical atherosclerosis[3]. In studies performed in different populations, microalbuminuria (MA), which is known to be associated with kidney disease, has been associated with an increased incidence of coronary artery disease (CAD) events, and elevated risk of all-cause and CAD mortality in diabetes mellitus (DM) and hypertensive (HT) patients, as well as individuals without these clinical

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Received for publication: 06-09-2016
Accepted for publication: 28-10-2016

conditions[4]. The association between urinary albumin excretion and CAD has been observed at albumin-to-creatinine ratios even below 30 mg per gram of creatinine, a value usually used to define microalbuminuria[5,6].

Coronary artery calcification (CAC) is a specific marker of the presence and magnitude of atherosclerosis[7]. This marker strongly correlates with histologically atherosclerotic plaque area[8]. Moreover, CAC may be a predictor of future coronary events in asymptomatic patients[9] and may be identified and quantified rapidly, easily, and non-invasively using computed tomography<. Several studies have found an association between MA and subclinical atherosclerosis, defined by the presence of CAC[10]. However, ethnic variations in the prevalence of CAC[11-14] as well as albuminuria[15,16] suggest that the degree of this association depends on the analyzed population. There have been no studies that analyze the relationship between MA and subclinical atherosclerosis in Mexico. For this reason, our objective was to investigate the prevalence of MA and its association with subclinical atherosclerosis defined by the presence of CAC in a "healthy" (no evidence of DM or HT) Mexican-Mestizo population as well as type 2 diabetic and hypertensive patients.

METHODS

This investigation is part of the "Genetics of Atherosclerosis Disease" (GEA, for its initials in Spanish) study, designed at the National Institute of Cardiology Ignacio Chavez to examine the genomic basis of CAD and to evaluate its relationship with traditional and emerging cardiovascular risk factors in an adult Mexican population. The study included a group of 1,000 patients with CAD and a control group of 1,500 individuals without CAD, who were all between the ages of 35-75 years and residents of Mexico City. Subjects from the control group were volunteers with no clinical or family history of CAD, who attended the blood bank of the National Institute of Cardiology Ignacio Chavez or were invited via written messages placed in social service centers. Exclusion criteria included a history or clinical evidence of cancer, liver or kidney disease, or corticosteroid use. Urine samples were unavailable in 28 participants; therefore this report includes only 1,472 control subjects. The GEA study was approved by the Ethics and Research Committee of the National Institute of Cardiology Ignacio Chavez following the guidelines of the Declaration of Helsinki. All subjects who participated in the study signed an informed consent form.

Clinical methods

Every participant was asked to complete a standardized questionnaire containing demographic information, personal background of cardiovascular risk, dietary habits, physical activity, alcohol and tobacco consumption, and use of medications. Weight (kg) and height (m) were recorded and body mass index (BMI) calculated as kg/m^2. Waist circumference was measured at the midpoint between the bottom of the lowest rib and the iliac crest with an approximation of 0.5 cm. Blood pressure was measured three times using a Welch Allyn digital sphygmomanometer after at least five minutes in a resting position. The average of the last two measurements was used for the analysis.

Laboratory procedures

Each participant was told to avoid vigorous exercise or smoking the morning of the study and there should be no evident infections during the previous two weeks. Venous blood samples were collected after a 12-hour fasting and 20 minutes in a resting position. K_2EDTA (1.8 mg/ml) plasma or serum were prepared by centrifugation at 4° C at 2,500 rpm for 20 minutes. Total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured in plasma, while uric acid and creatinine were determined in serum within three days after obtaining the sample. The measurements were made in a Hitachi 902 automated analyzer (Hitachi LTD, Tokyo, Japan) using enzymatic-colorimetric reagents (Roche Diagnostics, Mannheim, Germany). The reproducibility and reliability of the lipid and lipoprotein determinations were evaluated periodically by the Lipid Standardization Program of the Centers for Disease Control and Prevention (LSP-CDC, Atlanta, GA, USA). Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formula as modified by De Long, et al. Albuminuria was determined in a spot urine sample that was kept at -70° C (boric acid 10 mg/ml) until analyzed by immunonephelometry in a BN ProSpec® nephelometer (Dade Behring, Marburg GmbH, Germany). Creatinuria was determined by the Jaffe method in a Hitachi 902 automated analyzer (Hitachi LTD, Tokyo, Japan). Renal function was estimated with the

glomerular filtration rate (GFR)[17], using the following formula: MDRD GF (ml/min/1.73 m^2) = 186 × serum creatinine$^{-1.154}$ × age$^{-0.203}$ × 0.742$^{(women\ only)}$.

Computed tomography study

Coronary artery calcification and visceral (VAF) and subcutaneous (SAF) abdominal fat were quantified using an axial computed tomography scan. We used a 64-channel multidetector scan (Somatom Sensation, Siemens) to obtain the images that were subsequently interpreted by a specialized radiologist. The VAF was quantified as described by Kvist, et al.[18]. The Agatston method was used to quantify CAC[19]. Subclinical atherosclerosis was considered to be present when CAC levels exceeded zero Hounsfield units (CAC > 0).

Cardiovascular risk factor definitions

To classify the degree of albuminuria, we used the albumin/creatinine ratio with the following cut-off points[20]: normal albuminuria (< 10 mg/g in men and < 15 mg/g in women), normal-high (10 to < 20 mg/g in men and 15 to < 30 mg/g in women), microalbuminuria (20 to < 200 mg/g in men and 30 to < 300 mg/g in women), and macroalbuminuria (> 200 mg/g in men and > 300 mg/g in women). Hypertension was defined with systolic and/or diastolic blood pressure readings ≥ 140/90 mmHg, or the use of antihypertensive medication. Impaired fasting glycemia was defined as fasting glucose levels between 100 and 125 mg/dl, while diabetes was considered when glucose levels were ≥ 126 mg/dl, when there was a previous diagnosis, or the patient used hypoglycemic treatment.

Statistical analysis

General characteristics of the population were reported as mean ± standard deviation, median (interquartile range) or prevalences. Comparisons were performed using ANOVA, Kruskal-Wallis or chi-squared, as appropriate. Coronary artery calcification > 0 and the degree of albuminuria were analyzed as simple prevalence. To identify the independence of the association between these variables, a logistic regression analysis adjusted for age, gender, VAF, uric acid, LDL-C, HDL-C and glomerular filtration rate was performed. All $p < 0.05$ values were considered statistically significant. Statistical analysis was performed using SPSS, version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Our investigation included 1,472 subjects (50.3% women), with an average age of 53.3 ± 94 years and BMI of 28.5 ± 4.4 kg/m^2. Hypertension was identified in 25%, DM in 13.0%, and obesity in 31.7% of the population studied. Overall MA prevalence was 9.3%: 12.0% in subjects with HT, 25% in those with DM, 11.6% among obese, and 5.4% in "healthy" subjects. Clinical and biochemical characteristics of the population stratified by albuminuria concentration are shown in table 1. Age, proportion of men, BMI, and waist circumference increased gradually and significantly as the value of urinary albumin increased. It is worth noting that the highest values of waist circumference present in MA subjects was determined by excess VAF, given that SAF showed no statistically significant differences across groups. In general, subjects with normal-high albuminuria and MA had a more adverse cardiometabolic profile than those with normal degree of albuminuria. This profile was characterized by an HT prevalence twice as high, and a DM prevalence 3-4-times higher. These subjects also presented higher LDL-C, TG, and uric acid levels, as well as lower HDL-C concentrations. This adverse cardiometabolic profile was associated with a higher prevalence of subclinical atherosclerosis (23.7 vs. 33.8 and 51.5%; $p < 0.001$).

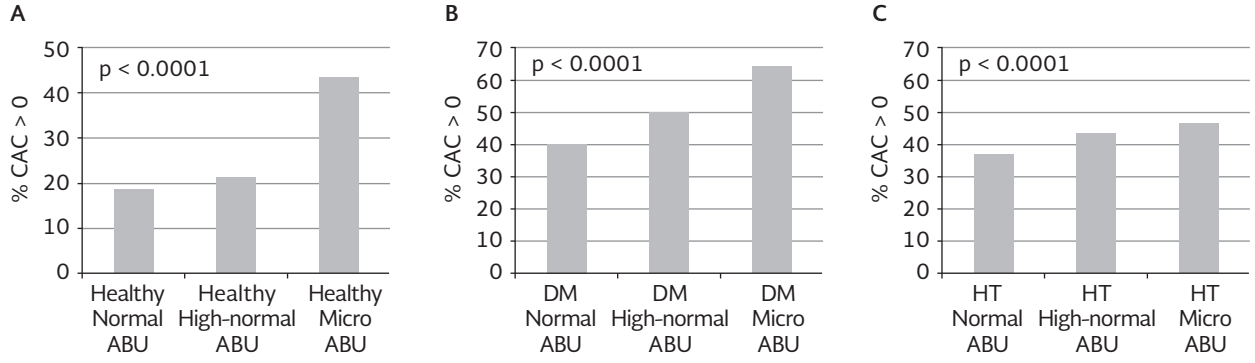
The association between microalbuminuria and subclinical atherosclerosis (Fig. 1) was analyzed separately: "healthy" subjects (panel A), DM patients (panel B), and HT patients (panel C). As expected, DM and HT patients had a CAC > 0 prevalence twice as high as the "healthy" subjects and this prevalence increased gradually and significantly as albuminuria concentrations increased in all three groups ($p < 0.0001$). The independence of this association was investigated through a logistic regression analysis (Table 2) adjusted for age, gender, VAF, uric acid, LDL-C, HDL-C, GFR, and patients receiving angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers (n = 128 and 82, respectively). To exclusively analyze the effect of albuminuria, in each group, subjects with normal albuminuria were considered as the reference group. In healthy subjects, the independent risk of subclinical atherosclerosis was three times as high for subjects with MA. In DM patients, normal-high albuminuria was associated with an independent risk, 1.8-times higher, while MA showed a risk 2.6-times higher. These associations were not independent for HT patients (Table 2).

Table 1. Clinical and biochemical characteristics of study population stratified by albuminuria concentration

	Normal n = 1176 (80%)	Normal-high n = 143 (9.7%)	Microalbuminuria n = 137 (9.3%)	Macroalbuminuria n = 16 (1%)	p*
Age (years)	52.6 ± 9.2	55.7 ± 9.6	55.5 ± 10.0	57.9 ± 9.0	< 0.0001
Men (%)	46.1	55.9	70.8	75.0	< 0.0001
BMI (kg/m^2)	28.3 ± 4.3	29.5 ± 4.5	29.4 ± 4.9	28.3 ± 3.5	< 0.01
Waist (cm)	94.1 ± 11.3	98.5 ± 11.5	98.3 ± 12.5	97.4 ± 9.5	< 0.0001
SAF (cm^2)	296 ± 112	306 ± 116	282 ± 118	256 ± 99	0.182
VAF (cm^2)	151 ± 61	181 ± 67	179 ± 71	186 ± 78	< 0.0001
SBP (mmHg)	115 ± 15	125 ± 21	130 ± 22	134 ± 19	< 0.0001
DBP (mmHg)	71 ± 8	75 ± 11	78 ± 12	7 7± 7	< 0.0001
HT (%)	20.9	39.9	41.6	50.5	< 0.0001
DM (%)	8.3	23.8	35.0	75.0	< 0.0001
Smoking (%)	21.9	22.4	23.5	31.3	0.814
LDL-C (mg/dl)	119 ± 31	120 ± 35	111 ± 31	111 ± 38	< 0.05
HDL-C (mg/dl)	47 ± 13	45 ± 13	43 ± 12	44 ± 8	< 0.05
TG (mg/dl)	144 (108-197)	162 (121-208)	167 (120-230)	183 (139-187)	< 0.0001
UA (mg/dl)	5.6 ± 1.5	5.6 ± 1.6	6.0 ± 1.6	5.7 ± 1.7	< 0.05
GF_{MDRD} (ml/min/1.73 m^2)	90 ± 20	97 ± 26	91 ± 19	86 ± 25	< 0.05
Agatston (HU)	24 ± 137	34 ± 115	86 ± 259	189 ± 601	< 0.0001
CAC > 0 (%)	23.7	33.8	51.5	31.3	< 0.001

*ANOVA.
BMI: body mass index; CAC: coronary artery calcium; DBP: diastolic blood pressure; DM: diabetes; GF_{MDRD}: glomerular filtration rate; HDL-C: High-density lipoprotein cholesterol; HT: hypertension; LDL-C: low-density lipoprotein cholesterol; SAF: subcutaneous abdominal fat; SBP: systolic blood pressure; TG: triglycerides; UA: uric acid; VAF: visceral abdominal fat.

Figure 1. Proportion of subjects with subclinical atherosclerosis (CAC > 0) in: **A.** Healthy subjects (without hypertension or diabetes mellitus) **B.** Subjects with diabetes mellitus, **C.** Subjects with hypertension without diabetes mellitus, by albuminuria concentration. chi-square.



DISCUSSION

The results of this investigation demonstrate that the prevalence of MA in patients without diabetes or arterial hypertension is 5.4%, while this prevalence increased to 12.0, 25.0, and 11.6%, for HT, diabetic, and obese subjects, respectively. Our most important finding was identified in the logistic regression analysis, adjusted for CAC confounding factors, including renal function. This analysis showed that even normal-high levels of albuminuria, and not MA exclusively, may be associated with subclinical coronary atherosclerosis, defined as CAC > 0. Despite their expected higher cardiovascular risk, the finding of a

Table 2. Odds ratios (95% CI) for coronary artery calcification (> 0) by albuminuria concentration in healthy, diabetic, and hypertensive subjects

	Normal albuminuria	Normal-high albuminuria	Microalbuminuria	p*
Healthy	Referent	0.7 (0.4-1.5)	2.9 (1.5-5.8)	< 0.05
Diabetes mellitus	Referent	1.8 (1.09-3.1)	2.6 (1.5-4.6)	< 0.001
Hypertension	Referent	1.4 (0.9-2.4)	1.4 (0.7-2.6)	0.319

*Logistic regression analysis adjusted for age, sex, visceral abdominal fat, uric acid, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, glomerular filtration rate, and treatment with angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers.

lower prevalence of CAC in the participants with macroalbuminuria may be explained by the much smaller number of subjects in this subgroup, thus potentially biasing this observation toward the null.

Differences in renal albumin excretion have been reported between Hispanic and Caucasian subjects[10]. Some studies have documented that MA is approximately twice as common in Hispanic populations of Mexican origin[16,21]. Our results are not consistent with these observations. The prevalence of MA in our healthy population was 5.4%, close to the 6% detected in the Framingham study performed on Caucasians with similar clinical characteristics[6]. On the other hand, previous data showed MA in ~30% of patients with type 1 or 2 DM[22], which agrees with our finding of MA in a quarter of the DM patients in this investigation.

Obesity is a condition with an important role in the development of MA. Epidemiologic studies have found an independent association between increased BMI and MA[23]. It has been suggested that excess visceral abdominal fat and the accompanying insulin resistance result in a state of oxidative stress and endothelial dysfunction that leads to MA[24]. In the PREVEND study, the prevalence of MA was 29.3% in obesity, while in normal-weight subjects it was 9.5%[23]. In the present study, the prevalence was 13.7 and 7.5%, respectively.

Despite the fact that several studies have not found a significant relationship between MA and CAD[25,26], many investigations have reported an independent association between these two conditions. Gerstein, et al.[27] identified a relationship between MA and the presence of stroke, myocardial infarction, and cardiovascular mortality in patients with and without diabetes. Other population-based studies have demonstrated an association of MA with a higher incidence of coronary events, as well as an elevated risk of cardiovascular morbidly and mortality[4,28,29]. Furthermore, normal-high concentrations of urinary albumin have been reported to be independently and strongly associated with coronary disease[5], even in individuals without DM or HT[6]. Conversely, some[30,31] but not all intervention studies[32] have observed that the incidence of cardiovascular events is reduced as albuminuria levels are decreased. Altogether, these findings show that the presence of MA may be associated with higher risk of cardiovascular events and mortality in high-risk patients, as well as those without DM or HT.

Microalbuminuria has also been shown to be associated with subclinical atherosclerosis, defined by the presence of CAC[10]. In the multi-ethnic study of atherosclerosis (MESA) carried out in 6,814 adults, with an average age of 62.7 years and with no clinical manifestations of cardiovascular disease, participants with MA and normal-high levels of albuminuria exhibited a higher frequency of elevated CAC scores[10]. In a more recent, cross-sectional study that included 1,318 asymptomatic subjects, without DM or HT, high CAC values of 100-400 and even > 400 Agatston units were significantly more common in subjects with MA[33]. Moreover, previous studies examined the influence of MA in the progression of coronary calcification. Subjects with MA were shown to have a higher degree of CAC progression even after adjusting for basal CAC and other confounding factors, in type 1[34] and 2[35] DM patients without clinical cardiovascular disease, followed during four years. The association between MA and the development and progression of atherosclerosis was also investigated in MESA[36]. The authors found a significantly higher risk in the incidence and progression of CAC in participants with MA. The results of the present study performed in a Mexican-Mestizo population

are consistent with the findings of previous studies[5,10] when demonstrating that normal-high levels of albuminuria, as well as MA, may be associated with subclinical coronary atherosclerosis, defined by the presence of CAC. These results were found even after adjusting for age, gender, visceral abdominal fat, uric acid, LDL-C, HDL-C, and GFR. This association was identified in apparently healthy subjects, as well as in patients with arterial hypertension and DM. Since the GEA study is currently in the follow-up stage, the effect of MA on the progression of atherosclerosis, as well as cardiovascular morbidity and mortality, may be established in the future.

The mechanisms that take part in the association between MA and CAD are not well known. Some factors that associate MA with atherogenesis, such as low-grade systemic inflammation[37] and endothelial dysfunction[38], have been identified. In addition, some authors have suggested that albuminuria may be a sign of endothelial dysfunction that affects the vascular beds in the brain, eyes, heart, and kidneys[39]. Furthermore, they have suggested that MA may represent the renal manifestation of microvascular disease[40] produced by a variety of hemodynamic, metabolic, and inflammatory processes. In the early stages of these conditions, microvascular alterations and MA are reversible by controlling the processes from which they originate. However, in later stages, the movement of albumin towards the arterial wall may result in an inflammatory response, accumulation of lipids, and development of atherosclerosis[41]. The results of the present and previous studies[10,33] are consistent with the hypothesis that MA and normal-high levels of albuminuria reflect glomerular endothelial dysfunction. Therefore, albuminuria may be an important marker for the presence of coronary atherosclerosis and future cardiovascular events, even in individuals without diabetes or hypertension[6].

Limitations

The authors of the present study recognize that the study has several limitations. First, as is the case with every cross-sectional study, our results cannot establish a cause-effect relationship. Second, albumin was quantified from a single urine sample. Previous studies have suggested that urinary albumin levels may have important, intra-individual variability[42]. Nevertheless, measuring the albumin/creatinine relationship from a single, random urine sample has been recommended because it is a simple procedure and the results often closely correlate with those obtained after collecting urine for 24 hours[43]. Third, the subjects of the GEA study were volunteers and therefore may not fully or correctly represent the general population. However, due to the unlikelihood that the studied population knew their coronary vascular bed score, we expect the risk relationship to be similar to one obtained from a random sample.

The results obtained in the present study allow us to identify the prevalence of microalbuminuria in the Mexican-Mestizo population. They also confirm that normal-high levels of urinary albumin and the presence of MA are associated with subclinical coronary atherosclerosis in arterial hypertension and DM patients, as well as in healthy subjects. The ongoing monitoring of the GEA study's subjects will allow us to identify the usefulness of MA in the prediction of cardiovascular events in the studied population.

ACKNOWLEDGEMENTS

The "Genetics of Atherosclerosis Disease" (GEA) study has been supported by the National Council of Science and Technology (CONACYT) (Project number: SALUD-2014-1-233727). The authors thank the personnel of the Endocrinology and the Tomography and Imaging departments of the National Institute of Cardiology "Ignacio Chavez" as well as the study's participants.

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Rev Inves Clin. 2016;68:269-74 ORIGINAL ARTICLE

Clinical and Genetic Findings in Mexican Patients with Duane Anomaly and Radial Ray Malformations/Okihiro Syndrome

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ABSTRACT

Background: Okihiro syndrome is an autosomal-dominant condition characterized by radial ray malformations associated with Duane anomaly and other clinical characteristics. *SALL4* mutations have been identified in 80-90% of patients with Duane-Radial ray defects/Okihiro syndrome. We report the clinical findings and results of *SALL4* sequencing from a group of Mexican patients with this disorder. **Objective:** Clinical description and identification of *SALL4* mutations in Mexican subjects with radial defects and Duane anomaly. **Materials and methods:** Five unrelated index cases were studied. Complete ophthalmologic and general physical examination was performed in all patients. Polymerase chain reaction amplification and automated nucleotide sequencing of coding exons and intron-exon junctions of *SALL4* gene were carried out in genomic DNA. **Results:** A novel heterozygous deletion was identified in one patient. Intragenic heterozygous single nucleotide polymorphisms on *SALL4* gene ruled out deletions of some exons in other affected patients in whom non-pathogenic variants were identified by Sanger sequencing. Likewise, multiplex ligation-dependent probe amplification analysis ruled out large deletions in this gene. **Conclusion:** We observed a low frequency of *SALL4* mutations in Mexican patients with clinical criteria of Okihiro syndrome. (REV INVES CLIN. 2016;68:269-74)

Key words: Duane anomaly. DRS. Okihiro syndrome. Radial ray defects. *SALL4* disorder. *SALL4* gene. Strabismus.

INTRODUCTION

Duane retraction syndrome (DRS) is a rare congenital eye movement disorder, accounting for around 1-5% of all strabismus cases. This anomaly occurs almost always sporadically and is defined by the combination of limited abduction, variable limited adduction, and globe retraction with palpebral fissure narrowing in attempted adduction[1]. Numerous associated anomalies have been described in patients with DRS, including dysmorphic ears and hearing dysfunction, vertebral defects, and variable degrees of

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Received for publication: 05-07-2016
Accepted for publication: 16-10-2016

limb malformations[2,3]. Duane-radial ray syndrome/Okihiro syndrome is a syndromic form of DRS, predominantly defined by the presence of radial ray malformation and other clinical findings such as fused cervical vertebrae, spina bifida, renal and gastrointestinal anomalies, heart atrial or ventricular septal defects, and facial asymmetry[2-6]. Duane retraction syndrome/Okihiro syndrome has an autosomal-dominant inheritance with a highly variable clinical presentation and reduced penetrance. The disease has an unknown frequency and is caused by mutations in the *SALL4* gene on human chromosome 20q13.13-q13.2[7,8]. Gene *SALL4* encodes a transcription factor playing critical roles during the embryonic development of abducens motoneurons[8]. The *SALL4* mutations can be demonstrated in approximately 80-95% of patients with *SALL4*-related disorders: Okihiro syndrome and the allelic acro-renal-ocular syndrome. In this study, we reported a low frequency (1/5 patients) of *SALL4* mutations in Mexican patients with clinical criteria for Okihiro syndrome. The ophthalmologic and systemic findings of five index cases with this syndrome are described and a novel *SALL4* heterozygous mutation is reported.

MATERIALS AND METHODS

Subjects and diagnostic criteria

This study was approved by the Institutional Review Board of the Institute of Ophthalmology "Conde de Valenciana". Informed consent was obtained from each subject/parents. The defining characteristics of Okihiro syndrome in our study were Duane retraction anomaly and radial ray malformations. All subjects underwent a complete ophthalmologic/strabologic examination and a general physical assessment.

Polymerase chain reaction amplification and Sanger sequencing analysis of *SALL4* gene

Genomic DNA was extracted from venous blood leukocytes using the QuickGene system (Fujifilm, Tokyo, Japan). The complete *SALL4* coding region (four exons) and adjacent intronic boundaries were amplified by polymerase chain reaction (PCR) using pairs of primers derived from the normal gene sequence. Each 25 µl PCR amplification reaction contained 1 × buffer, 100 ng of genomic DNA, 0.2 mM of each dNTP, 2 U taq polymerase, 1 mM of forward and reverse primers, and 1.5 mM MgCl2. The PCR products were analyzed in 1.5% agarose gels from the bands with the amplified templates, were excised, and the DNA was subsequently purified with the QIAEX® II kit (Qiagen, Hilden, Germany). Direct automated sequencing of all exons was performed with the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All samples were analyzed on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). Wild-type (ENST 00000217086) and mutated *SALL4* sequences were compared manually. The novel *SALL4* mutation was investigated in the 1000 Genomes Project (www.1000genomes.org), Exome variant Server (www.evs.gs.washington.edu), and Exome Aggregation Consortium (ExAC) (www.exac.broadinstitute.org) databases. A total of 100 unrelated DNAs from Mexican Mestizo individuals without strabismus and/or radial ray defects were included as a mutational control group.

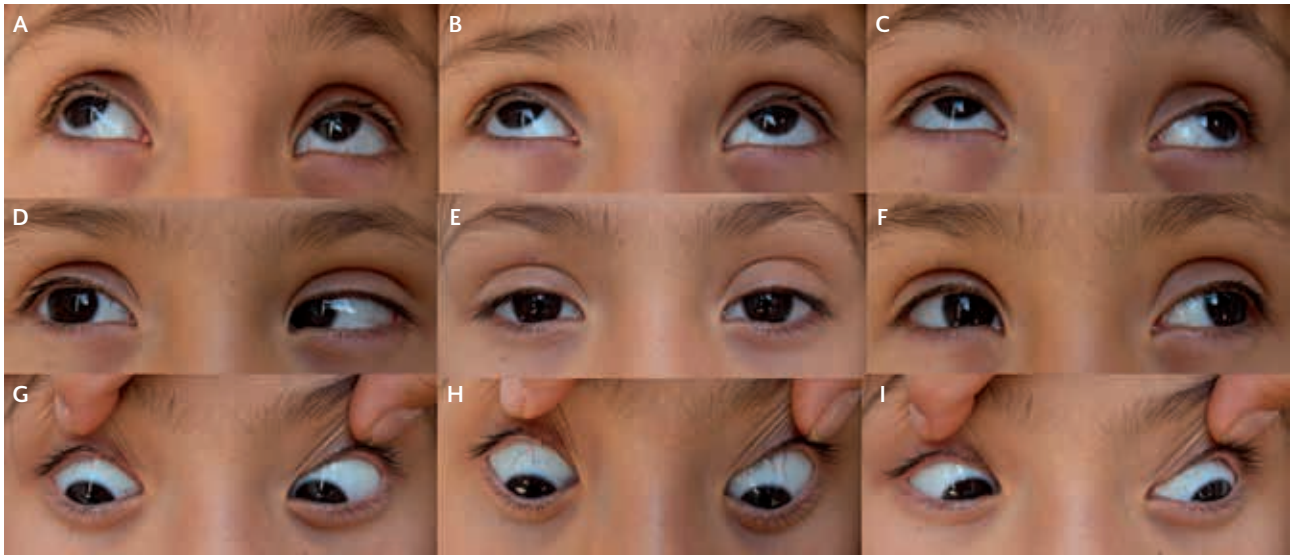
Multiplex ligation-dependent probe amplification analysis of *SALL4* gene

Multiplex ligation-dependent probe amplification (MLPA) was used to exclude gene deletions or duplications. The commercially available SALSA® MLPA® P180-B3 Limb-2/Heart probe mix (MRC-Holland, Amsterdam, Netherlands) contains four probes targeting *SALL4* gene exons 1, 2, 3, and 4.

The DNA samples were diluted to 50 ng/µl in deionized water, re-purified by 3M sodium acetate precipitation in 100% ethanol, washed in 70% ethanol, and rehydrated in Tris-EDTA pH 8.5 buffer to 20 ng/µl. The MLPA® assay was then completed according to the standard protocol supplied by MRC-Holland (MLPA® DNA Protocol version MDP-005; http://www.mrc-holland.com). The MLPA products (1 µl) were mixed with 13 µl of HiDi® formamide and 0.6 µl of GeneScan™ 600 LIZ® Size Standard (Applied Biosystems®, Foster City, USA). The fragment separation was performed on capillary electrophoresis system 3500 Genetic Analyzer (Applied Biosystems®) using POP7® polymer under the following conditions: run time 1,330 seconds at 19.5 kV, injection time 8 seconds at 1.6 kV, run current 6 µA and 60 °C run temperature.

The raw data analysis and the copy number ratio determination were performed using the Coffalyser. NET v.140721.1958 free software (MRC-Holland).

Figure 1. Eye movement examination in nine cardinal gaze positions in patient #1. **A.** Upshot at oblique right superior position. **B.** Upgaze divergence. **C.** Upshot evidenced at oblique left superior position. **D.** Moderate abduction limitation of right eye with narrowing of palpebral fissure on adduction with mild globe retraction of left eye. **E.** Both eyes are straight at primary position. **F.** Mild abduction limitation of left eye with narrowing of palpebral fissure on adduction with marked globe retraction in the right eye. **G, H, I.** Normal downgaze positions.



Nine reference probes included in the probe mix were used for intra-normalization. Three DNA samples of healthy individuals were included as reference for inter-normalization.

RESULTS

Five unrelated index cases born from healthy and non-consanguineous parents were studied. Their family histories were uneventful, and mothers of probands received regular prenatal care. Exposure to teratogenic agents was denied in all cases.

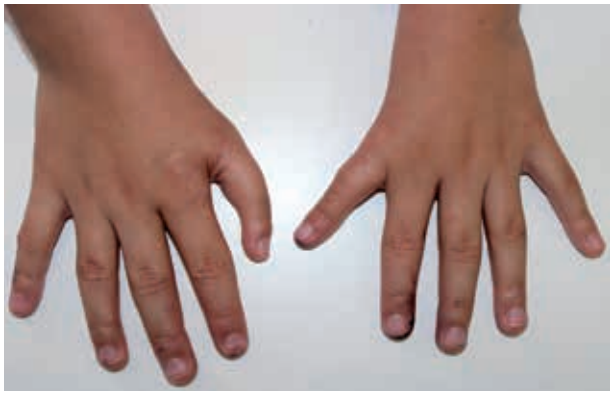
Patient #1 (mutation positive)

An 11-year-old female was referred to the Genetics Department due to esotropia and malformations in both hands. She was born by natural childbirth and her birth weight was 3,700 g; no other birth measurements were documented. Esotropia was noted since birth, although it appeared to decrease over time. Ophthalmic examinations by slit-lamp biomicroscopy and by fundoscopy were unremarkable in both eyes. Visual acuity measurement using Snellen chart revealed 20/20 for both eyes, while refraction was +0.25 +0.25 × 90º in both eyes. Stereopsis test measured by random-dot test revealed 20 seconds of arch. Eye movement examination disclosed a moderate abduction limitation of right eye and mild abduction limitation of left eye. Narrowing of palpebral fissure on adduction with marked globe retraction in right eye and mild in left eye were noted. An upshoot was found in superior oblique right and left positions during examination, and divergence was seen in upper gaze, supporting a bilateral Duane syndrome diagnosis (Fig. 1). Systemic examination disclosed hypertelorism, bilateral epicanthal folds, and midfacial hypoplasia (Table 1). Bilateral triphalangeal thumb or digitalized thumb was also found in the affected hands. Her right hand had a previous surgery (Fig. 2). Nucleotide analysis in this patient disclosed a novel heterozygous mutation c.1427delC in *SALL4* exon 2 (Table 2; Fig. 3). This one-base deletion originated a frame shifting and caused a premature stop signal (TAA) 4 codons downstream (p.P476L*4). Three synonymous changes including c.540T>C (p.N179=, homozygous), c.1056G>A (p.A351=, heterozygous), and c.2640G>C (p.S879=, heterozygous), were also identified in DNA from this subject. All *SALL4* nucleotide variants identified in this study are summarized in table 2.

Patients #2 to #5 (mutation negative)

Patient #2 is a one-year-old girl who had facial asymmetry, Duane syndrome, right thenar hypoplasia, and

Figure 2. Digitalized thumb in both hands (patient #1).



absence of left radius. Two nonpathogenic nucleotide variants, c.1520T>G (p.L507R) and c.1860A>G (p.T620=) were identified in heterozygous state in DNA from this subject. Patient #3 is an 18-year-old male who presented facial asymmetry, congenital strabismus (Duane syndrome), short neck, low posterior hair line, cervicothoracic kyphosis, ventricular septal defect, hypoplastic right humeral head, absence of right radius, absent thumbs, left radioulnar synostosis, syndactyly, and horseshoe kidney. Two heterozygous synonymous variants were found in DNA from this patient: c.1056G>A (p.A351=), and c.2037C>T (p.T679=). Patient #4 is a 17-year-old male referred due to

Table 1. Clinical findings in patients with Okihiro syndrome

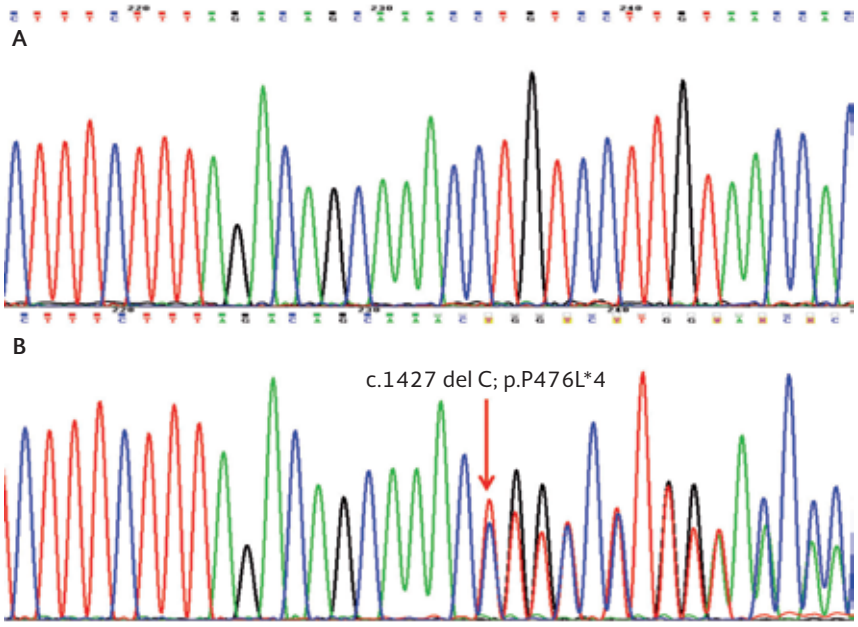
Finding	Patient #1	Patient #2	Patient #3	Patient #4	Patient #5
Duane anomaly	+	+	+	+	+
Radial ray anomaly	+	+	+	+	+
Facial asymmetry	+	+	+	+	–
Hypertelorism	+	–	–	–	–
Epicanthal folds	+	–	–	–	–
Ptosis	–	–	–	+	–
Midfacial hypoplasia	+	–	–	–	–
Microtia	–	–		+	–
Dental anomalies	–	–	–	+	–
Short neck	–	–	+	–	–
Congenital heart defects	–	–	+	–	–
Renal anomalies	–	–	+	+	–
Spine anomalies	–	–	+	+	–
Other limb anomalies	–	–	+	–	–
Syndactyly	–	–	+	+	–
Intellectual disability	–	–	–	+	–

+: present; –: not present.

Table 2. Nucleotide variants identified in this study

Patient #	Pathogenic variant	Non-pathogenic variants (Genotype) (Exon)
1	c.1427delC; p.P476L*4	c.540T>C; p.(N179=) (homozygous) (E2) c.1056G>A; p.(A351=) (heterozygous) (E2) c.2640G>C; p.(S879=) (heterozygous) (E3)
2		c.1520T>G; p.L507R (heterozygous) (E2) c.1860A>G; p.(T620=) (heterozygous) (E2)
3		c.1056G>A; p.(A351=) (heterozygous) (E2) c.2037C>T; p.T679=) (heterozygous) (E2)
4		c.540T>C; p.(N179=) (homozygous) (E2) c.1520T>G; p.L507R (heterozygous) (E2) c.1860A>G; p.(T620=) (heterozygous) (E2)
5		c.540T>C; p.(N179=) (homozygous) (E2) c.1056G>A; p.(A351=) (heterozygous) (E2) c. 2640G>C; p.(S880=) (heterozygous) (E3)

Figure 3. Partial DNA sequence in *SALL4* exon 2 from control father (A) and patient #1 carrying a heterozygous c.1427delC (p.P476L*4) mutation (B). The arrow indicates the frameshift mutation.



intellectual disability and dysmorphic features including facial asymmetry, right Duane anomaly, right palpebral ptosis, right microtia, absence of mandibular incisors, thoracic scoliosis, telethelia, segmentation defect of the cervical vertebra (C5-C7), left kidney agenesis, hypoplastic right radius, triphalangeal thumb in right hand, syndactyly in left hand, and sandal gap between first and second toes bilaterally. Three nonpathogenic variants were identified in DNA from this patient: c.540T>C (p.N179=, homozygous), c.1520T>G (p.L507R, heterozygous), and c.1860A>G (p.T620=, heterozygous). Patient #5 is a six-year-old female who was referred due to Duane syndrome and bilateral radial ray defects (radioulnar synostosis, first metacarpal hypoplasia, thumb hypoplasia, and thenar hypoplasia). One homozygous c.540T>C (p.N179=) and two heterozygous c.1056G>A (p.A351=) and c.2640G>C (p.S880=) nonpathogenic variants were identified in DNA from this patient.

The MLPA analysis did not detect the presence of deletions or duplications in *SALL4* gene in any of the patients (patients #2 to 5).

DISCUSSION

SALL4-related disorders comprise a spectrum of phenotypes previously recognized as distinct entities including Duane-radial ray syndrome or Okihiro syndrome, acro-renal-ocular syndrome, and rarely, Holt-Oram syndrome[6]. In this study, we have selected as key inclusion criteria Duane anomaly and ray radial malformation due to the fact that these two features are present in up to 45% of all the subjects carrying *SALL4* mutations[9]. Similarly, previous studies have shown a *SALL4* mutational frequency of 90% in patients associating Duane syndrome and radial ray defects, which sharply contrasts with a 20% mutation frequency (1/5 cases) in our group of Mexican patients with these anomalies[9].

Okihiro syndrome presents high phenotypic variability and anomalies of the eyes (microphthalmia, cataracts, optic disc hypoplasia and dysplasia, retinal coloboma, epicanthal folds, and mild hypertelorism)[10,11], upper extremities (hypoplastic humerus and ulnae, syndactyly, hypoplastic deltoid muscle)[11,12], kidney (renal agenesis, and dystopian or ectopic kidney)[10,12], ears (sensorineural and/or conductive deafness, abnormal pinnae, microtia)[4,12], and heart (atrial or ventricular septal defects, tetralogy of Fallot)[6] have been reported. Patients #2 to #4 presented facial asymmetry, and one of them (patient #4) had also microtia, fused vertebrae, and renal agenesis, features that may resemble oculo-auriculo-vertebral spectrum. Terhal, et al. described a

family with similar characteristics and carrying a *SALL4* stop mutation, raising the possibility that some patients with hemifacial microsomia might have pathogenic *SALL4* mutations[13]. Patient #3 had a history of congenital strabismus, congenital heart defect, and renal malformation (horseshoe kidney), a phenotype that is similar to the Holt-Oram syndrome. However, the Holt-Oram syndrome does not include eye anomalies[14]. Thus, all our patients had phenotypic features compatible with the Okihiro syndrome, and although a *SALL4* mutation was identified only in one patient, other molecular mechanisms, such as whole or partial deletion of the *SALL4* gene, could explain the low mutation frequency. Several patients and families with SALL4-related phenotypes have no identifiable *SALL4* mutations by direct sequencing[7], but subsequent deletion/duplication analysis demonstrated causal heterozygous deletions of the whole gene, of exons 1-3, of exon 1, or of exon 4[15]. In our patients, some identified variants (single nucleotide polymorphisms) were used to exclude heterozygous deletions of some exons (Table 2). Thus, two heterozygous variants, c.1520T>G and c.1860A>G, in exon 2 in patients #2 and #4 excluded deletion of this exon. Similarly, three heterozygous variants, c.1056T>G, c.2037C>T, and c.2640G>C, ruled out deletion of *SALL4* exon 2 in DNA from patients #3 and #5. Finally, a heterozygous c.2640G>C nucleotide change in patient #5 excluded deletion of *SALL4* exon 3 (Table 2). Mental retardation, which was observed in patient #4, is a feature described in patients carrying large deletions in the *SALL4* gene[16]. Nevertheless, MLPA analysis of *SALL4* gene completely ruled out any deletion or duplications in this gene in patients #2 to #5.

SALL4 encodes sal-like protein 4, a zinc finger transcription factor essential for developmental regulation that cooperates with *SALL1* and *TBX5* in anorectal, heart, brain, and kidney development[17]. In our study, we reported a novel c.1427delC heterozygous one-base deletion, which predicts premature protein truncation that probably led to *SALL4* haploinsufficiency. To date, 48 *SALL4* mutations have been reported, most of which are non-sense mutations and gross deletions[18]. Locus heterogeneity or a misdiagnosis of the syndrome may be other possibilities when mutations in *SALL4* are not identified in individuals with a clinical diagnosis of Okihiro syndrome.

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Acknowledgments

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