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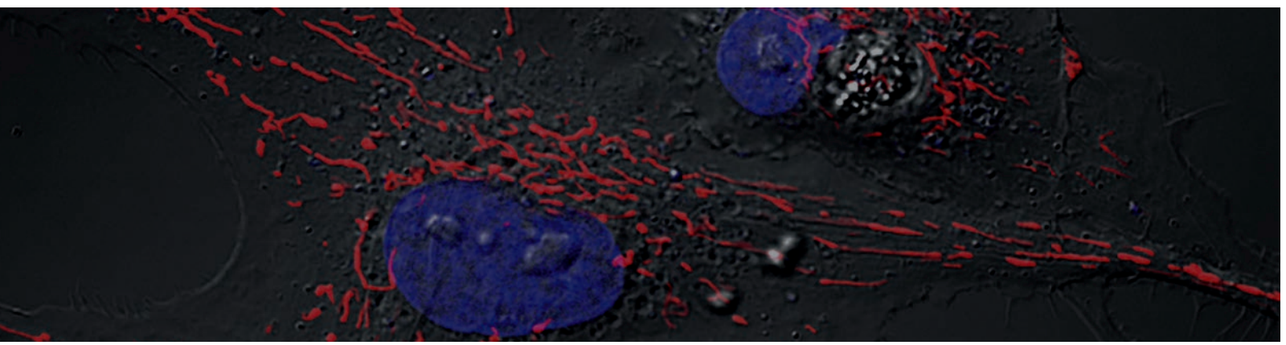

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Mallorca, 310
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España
Tel.: +34 93 207 59 20
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Cover figure: Human fibroblasts showing mitochondria stained with MitoTracker (red) and nuclei stained with DAPI (blue). Imagen was captured with an Olympus FV1000 IX81 confocal microscope, using a Plan Apo 60X objective. Courtesy of Dr. Alfonso R. Salgado Aguayo, Research Laboratory in Rheumatic Diseases, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas" (INER), Mexico City, Mexico.

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Rev Inves Clin. 2016;68:105-11 ORIGINAL ARTICLE

Effect of Human Breast Milk on the Expression of Proinflammatory Cytokines in Caco-2 Cells after Hypoxia/Re-Oxygenation

Wei-Yong Ruan[1]*, Ming-Yuan Bi[2], Wei-Wei Feng[1], Yu-Jun Wang[1], Wei-Quan Bu[1] and Ling Lu[2]

[1]Jiangsu Traditional and Western Medicine Hospital; [2]Nanjing University of Chinese Medicine, Nanjing, China

ABSTRACT

Background: Neonatal necrotizing enterocolitis is a common and often fatal gastrointestinal disease, especially in premature infants. To study potential mechanisms underlying the protective effect of breast milk on neonatal necrotizing enterocolitis, we induced intestinal inflammation in a Caco-2 cell model of neonatal necrotizing enterocolitis by hypoxia/re-oxygenation to investigate whether breast milk supernatant fluid inhibited the expression of proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α. **Methods:** Caco-2 cells were divided into normal (control) and neonatal necrotizing enterocolitis groups. Neonatal necrotizing enterocolitis was mimicked by exposing Caco-2 cells to hypoxia/re-oxygenation. Cells were independently maintained in minimal essential medium alone, minimal essential medium containing 5% breast milk supernatant, or 5% boiled breast milk supernatant. Production of interleukin-1β, interleukin-6, and tumor necrosis factor-α was investigated in cell culture supernatants by ELISA, reverse transcription polymerase chain reaction, and immunofluorescence. **Results:** Hypoxia/re-oxygenation significantly increased the expression of interleukin-1β, interleukin-6, and tumor necrosis factor-α. In the normal group, breast milk supernatant and boiled breast milk supernatant markedly downregulated the expression of interleukin-1β, interleukin-6, and tumor necrosis factor-α when compared with the minimal essential medium group, with the reduction in interleukin-1β expression being more pronounced in the breast milk group. In Caco-2 cells undergoing hypoxia/re-oxygenation, both breast milk supernatant and boiled breast milk supernatant significantly reduced the expression of interleukin-1β, interleukin-6, and tumor necrosis factor-α, where the decrease in interleukin-1β expression was greater in the breast milk group. **Conclusions:** Breast milk supernatant fluid inhibited the expression of proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α in Caco-2 cells, especially after hypoxia/re-oxygenation. This may be one of the mechanisms underlying the protective effect of breast milk on neonatal necrotizing enterocolitis. (REV INVES CLIN. 2016;68:105-11)

Key words: Necrotizing enterocolitis. Human milk. Interleukin-1. Interleukin-6. Tumor necrosis factor-α.

Corresponding author:
*Wei-Yong Ruan
Jiangsu Traditional and Western Medicine Hospital
Nanjing 210028, China
E-mail: ruanweiyongrwy@163.com

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease of neonates, especially premature infants, with a poor prognosis and a mortality of 10-50% in China[1,2]. Apoptosis is the main type of epithelial cell death and a necessary stage in the progression of NEC[3,4]. Cytokines including tumor necrosis factor-α (TNF-α), lipopolysaccharide-binding protein, and platelet-activating factor play important roles in the pathogenesis of NEC[5,6]. A number of studies have demonstrated that multiple cytokines and the signaling pathway mediated by them play important roles in systemic inflammatory responses in children with NEC. Most of these studies focused on proinflammatory cytokines, including interleukin (IL)-1, IL-6, TNF-α, and interferon (IFN)-γ, while few studies were about protective cytokines such as tumor growth factor (TGF)-β and IL-10. Ischemia/reperfusion injury of the intestine, a common cause of intestinal mucosal barrier function injury, has been proved to activate endothelial cells to cause an imbalance of the local cytokine network, with overexpression of some cytokines and adhesion molecules, resulting in tissue injury mediated by white blood cells[7].

Nuclear transcription factor-κB (NF-κB) is one of the hubs of proinflammatory cytokines gene expression[8], including IL-1, IL-6, TNF-α, and INF-γ. One study[9] showed that activated NF-κB could enhance gene expressions of TNF-α and IL-6, increase TNF-α and IL-6 release, and thus upregulate the inflammatory response. In addition, TNF-α and IL-6 could activate NF-κB in a feed-back way and further amplify primary inflammatory signals, causing a cascade of inflammatory responses. A moderate intervention of NF-κB could have positive effects on the clinical therapy of ischemic injury of the bowel mucosa and provide a new direction for research on the prevention of hypoxic/ischemic bowel mucosa injuries.

Although great progress has been made in elucidating the potential mechanisms underlying the pathogenesis of NEC, its molecular biology has not been fully understood and thus effective treatments have not been developed[8,9].

It has been reported that the incidence of NEC in formula-fed babies is higher than that in infants exclusively fed breast milk. The basic mechanism for this protective role of human milk, however, has not been extensively investigated. In the present study, in an *in vitro* model of NEC, Caco-2 cells underwent hypoxia/re-oxygenation and were then treated with breast milk[10]. Expression of proinflammatory factors was detected in these cells to explore the potential mechanism underlying the protective effect of breast milk on NEC.

MATERIALS AND METHODS

Breast milk samples

Colostrum was collected from 12 mothers at 2-4 days after normal deliveries at the Department of Obstetrics in the Combined Traditional Chinese and Western Medicine Hospital in Jiangsu Province, from March to June 2013. Approximately 15-20 ml of colostrum was collected from each mother, stored at 4 °C, and centrifuged within 24 hours at 12,000 rpm/min for 10 minutes to remove fat and cell fragments. Milk supernatants were randomly pooled and assigned to one of two groups of six samples each. One group was stored (un-boiled) at 4 °C for further use, and the other was boiled for five minutes to inactivate the proteins or peptides (boiled). The boiled supernatant was centrifuged for 10 minutes at 12,000 rpm/min and the inactivated milk supernatant was collected. Both un-boiled and boiled milk supernatants were stored at −20 °C for further use.

Culture of Caco-2 cells

Caco-2 cells (Shanghai Cell Bank of the Chinese Academy of Sciences) were cultured in a 75 cm^2 dish and maintained at 37 °C in a humidified environment with 5% CO_2 in a medium containing 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin-streptomycin; cells were untreated (control) or treated with boiled or un-boiled milk supernatant. Passaging was performed at a ratio of 1:3 once every five days, reaching cell fusion seven days later. When cell confluence reached approximately 80%, 6-7 days later, cells were harvested and processed for further analysis.

Hypoxia/re-oxygenation of Caco-2 cells

Six days after passaging, 90% of medium was removed to minimize the gas-diffusion distance, leaving

Table 1. Concentration of proinflammatory cytokines in the supernatant of the control and hypoxia/re-oxygenation Caco-2 cell groups

Group		IL-1β	IL-6	TNF-α
Normal	MEM	146.87 ± 7.95	126.37 ± 1.38	938.46 ± 180.80
	BM	65.45 ± 3.83	99.27 ± 6.07	694.29 ± 49.58
	BMb	76.38 ± 2.59	111.48 ± 10.22	704.51 ± 74.31
Hypoxia/re-oxygenation	MEM	277.38 ± 11.56	363.87 ± 43.42	3,918.01 ± 761.54
	BM	117.30 ± 23.16	197.21 ± 6.59	1,751.92 ± 6.95
	BMb	167.43 ± 28.53	230.46 ± 24.47	1,896.24 ± 86.55

IL: interleukin; TNF-α: tumor-necrosis factor α; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

the remaining medium to keep cells alive. Hypoxia/re-oxygenation was induced as follows: cells were placed in a closed container connected to a negative-pressure generator and flushed with high purity nitrogen, followed by incubation for another 90 minutes (hypoxia). Then the medium was added to reach the original volume and cells were grown for another 30 minutes (re-oxygenation)[11].

Cell groups

Caco-2 cells were seeded into a six-well plate at a density of 10^5 cells per well. Cells were divided into two groups: normal group and hypoxia/re-oxygenation group; cells were further subdivided into three subgroups: (i) minimal essential medium (MEM) group; (ii) 5% breast milk supernatant fluid (BM); (iii) 5% boiled breast milk supernatant fluid (BMb). The contents of proinflammatory factors (IL-1β, IL-6, TNF-α) secreted by Caco-2 cells were determined by ELISA in culture supernatants in the six subgroups after a six-hour culture.

Detection of nuclear factor-κB p65 messenger RNA expression by reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed following the manufacturer's instructions (Reverse Transcription System, MBI Fermentas Co., USA), and repeated three times. The PCR primers were as follows: (i) GAPDH primers: F:5'-ACCACAGTCCATGCCATCAC-3', R:5'-TCCACCACCCTGTTGCTGTA-3'; (ii) NF-κB p56 primers: F:5'-AGGCTCCTGTGCGTGTCTCC-3', R:5'-GGGTGGGCTTGGGGGCAGGT-3'.

Detection of nuclear factor-κB p65 messenger RNA expression by immunofluorescence

Cells in the logarithmic phase were inoculated in a 24-well plate, with a density of 70-80% per well. Cells were fixed with 4% iced paraformaldehyde for 20 minutes, washed with phosphate buffered saline (PBS) for 15 minutes, and incubated with 0.1% Triton X-100 at room temperature for 10 minutes. The first antibody (NF-κB p65 antibody, CST Ltd., Chicago, IL, USA) was incubated overnight at 4 °C, and the second antibody, labeled with Texas red, was incubated for one hour. After washing with PBS, cells were sealed with 3% bovine serum albumin for one hour. The slides were dyed with the fluorescent stain DAPI and sealed; cells were observed under fluorescence microscope and photographed.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0. Quantitative data were expressed as means ± standard deviation ($\bar{X}$ ± S); one-way analysis of variance was performed for comparisons between groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Concentration of proinflammatory cytokines in Caco-2 cells

When compared with the normal group, the concentration of IL-1β, IL-6, and TNF-α markedly increased in the hypoxia/re-oxygenation group (t = 16.112,

Figure 1. Interleukin-1β concentration in Caco-2 cells. Milk supernatant inhibited the expression of proinflammatory cytokine IL-1β in Caco-2 cells, especially the un-boiled supernatant, which induced a more potent inhibition of IL-1β compared to boiled supernatant. *Compared with the normal group, the concentration of IL-1β markedly increased in hypoxia/re-oxygenation group (t = 16.112, p= 0.000). #In Caco-2 cells undergoing hypoxia/re-oxygenation, the concentration of IL-1β markedly decreased in the BM group (p = 0.000) and BMb group (p = 0.001) compared with the control (MEM) group. ##The decrease in IL-1β in the BM group was significantly greater than in the BMb group (p = 0.033).
IL: interleukin; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

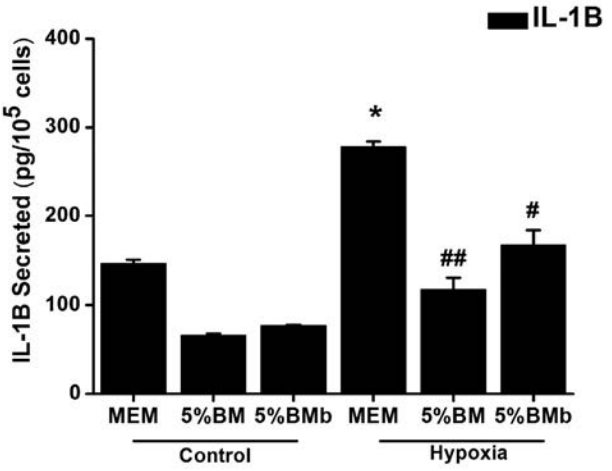
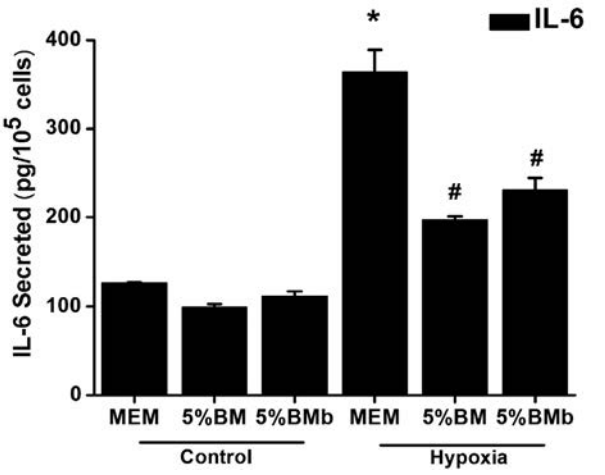


Figure 2. Interleukin-6 content of Caco-2 cells. Milk supernatant inhibited the expression of proinflammatory cytokine IL-6 in Caco-2 cells. *Compared with the normal group, the concentration of IL-6 significantly increased in the hypoxia/re-oxygenation group (t = 9.468, p = 0.001). #In Caco-2 cells undergoing hypoxia/re-oxygenation, the concentration of IL-6 significantly decreased in the BM (p = 0.000) and BMb (p = 0.001) groups compared with the control (MEM) group.
IL: interleukin; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.



p = 0.000; t = 9.468, p = 0.001; t = 6.593, p = 0.003, respectively) (Table 1, Fig. 1-3). This suggests that the expression of proinflammatory cytokines increases significantly after hypoxia/re-oxygenation.

In the normal group, one-way analysis of variance showed that IL-1β, IL-6, and TNF-α concentrations reduced significantly in the BM and BMb groups when compared with the MEM group (p < 0.05). Further paired comparisons revealed that the IL-1β decrease in the BM group was greater than in the BMb group (p < 0.05), but the reductions in IL-6 and TNF-α were similar in both groups. This suggests that milk supernatant may inhibit the expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in Caco-2 cells, and especially the un-boiled supernatant has a more potent ability to inhibit IL-1β as compared to boiled supernatant.

Figure 3. Tumor necrosis factor-α content of Caco-2 cells. Milk supernatant inhibited the expression of proinflammatory cytokine TNF-α in Caco-2 cells. *Compared with the normal group, the concentration of TNF-α significantly increased in the hypoxia/re-oxygenation group (t = 6.593, p = 0.003). #In Caco-2 cells undergoing hypoxia/re-oxygenation, the concentration of TNF-α significantly decreased in the BM (p = 0.001) and BMb (p = 0.001) groups compared with the control group.
TNF: tumor necrosis factor; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

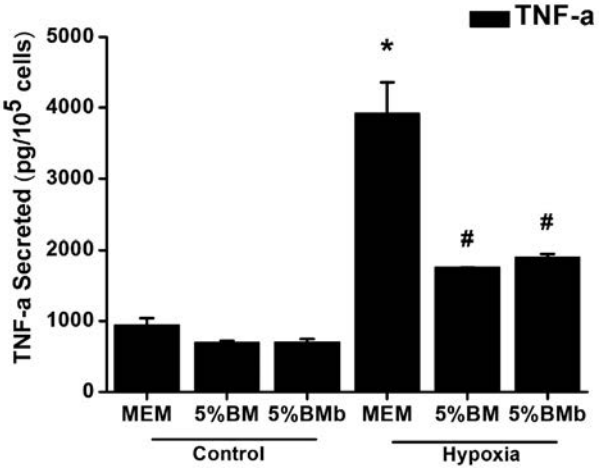


Table 2. Relative nuclear factor-κB p65 messenger RNA expression in Caco-2 cells ($\bar{X} \pm S$, n = 3)

Group		Relative NF-κB p65 mRNA expression
Control group	MEM	1.00 ± 0.10
	BM	0.48 ± 0.05[#]
	BMb	0.45 ± 0.08[#,§]
Hypoxia/re-oxygenation group	MEM	2.12 ± 0.22[*]
	BM	0.63 ± 0.07[†,‡]
	BMb	0.97 ± 0.25[†]

*p < 0.01 when compared with MEM group in control group; †p < 0.01 when compared with MEM group in hypoxia/re-oxygenation group; ‡p < 0.05 when compared with BMb group in hypoxia/re-oxygenation group; #p < 0.05 when compared with MEM group in control group; §p > 0.05 when compared with BMb group in control group.
NF-κB p65: nuclear transcription factor κB p65 protein; mRNA: messenger RNA; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

In Caco-2 cells undergoing hypoxia/re-oxygenation, one-way analysis of variance revealed that the concentration of IL-1β, IL-6, and TNF-α markedly decreased in the BM and BMb groups when compared with the MEM group (p < 0.05). Further paired comparisons indicated that the IL-1β reduction in the BM group was greater than in the BMb group (p < 0.05), but the reductions in IL-6 and TNF-α were similar in the BM and BMb groups. This indicates that milk supernatant may inhibit the expression of proinflammatory cytokines in Caco-2 cells after hypoxia/re-oxygenation, and especially un-boiled supernatant had a more potent ability to inhibit IL-1β as compared to boiled supernatant.

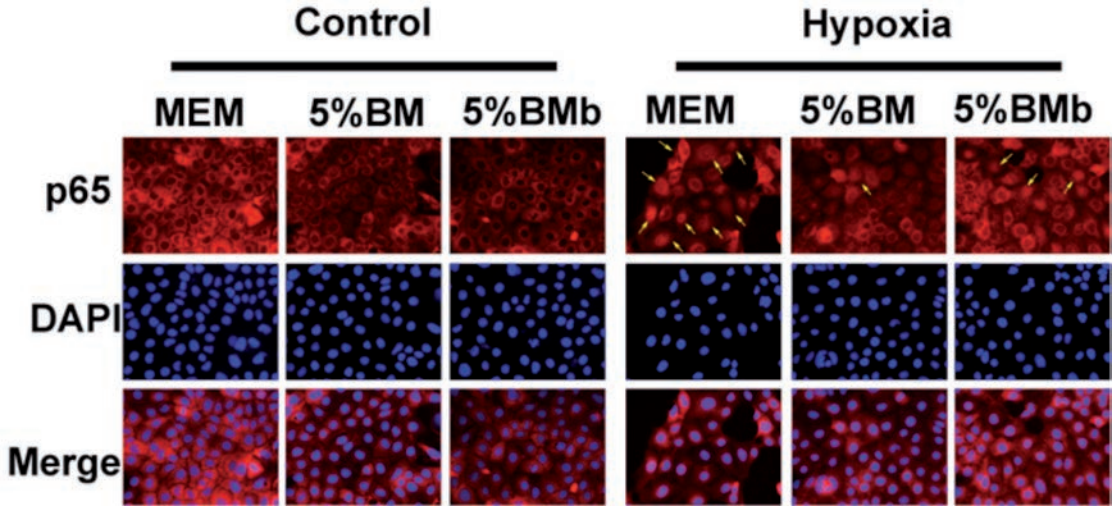
Reverse transcription polymerase chain reaction for detecting nuclear factor-κB p65 messenger RNA expression in Caco-2 cells

Results of RT-PCR (Table 2) show that the relative expression of NF-κB p65 mRNA in the MEM subgroup of hypoxia/re-oxygenation increased significantly when compared with the MEM subgroup of the control group (p < 0.01), indicating that NF-κB p65 mRNA was highly expressed after hypoxia/re-oxygenation, which proved a successful establishment of the model. When the BM and BMb subgroups in the hypoxia/re-oxygenation group were compared with those in the MEM group, the expression of NF-κB p65 mRNA significantly decreased (p < 0.01); and when the BM and BMb sub-groups in the control group were compared with the MEM group, the expression of NF-κB p65 mRNA also decreased significantly (p < 0.01), indicating that BM and BMb could downregulate NF-κB p65 mRNA expression in both control and hypoxia/re-oxygenation groups, and that both BM and BMb could downregulate NF-κB p65 mRNA expression in Caco-2 cells before and after hypoxia/re-oxygenation. Relative NF-κB p65 mRNA expression in the BM subgroup in the hypoxia/re-oxygenation group was lower than that in the BMb group (p < 0.05), while there was no significant difference in relative NF-κB p65 mRNA expression between BM and BMb subgroups in the control group (p > 0.05), suggesting that the downregulation effect of BM on NF-κB p65 mRNA expression was more significant than that of BMb after hypoxia/re-oxygenation of Caco-2 cells.

Immunofluorescence to detect nuclear factor-κB p65 messenger RNA expression in Caco-2 cells

Results of immunofluorescence (Fig. 4) showed that there was almost no red fluorescence in Caco-2 cell nuclei in the control group, while there was red fluorescence in all Caco-2 cell nuclei in the hypoxia/re-oxygenation group, which was most significant in the MEM group and least in the BM group. This indicated that NF-κB p65 in the plasma of Caco-2 cells after hypoxia/re-oxygenation was activated and entered and expressed in cell nuclei; BM could inhibit the entrance and expression of NF-κB p65 in nuclei, which was more significant than the BMb. These were consistent with the results of RT-PCR.

Figure 4. Nuclear factor-κB p65 messenger RNA expression in Caco-2 cells by immunofluorescence.
NF-κB p65 dye gives a red fluorescence; the DAPI dye of cell nuclei gives a blue fluorescence. Activation of NF-κB may be identified by its entrance to the nucleus. There was almost no red fluorescence (NF-κB p65) in cell nuclei in the control group, while red fluorescence was seen in all Caco-2 cell nuclei in the hypoxia/re-oxygenation group. This was most intense in the control (MEM) group and least in the BM group.
NF-κB: nuclear factor-κB; MEM: minimal essential medium; BM: 5% breast milk supernatant.



DISCUSSION

Neonatal NEC is a common but severe digestive tract disease in which a number of unanswered questions regarding its diagnosis and treatment still remain. To date, the etiology and pathogenesis of NEC are still poorly understood. Studies have proposed that a variety of factors, such as premature birth, hypoxia, hypothermia, improper feeding, intestinal ischemia, and infections, synergistically cause NEC. On the other hand, breast-feeding is a protective factor. For neonates, breast-feeding is recommended for adequate growth and development and the prevention of diseases. It has been reported that the incidence of NEC in formula-fed neonates is higher than among those receiving breast milk. The osmotic pressure of breast milk is 286 mmol/l and it contains a large amount of epidermal growth factors, which are beneficial in the prevention of NEC[1,12]. In the present study, Caco-2 cells, human colon cancer cells with characteristics similar to human intestinal epithelial cells, were cultured *in vitro* to investigate at the cellular and molecular levels the potential mechanisms underlying the protective effect of breast milk on NEC. In this study, we tested colostrum collected after 2-4 days of delivery, which contains significantly more adequate cytokines than mature breast milk, making it more suitable for our research purposes.

Caco-2 cells are derived from human colon adenocarcinoma and have homology with human intestinal cells. Caco-2 cells have spontaneous epithelial differentiation and may form tight junctions between cells. In addition, the morphology, expression of functional enzymes, and osmolality of Caco-2 cells are similar to those of intestinal cells. Thus, these cells can be used to investigate drug transport, absorption, and metabolism of intestinal epithelial cells *in vitro* and have been widely used in studies. Cytokines refer to small molecular polypeptides secreted by immune cells, mainly mediate and regulate immunity, inflammation, and hematopoiesis, and are important for the regulation of infection, immune response, inflammation and trauma. Proinflammatory cytokines include IL-1, IL-6, TNF-α, and IFN-γ, among others. Intestinal ischemia/reperfusion is a common cause of mucosal barrier dysfunction of the intestine. It has been confirmed that ischemia/reperfusion may activate endothelial cells and cause an imbalance of the cytokine network and overexpression of cytokines and adhesion molecules, resulting in leukocyte-mediated injury[7]. In the

present study, results showed that hypoxia/re-oxygenation significantly increased the expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α), which was consistent with previous findings[11], and further confirms that the imbalance of the cytokine network is related with the pathogenesis of NEC. In the cytokine network, the corresponding anti-inflammatory cytokines to proinflammatory cytokines, such as TGF-β and IL-10, also play important roles in the pathogenesis of NEC. In this study, both un-boiled and inactivated milk supernatant could downregulate levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in cells with hypoxia/re-oxygenation injuries, suggesting that there may be other active substances involved. In future studies we will aim to clarify the regulation mechanisms from both pro- and anti-inflammatory cytokines.

In previous studies, 1 and 5% breast milk supernatants could exert inhibitory effects on Caco-2 cells, with the effects of 5% supernatant being more pronounced, but no significant difference was observed ($p > 0.05$). In the present study, 5% breast milk supernatant was used. In the normal group (not subjected to hypoxia/re-oxygenation), BM and BMb could downregulate the expression of IL-1β, IL-6, and TNF-α. Moreover, IL-1β reduction was greater in the BM than in the BMb group. In the hypoxia/re-oxygenation group, BM and BMb were found to markedly reduce the expression of IL-1β, IL-6, and TNF-α, and the reduction of IL-1β was greater in the BM than in the BMb group ($p < 0.05$). These results show that boiling the supernatants decreases, but does not eliminate, their protective function, implying that not only heat denaturalizing-prone elements are participating in this phenomenon. It is important to consider that BM also contains other substances besides cytokines, for example, soluble human milk oligosaccharides that strongly attenuate inflammatory processes in the intestinal mucosa. These findings suggest that breast milk is able to downregulate the expressions of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in Caco-2 cells, especially after hypoxia/re-oxygenation. This may be one of the mechanisms underlying the protective effect of breast milk on NEC. Breast milk has many components with biological activities, and boiling may affect the biological activities of heat-sensitive proteins and polypeptides. When compared with boiled milk supernatant, reduction of IL-1β was greater after treatment with un-boiled milk supernatant. In addition, the concentration of breast milk supernatant may be another contributing factor, and the influence of milk supernatant concentration on NEC will be further investigated in our future studies[13].

The pathogenesis of NEC is complex. Studies have shown that it is associated with the abnormal activation of toll-like receptor and nuclear transcription factor signal transduction[14,15]. New studies have been conducted to investigate the molecular mechanisms regulating the expression of nuclear transcription factors and toll-like receptor in intestinal epithelial cells after breast milk intake.

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Rev Inves Clin. 2016;68:112-8 ORIGINAL ARTICLE

Clinical Benefit of 3 Tesla Magnetic Resonance Imaging Rescanning in Patients With Focal Epilepsy and Negative 1.5 Tesla Magnetic Resonance Imaging

Lady D. Ladino[1,2,3]*, Pedro Balaguera[4], Simon Rascovsky[2], Jorge Delgado[2], Juan Llano[2], Lizbeth Hernández-Ronquillo[3], Bety Gómez-Arias[5] and José F. Téllez-Zenteno[3]

[1]Section of Neurology, Hospital Pablo Tobón Uribe, University of Antioquia; [2]Instituto de Alta Tecnología Médica (IATM), Medellín, Colombia; [3]Division of Neurology, University of Saskatchewan, Saskatoon, Canada; [4]Clínica Medellín; [5]Hospital Universitario San Vicente Fundación, Medellín, Colombia

ABSTRACT

Background: Magnetic resonance imaging is an essential tool in the pre-surgical evaluation of patients with drug-resistant epilepsy. **Objective:** Our aim was to assess the value of re-imaging patients with focal drug-resistant epilepsy. **Methods:** Thirty patients with negative or non-conclusive 1.5 Tesla magnetic resonance imaging were rescanned with 1.5T and 3T. All of them had previous 1.5 scans with no seizure protocol in a non-specialized center. Two neuroradiologists who were blinded to prior imaging results randomly reviewed the magnetic resonance images. Kappa score was used to assess the reliability. **Results:** Mean age of patients was 30 (SD ± 11) years. The intra-observer agreement for the first radiologist was 0.74 for 1.5T and 0.71 for 3T. In the second radiologist it was 0.82 and 0.66, respectively. Three lesions (10%) were identified by general radiologists in non-specialized centers using a 1.5T standard protocol. In our center a consensus between two neuroradiologists using epilepsy protocol identified seven lesions (23%) using 1.5T and 10 (33%) using 3T ($p < 0.01$). In 28% of patients this additional information resulted in a change in clinical management. **Conclusions:** 3T magnetic resonance imaging rescanning improves the diagnostic yield in patients with focal epilepsy and previous negative 1.5T magnetic resonance imaging. Use of 3T magnetic resonance imaging, epilepsy protocols, and interpretation by experienced neuroradiologists is highly recommended. (REV INVES CLIN. 2018;68:112-8)

Key words: Epilepsy surgery. Lesional epilepsy. Magnetic resonance imaging. Partial epilepsy. Refractory epilepsy. 3 Tesla.

Corresponding author:
*Lady Diana Ladino
Servicio de Neurología
Hospital Pablo Tobón Uribe
Universidad de Antioquia
Street 78 B No. 69-240
Medellín, Colombia
E-mail: lladino@hptu.org.co, lady.ladino@gmail.com

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INTRODUCTION

One of the main challenges of epilepsy surgery is precise localization of the epileptogenic zone, defined as the minimum amount of cortical tissue that must be resected to provide seizure freedom[1]. Thirty[2] to 60%[3] of patients with focal epilepsy have normal magnetic resonance imaging (MRI) and are denominated MRI-negative or non-lesional epilepsy cases. The presence of an MRI lesion and the reclassification of a patient with epilepsy from a "non-lesional" to a "lesional" category triples the likelihood of achieving seizure freedom after epilepsy surgery[4]; therefore, all efforts should be made to detect a lesion.

An MRI is the preferred imaging modality to detect structural lesions and determine a potential surgery. High-field MRIs provide an improved signal-to-noise ratio, which could theoretically result in higher image resolution, possibly providing better definition of subtle epileptogenic lesions missed on standard field MRIs[5]. Detection of structural lesions requires a high-strength field, but also a dedicated epilepsy protocol and meticulous examination of the images by the interpreting radiologist[6], as well as post-processing analysis tools.

This is a prospective study assessing the value of re-imaging patients with drug-resistant focal epilepsy (DRFE) who were initially scanned with 1.5 Tesla (1.5T), using 3 Tesla (3T) MRI. Our hypothesis was that re-scanning patients in a specialized radiology center using an appropriate epilepsy protocol with a review by neuroradiologists would increase the possibility of detecting new lesions.

METHODS

This is a cross-sectional study performed at the Instituto de Alta Tecnología Médica (IATM) in Medellín, Colombia, between January 2012 and January 2014. The IATM is a center specialized in radiology. The Institutional Review Board of this institution approved the research protocol.

Patients

Patients with DRFE were recruited in two neurology clinics (Clínica Medellín and Hospital Universitario San Vicente Fundación in Medellín, Colombia) by two neurologists specialized in epilepsy. The medical history of each patient was obtained from clinical charts, a standardized diagnostic interview, and neurological examination. Epilepsy type, seizure type, and epilepsy syndrome were classified according to recommendations of the International League Against Epilepsy (ILAE)[7]. Definition of drug-resistant epilepsy was determined by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies[8].

Inclusion criteria

Patient inclusion criteria were as follows; (i) patient older than 14 years; (ii) clear diagnosis of DRFE by clinical history and electroencephalogram (EEG) findings; (iii) having normal or equivocal 1.5T MRI that failed to identify a relevant epileptogenic lesion; (iv) having been considered ineligible for surgery because of an inability to localize the potential epileptogenic zone; and (v) having already undergone a pre-surgical epilepsy evaluation, including at least a long-term video-EEG monitoring (120 hours or more). In some cases Positron Emission Tomography (PET), single-photon emission computerized tomography (SPECT), and psychiatric and neuropsychological evaluation were also part of the pre-surgical evaluation. Patients were asked to voluntarily participate in the study. A full explanation of the nature of the study was provided to them. Patients with previous epilepsy surgery or intracranial EEG evaluation, as well as patients with an implanted vagal nerve stimulator and absolute contraindications for undergoing MRI, were excluded.

Magnetic resonance imaging

All patients had a 1.5T scan prior to the study. This imaging was done without an epilepsy protocol in a non-specialized radiology center and was interpreted by a general radiologist. The entire sample of patients underwent a new 1.5T and 3T MRI in our center using an epilepsy protocol. Informed consent of participants was obtained before the MRIs.

All subjects were scanned on a Philips Ingenia MRI System with 3T and a Philips Achieva Nova Dual Series MRI System with 1.5T (Philips Medical Systems, Best, Netherlands) at the IATM, both with an 8-channel phased array sense head coil for signal reception. Sequences acquired for each patient in both machines included the following: Sagittal 3D

FLAIR (fluid attenuated inversion recovery); repetition time (RT) = 4,800, echo time (ET) = 344, NSA (number of signals averaged) = 2; voxel size = 1 x 1 x 1 mm; sagittal 3D T1-weighted, RT = 9.7, ET = 4.5; NSA = 2, voxel size = 0.8 x 0.8 x 0.8 mm; coronal T2-weighted drive, RT = 3,000, ET = 80, NSA = 2, voxel size = 0.7 x x 1.0 mm; coronal T1-weighted IR (inversion recovery), RT = 1,400, ET = 15, NSA =1, voxel size = 0.8 x x 1.0 mm; Axial DWI-HR (diffusion-weighted imaging in high-resolution), RT = 4,382, ET = 126, NSA = 3, voxel size = 1.3 x 1.8 mm and sagittal 3D T2-weighted, RT = 2,200, ET = 262, NSA = 2, voxel size = 1.0 x x 1.0 x 0.5 mm. With isovolumetric images the radiologists were able to assess areas suggestive of cortical thickening in directly orthogonal or perpendicular planes to rule out artifacts related to in-plane cortex. Intravenous contrast agents were not given. Total scanning time for the 1.5T and 3T MRI was about 80 minutes.

Image review

OsiriX (Geneva, Switzerland) version 4.1.1 was used by two neuroradiologists experienced in epilepsy imaging who independently and blinded assessed the scans. One neurologist summarized in a short sentence the clinical information of patients including age, gender, seizure semiology, and EEG information. Wording of the description was changed to strengthen the process of blinding. All scans were reviewed on a workstation running OsiriX software. The image sets were randomly coded with a random number. The two radiologists received the codes from the patients that they should read. The reviewers classified the MRI as normal or abnormal. An MRI was considered abnormal when a structural epileptogenic brain lesion was identified. Incidental lesions such as pituitary microadenomas or unspecific white matter hyperintensities were not considered significant. An MRI was considered abnormal when a focal lesion was identified with the standardized protocol such as mesial temporal sclerosis (MTS), focal cortical dysplasia (FCD), tumors, migration disorder, and others. Review by the two neuroradiologists was done with a timeframe of five days. Previous 1.5T scan reports were blinded to neuroradiologists. All the data were stored in a database called SQLite (http://www.sqlite.org). Finally, a team of neurologists and neurosurgeons determined if the new information resulted in a change in clinical management.

Statistical analysis

All analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL., USA). We used descriptive statistics to assess frequencies and distributions. We compared the final consensus reviews of 1.5T and 3T MRI. Inter-observer and intra-observer agreement was calculated using Kappa. We used a Chi-Square analysis to compare the final reading of MRIs from the IATM with previous 1.5T scan reports from non-specialized institutions. A p value < 0.05 was considered significant. All reliability estimates were presented with a 95% confidence interval (CI).

RESULTS

Thirty patients with DRFE were included. Sixteen (53%) were males; 25 (83%) were adults, and five (17%) were patients under the age of 18. The EEG and clinical focus was localized in the temporal lobe in 16 (53%), frontal in 10 (33%), insular in two (7%), and parieto-occipital in the other two (7%) cases (Table 1). The majority of patients (73%) had a psychiatric comorbidity, with depression being the most frequently reported (82%). All the patients were evaluated at least once for epilepsy surgery without success before the assessment in our center.

Structural lesions

Three lesions (10%) were identified by general radiologists in non-specialized centers using a 1.5T MRI standard protocol. The radiologic diagnoses were as follows: left parieto-occipital ulegyria, diffuse leukodystrophy, and a left temporal dysembryoplastic neuroepithelial tumor (DNET). These patients were not eligible for epilepsy surgery because the lesion detected in the MRI was discordant with the clinical and EEG findings. In these three patients, the new 1.5T and 3T MRIs with the special epilepsy protocol allowed a better anatomic definition of the pathologies; however, the new MRIs did not add any additional information and did not alter patient management in these cases. None of the patients have progressed to surgery at this time (Fig. 1).

In our center a consensus between the two neuroradiologists using an epilepsy protocol identified in total seven lesions (23%) using 1.5T, and 10 lesions (33%)

Table 1. Clinical characteristics of the patients

Variable n = 30		N (%)	Mean (SD)
Gender (male)		16 (53)	–
Age, years		–	30.2 (11.4)
Years of evolution		–	17.2 (11.4)
Frequency of seizures per month		–	25.3 (56.0)
Febrile seizures		5 (17)	–
Neonatal hypoxia		10 (33)	–
Family history of epilepsy		12 (40)	–
Developmental delay		14 (47)	–
Psychiatric comorbidity	Depression	18 (60)	–
	Anxiety	5 (17)	–
	Personality disorder	2 (7)	–
Semiology and EEG foci	Temporal	16 (53)	–
	Frontal	10 (33)	–
	Parietal	2 (7)	–
	Insula	2 (7)	–
AEDs in the past		–	5.4 (1.8)
Actual number of AEDs		–	2.1 (0.7)

AED: antiepileptic drug.

Figure 1. Comparison of imaging definition of a dysembryoplastic neuroepithelial tumor case in 1.5T and 3T magnetic resonance imaging.
T2-weighted coronal sequence showing a DNET with high signal and 'bubbly appearance'. The lesion is better defined on 3T-MRI (on the right).
MRI: magnetic resonance imaging; DNET: dysembryoplastic neuroepithelial tumor.

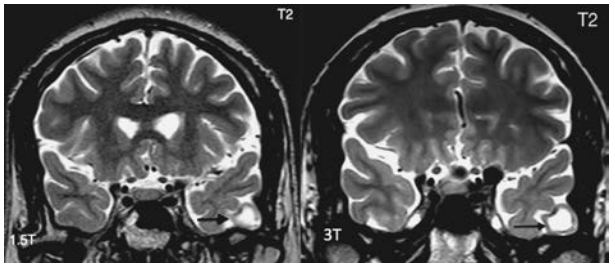
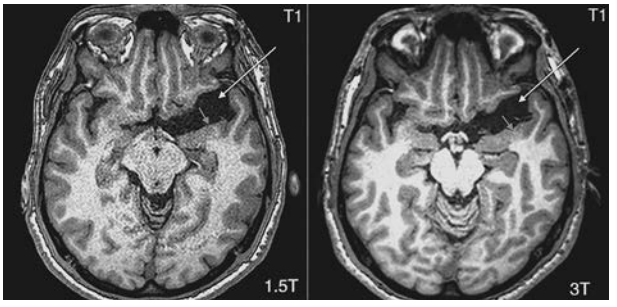


Figure 2. Comparison of imaging definition of a focal cortical dysplasia case in 1.5T and 3T magnetic resonance imaging.
Axial 3D T1-weighted sequence showing a left temporal arachnoid cyst (white arrow) with an area of cortical dysplasia in the anterior temporal cortex (yellow arrow). The FCD was also detected on the new 1.5T-MRI (on the left). This finding is more clearly appreciated on 3T-MRI (on the right). The old 1.5T-MRI was interpreted as normal.
MRI: magnetic resonance imaging; FCD: focal cortical dysplasia.



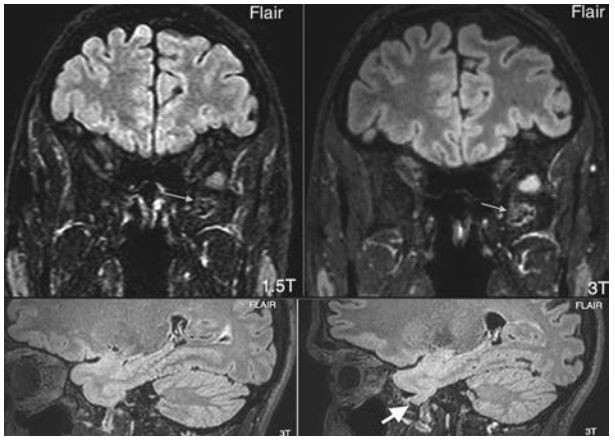
using 3T MRI. The 1.5T and 3T scans identified the same lesions in seven cases. The 3T MRI identified three additional lesions. The four new lesions identified by a 1.5T MRI in our center were two cases of FCD, one heterotopia case, and one MTS case (Fig. 2). Two of the three additional lesions (67%) that were only identified by the 3T MRI were areas of FCD (Table 2). In total, half of FCD were located in the frontal lobe and the other half in the temporal lobe. One of the cases was detected only by one neuroradiologist as a small temporal pole encephalocele associated with FCD (Fig. 3). After the consensus meeting, the two neuroradiologists agreed with the diagnosis. According to the treating team, in two of these seven patients (28%) the additional information resulted in a change in clinical management. Finally, there was a significant difference ($p < 0.01$) between the abnormalities reported at non-specialized centers without epilepsy imaging protocol (3/30, 10%) and the results of our center (10/30, 33%).

Table 2. Magnetic resonance imaging findings of the sample

Scan	New 1.5T		Cons 1.5T	New 3T		Cons 3T
Kappa	Intra-observer Kappa			Intra-observer Kappa		
	R1 0.74 (p < 0.009)	R2 0.82 (p < 0.001)		R1 0.71 (p < 0.01)	R2 0.66 (p < 0.07)	
Abnormal scans: n	11	9	7	10	10	10
Final diagnosis						
MTS	2	2	1	2	2	2
FCD	5	3	2	5	4	4
Tumor (DNET)	2	1	1	1	1	1
Ulegyria	1	1	1	1	1	1
Heterotopia		1	1		1	1
Leukodystrophy	1	1	1	1	1	1

The new 1.5T magnetic resonance imaging consensus at the IATM detected seven lesions (23%) and the 3T magnetic resonance imaging detected 10 lesions (33%).
Cons: consensus; DNET: dysembryoplastic neuroepithelial tumor; FCD: focal cortical dysplasia; MTS: mesial temporal sclerosis; R1: radiologist one; R2: radiologist two.

Figure 3. Small encephalocele and focal cortical dysplasia. At the top: a coronal 3D-FLAIR sequence showing a basal encephalocele on the left anterior temporal fossa with a small area of cortical dysplasia (white arrow). The finding was not detected in the new 1.5T-MRI (on the left), and is appreciated on the 3T-MRI (on the right). At the bottom: 3D-FLAIR technique showing comparative sagittal sequence of the normal right temporal lobe (on the left) and the abnormal left temporal lobe (on the right). The image shows a basal encephalocele on the left medial cranial fossa with protruding dysplastic cerebral cortex (white arrow). The imaging finding is consistent with the clinical and electrical suspicion in this patient.
MRI: magnetic resonance imaging; FLAIR: fluid attenuated inversion recovery.



Reliability

The intra-observer agreement for the first neuroradiologist was 0.74 for 1.5T and 0.71 for 3T. In the second neuroradiologist it was 0.82 and 0.66, respectively. The inter-observer agreement was 0.76 for 1.5T ($p < 0.001$) and 0.77 for 3T ($p < 0.001$) (Table 2).

DISCUSSION

The most important finding in our study is that the use of 3T MRI with an adequate epilepsy imaging protocol and the interpretation of a neuroradiologist yielded relevant new findings in seven patients (26%) with DRFE who had previous 1.5T scan in other centers that had been reported as normal (7/27). Only by using a 1.5T MRI with epilepsy protocol were we able to detect a new lesion in 15% of cases (4/27).

Patients with established DRFE have increased risks of premature death, psychosocial dysfunction, and a reduced quality of life; therefore, they should be evaluated early for surgical treatment[9]. Because of potential negative outcomes in patients with DRFE, a major effort has to be made to identify a lesion, especially when epilepsy surgery has been considered[4]. Additionally, when the MRI is negative, further work-up is critical to help localize the epileptogenic zone[10], and functional imaging (PET and SPECT) with or without invasive electroencephalographic monitoring is not always available and increases the costs considerably.

In the last three decades there has been a growing interest in the development of new techniques to identify structural lesions in the brain. The introduction of MRI for clinical use in the 1980s revolutionized the

diagnosis and surgical treatment of epilepsy. As a result of the improvement of technology, MRI has replaced CT in the work-up of patients with epilepsy[11,12]. The increase of the magnetic field strength (0.5T, 1.0T, 1.5T, 3T, and 7T) will potentially provide more benefit for patients. All the technological changes have generated a substantial impact on the quality and definition of MRI imaging; for instance, doubling the field strength from 1.5T to 3T is estimated to increase the signal-to-noise ratio by a factor of 6-8, which potentially can increase the diagnostic yield of identifying lesions in surgical candidates with epilepsy[13]. Moreover, implantation of head coils[14], the use of an epilepsy protocol including selected sequences, thin-slice thickness, and perpendicular orientation to the longitudinal axis of the hippocampal body[15], as well as the reading by expert neuroradiologists, can also improve the diagnostic yield[13,15].

In our study, standard 1.5T MRI without epilepsy protocol in non-specialized centers failed to detect a focal lesion in 70% (7/10) of patients in whom 3T MRI detected a clear epileptogenic lesion. Similar findings were reported in previous studies. In a university hospital, 1.5T standard MRI did not detect lesions identified by four-coil phased-surface array 1.5T MRI in more than half the children (56%)[16]. In another prospective study performed in adults, von Oertezen, et al. demonstrated that in almost 60% of cases, conventional MRI without a neuroradiologist's standardized interpretation approach failed to detect structural lesions[15].

In general, four (57%) of the seven new lesions detected in our study were areas of FCD. These results are similar to previous studies comparing 3T to 1.5T MRI, where 3T MRI was better in detecting areas of dysplasia[17-19], as well as several other studies that compared phased array to standard head coil imaging[13,20,21]. Our study shows that small areas of FCD may be missed with 1.5T MRI and the improved signal-to-noise of the 3T MRI studies, which can be translated in higher image resolution, results in better detection. The second type of new lesion detected in our study was hippocampal sclerosis, which is one of the most common radiological diagnoses in DRFE. This result corresponds well with previous studies by McBride, et al.[22], von Oertezen, et al.[15], and Winston, et al.[17].

Additionally, our study shows a benefit in performing a 3T MRI in patients who already had a normal 1.5T MRI. Three more lesions were identified using 3T MRI compared with 1.5T (10%). Similar percentages have been identified by other authors using 3T MRI compared with 1.5T MRI: Winston, et al. 5%[17] and Nguyen, et al. 5%[23]. Although it might be seen as a non-significant percentage, clinically it could be relevant because the decision to perform epilepsy surgery and the outcomes can be modified with the presence of a lesion[24]. Therefore, some patients may benefit from re-scanning.

When assessing the ability of a test to be helpful to clinicians, it is important that its interpretation be precise. To estimate reliability of the MRI interpretations, we used the kappa coefficient of agreement to evaluate inter-observer and intra-observer variability. We have followed the classification system used by Landis and Koch[25], where agreement is quantified as slight (0.01-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80), and almost perfect (0.81-0.99). As expected, the agreement between observers for identification of MRI lesions on 1.5T and 3T was substantial (0.76 and 0.77). The second radiologist 3T intra-observer agreement was almost perfect (0.82), and all the other intra-observer agreements were likewise substantial. The clinical significance of a kappa value depends upon its context, and the values cannot be always compared between studies as it is dependent on disease prevalence; nonetheless, a recent study of FCD detection by MRI (kappa 1.5T: 0.83, kappa 3T: 1.0) reported higher values than were seen in our study[19].

This study has some limitations. It is possible that because our readers were blinded to seizure semiology, lesions outside the lobe of interest may have been missed and lesions in the lobe of interest may be overcalled. This study is limited to findings on imaging; the final correlation of the lesion identified in MRI with the surgical outcomes is the most important. In the future, with a large sample size, it will be possible to make correlations with surgical outcomes. Finally, we believe that the differences between the studies from our center and the non-specialized center could be related with effects of higher field strength, a standardized epilepsy protocol, and an experienced reader, but we cannot rule out the possibility that some pressure and more expectations were given to the neuroradiologists to identify lesions during the study.

DECLARATION OF INTEREST

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PERMANYER

Rev Inves Clin. 2016;68:119-27 ORIGINAL ARTICLE

Effect of Passive Smoking on the Growth of Pulmonary Function and Respiratory Symptoms in Schoolchildren

Rosario Fernández-Plata[1], Rosalba Rojas-Martínez[2], David Martínez-Briseño[1], Cecilia García-Sancho[1] and Rogelio Pérez-Padilla[1]*

[1]Epidemiology and Social Science in Health, Instituto Nacional de Enfermedades Respiratorias (INER); [2]Population Health Research Center, Instituto Nacional de Salud Pública (INSP), Mexico City, Mexico

ABSTRACT

Background: Environmental tobacco smoke affects the current and future health of children. **Objective:** To determine whether schoolchildren aged 8-17 years old residing at an altitude of 2,240 m and exposed to tobacco smoke at home presented a reduction in the growth of pulmonary function and a greater problem of respiratory symptoms and infections compared with non-exposed children. **Materials and Methods:** We followed, with questionnaires and spirometry, 1,632 boys and 1,555 girls from Mexico City and its metropolitan area (the Metropolitan Study to Evaluate the Chronic Effects of Pollution in School-age Children [EMPECE]) every six months for six years. The impact of passive smoking was estimated by mixed-effects models and Generalized Linear and Latent Mixed Models (GLLAMM), stratifying by gender and adjusting for age, height, weight, and ozone levels. **Results:** Passive smoking (reported by one-half of participants) was associated with reduced spirometric lung function (log transformed or as Z-scores) and a higher frequency of self-reported respiratory symptoms and respiratory infections. Levels of forced expiratory volume in 1 second and forced vital capacity in individuals exposed to passive smoking were 6.8 and 14.1 ml, respectively, below those of non-exposed children, and these values decreased with increasing number of smokers at home and higher ozone levels. **Conclusions:** Passive smoking in children is a significant risk factor for respiratory disease and reduced lung function growth, which are additive with levels of air pollution, asthma, and the presence of respiratory symptoms. (REV INVES CLIN. 2016;68:119-27)

Key words: Pulmonary function testing. Epidemiology. Passive smoking. Respiratory symptoms. Asthma.

INTRODUCTION

Environmental tobacco smoke is a world health problem and part of the tobacco epidemic which is suffered mostly by children and imposes risks for their current and future health, unfortunately mainly caused by their parents and close relatives. Environmental tobacco smoke contains thousands of chemical substances, many of which are toxic, irritating, or pharmacologically active, and over 40 of these cause cancer[1,2].

The 2009 report of the World Health Organization (WHO) indicates that 700 million children worldwide (about 40% of all children) are exposed to second-hand

Corresponding author:
*Rogelio Pérez-Padilla
Instituto Nacional de Enfermedades Respiratorias
"Ismael Cosío Villegas"
Calzada de Tlalpan, 4502
Col. Sección XVI, Del. Tlalpan
C.P. 14080, Ciudad de México, México
E-mail: perezpad@gmail.com

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tobacco smoke (SHS) at home. Also, SHS is estimated to cause about 600,000 premature deaths each year worldwide. Of all deaths attributable to SHS, 31% occur among children[3]. Likewise, a study conducted in Scotland reported an association between salivary cotinine levels in children aged 2-12 years, the number of persons who smoke in the home, and the frequency with which they smoke[4]. The authors also found a positive association between passive smoking and respiratory problems in the children studied.

A decrease in pulmonary function has also been reported in children exposed to passive smoking[5], particularly in those with asthma[6] and those prenatally exposed[7,8]. Whether due to a direct exposure from their mothers or from environmental tobacco smoke, a reduction in birth weight has been observed, affecting the child's development and behavior[9,10], and increasing the risks for chronic obstructive pulmonary disease (COPD) or lung cancer at a mature age[11,12], as well as leukemias, lymphomas, and brain tumors during infancy[13]. In addition, boys exposed to tobacco smoke who reported wheezing and asthma showed greater functional deterioration than girls[14,15]. The SHS from parents and especially from the mother is associated with lower respiratory tract diseases in children[13].

In Mexico, surveys have shown that children's exposure to SHS is high[16], and smoking during pregnancy reduces the newborn's weight by 154 g and the size by 0.79 cm[17]. The 2002 National Drug Addictions Survey (ENA) in Mexico found that approximately 50% of the total population and up to 80% of those residing in urban areas were passive smokers[18].

The objective of the present study was to determine whether exposure to tobacco smoke at home in schoolchildren aged 8-17 years residing at 2,240 m above sea level is associated with a reduction in the growth of pulmonary function or with an increase in respiratory symptoms and infections, and whether there is a gender-related differential effect and an additive effect with air pollution.

MATERIALS AND METHODS

The Metropolitan Study to Evaluate the Chronic Effects of Pollution in School-age Children (EMPECE Study) was undertaken in Mexico City beginning on April 23, 1996, with children in third grade of primary school[19]. The protocol was approved by the Ethics Committee of the National Institute of Respiratory Diseases (INER) of Mexico. All parents provided written informed consent for their children to be study subjects.

We selected 10 fixed-site air-monitoring stations in Mexico City and randomly selected 39 elementary schools from among those located within a 2 km radius of the stations. The study cohort consisted of students at the selected schools who were eight years of age at the beginning of the study and whose parents had signed a letter of informed consent. Children could enter or leave the cohort during the course of the study. At baseline, a questionnaire was completed by the parents of 1,819 children, and a spirometric test was administered to each child (Phase 1). The subsequent evaluations (a total of six) were done every six months during the spring and autumn of each year until the end of the children's primary school education in 1999. Children remaining in the same school to study secondary level were followed for three additional years until 2002; thus, we collected information for a total of 12 evaluations[9,20,21]. A health questionnaire and written informed consent were filled out by parents and returned the day before each evaluation; it included self-reported diseases (asthma, respiratory infections, vaccination, previous pneumonias, ear infections, allergies, previous tonsillectomy, hyperactivity, neurologic diseases, heart diseases, gastroesophageal reflux, and obesity), and chronic respiratory symptoms (cough, wheezing, phlegm, and dyspnea). We also analyzed the reported frequency of respiratory infections (common cold, sore throat, and bronchitis). Exposure to SHS in children was evaluated with the most common questions, all bearing an independent association with cotinine levels in children[22]: Does the father smoke inside the house? Does the mother smoke inside the house? Do other persons smoke inside the house? During the last evaluation in secondary school (Phase 12), a question of direct smoking was included: Have you smoked a cigarette in the last month?

Spirometry tests were conducted using identical computerized dry-rolling seal spirometers (922 Spirometer; SensorMedics, USA) that were calibrated each morning prior to data collection with a 3 l syringe (SensorMedics). We recorded only the expiratory part of forced expiratory maneuvers and analyzed forced expiratory volume

Table 1. Description of variables by gender and phase of the study

		Exposed to passive smoking	Age (years)	Height (cm)	BMI
Girls	Phase*	n/total (%)	Mean (SD)	Mean (SD)	Mean (SD)
	1	406/929 (43.7)	8.7 (0.8)	130.6 (6.5)	17.2 (3.0)
	2	509/944 (53.9)	9.1 (0.8)	133.3 (6.5)	17.5 (3.0)
	3	637/1,161 (54.9)	9.6 (0.8)	137.3 (7.0)	18.2 (3.4)
	4	598/1,117 (53.5)	10.2 (0.8)	140.5 (7.5)	18.8 (3.6)
	5	515/942 (54.7)	10.6 (0.8)	143.4 (7.3)	19.2 (3.7)
	6	595/1,112 (53.5)	11.1 (0.8)	146.3 (7.1)	19.9 (3.8)
	7	573/1,115 (51.4)	11.7 (0.8)	149.1 (6.7)	20.1 (3.9)
	8	202/462 (43.7)	12.6 (0.7)	152.8 (5.9)	21.8 (4.4)
	9	186/426 (43.7)	13.1 (0.6)	154.2 (5.7)	22.0 (4.2)
	10	161/403 (40.0)	13.5 (0.6)	155.0 (5.5)	22.4 (4.2)
	11	141/350 (40.3)	14.0 (0.6)	155.7 (5.5)	22.7 (4.1)
	12	149/355 (42.0)	14.4 (0.7)	156.2 (5.6)	22.7 (4.2)
Boys	Phase	n/total (%)	Mean (SD)	Mean (SD)	Mean (SD)
	1	342/890 (42.9)	8.7 (0.8)	131.0 (6.2)	17.4 (3.1)
	2	540/962 (56.1)	9.2 (0.8)	133.7 (6.7)	17.8 (3.3)
	3	648/1,160 (55.9)	9.7 (0.8)	136.8 (6.8)	18.3 (3.6)
	4	546/1,087 (54.8)	10.2 (0.8)	139.8 (7.4)	19.0 (3.9)
	5	503/917 (54.9)	10.7 (0.8)	142.8 (7.6)	19.2 (3.9)
	6	598/1,105 (54.1)	11.2 (0.8)	145.5 (8.0)	19.6 (3.9)
	7	585/1,103 (53.0)	11.7 (0.8)	148.7 (8.1)	19.8 (4.1)
	8	185/466 (39.7)	12.6 (0.7)	155.1 (8.3)	21.0 (4.5)
	9	159/407 (39.1)	13.1 (0.6)	158.1 (8.2)	21.0 (4.3)
	10	141/389 (36.3)	13.5 (0.6)	160.7 (7.8)	21.3 (4.4)
	11	124/330 (37.6)	14.1 (0.6)	163.1 (7.4)	21.7 (4.4)
	12	148/343 (43.2)	14.5 (0.6)	164.9 (7.3)	22.0 (4.6)

*Study phase: 12 study evaluations, one every six months.
BMI: body mass index; SD: standard deviation.

in 1 second (FEV_1), forced vital capacity (FVC), and their ratio (FEV_1/FVC): forced expiratory flow (FEF) between 25-75% of the FVC ($FEF_{25-75\%}$).

Tests were performed at the school during morning and early afternoon hours. As many as eight maneuvers were conducted for each child to obtain three acceptable tests according to 1994 American Thoracic Society (ATS) criteria[23]. Additional details on spirometry methodology, including sustained quality control along the study, longitudinal variability, reference values and impact of socioeconomic status, were described in previous reports[17,18,24,25].

The impact of several air pollutants on the lung function of the cohort was previously reported[16]. As an indicator of air pollution, we included in the present analysis ambient ozone (O_3) from 10 government air-monitoring stations, assigning for each child data from the station closest to his/her school. We calculated 8 hour O_3 means (parts per billion, ppb, between 10 a.m. and 6 p.m.), whereas long-term exposure for each day of the study period was estimated as the average over the previous six months of the daily O_3 8 h mean[19].

Statistical analysis

We fitted multilevel mixed-effects linear models adjusted for age and gender to determine the association of SHS (the presence of any parent or person smoking inside the home) with spirometry variables (log-transformed FEV_1 and FVC) along time with and without height, and ozone concentrations (average over the previous six months of the daily O_3 8 h mean, as a general indicator of air pollution). Levels considered

in the models were the city zone (air pollution monitors) and repeated measurements. We also explored lung function (FEV_1 and FVC) expressed as the Z-score of height-, age-, and gender-predicted values[25] in models adjusted for ozone, presence of asthma, presence of respiratory symptoms, and frequent respiratory infections. Three-level Generalized Linear and Latent Mixed Models (GLLAMM) were fitted for dichotomous responses (yes/no) such as the presence of respiratory symptoms and the reported frequency of respiratory infections as a function of secondhand smoking. The order of levels included was as follows: the observations, the children, and the pollutant monitors reflecting the geographical area of Mexico City. Repeatability of passive smoking questions was estimated by the Kappa coefficient[26]. Secondhand smoking was included as adjustment variable in a previous report on the impact of socioeconomic status on lung function[24].

Analyses were conducted using STATA v.13 statistical software (Stata Corp., College Station, TX, USA).

RESULTS

We included a total of 1,819 participants (929 girls and 890 boys) in the first study phase; the number of children studied in each phase is described in table 1 as well as the proportion of passive smoking and anthropometric variables by gender. Kappa coefficient for multiple observations of passive smoking questions (up to a maximum of 12 evaluations) were for the father 0.520 (95% CI: 0.496-0.533), for the mother 0.605 (95% CI: 0.595-0.607), and for "other persons smoking at home" 0.477 (95% CI: 0.463-0.486), concordance classified usually between moderate and considerable[26]. The proportion of passive smoking ranged from 40.0 to 54.9% in girls and from 36.3 to 56.1% in boys in the repeated questionnaires applied, with no significant difference secondary to reported income or parents' education level. Reports of respiratory infections in the previous three months (bronchitis, common cold, and sore throat) in Phase 1 were as high as 69.6% in girls and 66.3% in boys.

Passive smoking, measured as the number of persons smoking inside the home and as dichotomous variable (yes/no), was associated significantly with respiratory symptoms and respiratory infections. For age-, height-, weight-, and ozone-level-adjusted GLLAMM models, we observed odds ratios (OR) of between 1.4 and 2.3 predicting respiratory symptoms in those exposed to passive smoking, exhibiting a trend to increase with the number of persons smoking inside the home both in boys and in girls; for recent respiratory infections, OR were between 1.2 and 1.5 in both boys and girls (Table 2).

Table 2. Multilevel linear logistic models by gender and the number of persons who smoked inside home

Number of persons smoking inside home	Girls				Boys			
	Crude		Adjusted*		Crude		Adjusted	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Respiratory symptoms (cough, wheezing, phlegm and dyspnea)								
One	1.6†	(1.21-2.05)	1.4†	(1.08-1.89)	1.4†	(10.07-10.83)	1.3	(0.94-1.66)
Two	1.6†	(1.18-2.20)	1.7†	(1.23-2.38)	1.6†	(1.18-2.18)	1.7†	(1.22-2.36)
Three	1.7‡	(1.09-2.72)	1.8†	(1.10-2.87)	2.2†	(1.41-3.42)	2.3†	(1.41-3.64)
Ozone			1.0	(0.98-1.01)			1.0	(0.97-0.99)
Respiratory infections (common cold, sore throat and bronchitis)								
One	1.4†	(1.21-1.54)	1.3†	(1.12-1.44)	1.3†	(1.14-1.45)	1.2‡	(1.03-1.33)
Two	1.3†	(1.17-1.54)	1.3†	(1.16-1.55)	1.5†	(1.32-1.74)	1.5†	(1.28-1.71)
Three	1.5†	(1.24-1.88)	1.5†	(1.19-1.83)	1.4†	(1.11-1.72)	1.4‡	(1.06-1.68)
Ozone			1.04†	(1.03-1.05)			1.04†	(1.04-1.05)

*Models adjusted for age, height, weight, and ozone levels; †$p < 0.01$; ‡$p < 0.05$.
OR: odds ratio; 95% CI: 95% confidence interval.

Table 3. Multilevel linear regression models of mixed effects by gender

Dependent variable	Girls		Boys	
Log-lung function models	β	**95% CI**	β	**95% CI**
		Number of persons who reported smoking within the home		
Ln(FEV_1)	−0.00412†	−0.0069, −0.0014	−0.00173	−0.0044, 0.0009
Ln(FVC)	−0.00757†	−0.0101, −0.0050	−0.00280‡	−0.0052, −0.0004
Ln(FEV_1/FVC)	0.00359†	0.0020, 0.0052	0.00106	−0.0006, 0.0027
		Ozone effect		
Ln(FEV_1)	−0.00096†	−0.0012, −0.0008	−0.00110†	−0.0013, −0.0009
Ln(FVC)	−0.00056†	−0.0008, −0.0004	−0.00065†	−0.0008, −0.0005
Ln(FEV_1/FVC)	−0.00039†	−0.0005, −0.0003	−0.00045†	−0.0006, −0.0003
Z score models	β	**95% CI**	β	**95% CI**
		Number of persons who reported smoking within the home		
FEV_1 (Z-score)	−0.04174†	−0.0629, −0.0206	−0.04076†	−0.0620, −0.0196
FVC (Z-score)	−0.07171†	−0.0920, −0.0514	−0.05016†	−0.0709, −0.0294
FEV_1/FVC (Z-score)	0.04488†	0.0233, 0.0665	0.00895	−0.0120, 0.0299
		Ozone effect		
FEV_1 (Z-score)	−0.01032†	−0.0118, −0.0088	−0.01016†	−0.0117, −0.0087
FVC (Z-score)	−0.00662†	−0.0081, −0.0052	−0.00660†	−0.0081, −0.0051
FEV_1/FVC (Z-score)	−0.00707†	−0.0086, −0.0055	−0.00593†	−0.0074, −0.0045

Lung function models: each model was adjusted for age, age squared, height, weight, ozone levels, asthma, respiratory symptoms, and respiratory infections. Z-score models were adjusted for age, height, and weight according to Martínez-Briseño, et al.[20] predicting equations. Each model was additionally adjusted for ozone levels, asthma, respiratory symptoms, and respiratory infections.
†$p < 0.01$; ‡$p < 0.05$.
β: coefficients; 95% CI: 95% confidence interval; Ln: lung; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity.

We also observed a decrease in log-lung function (log-FEV_1 and logFVC) according to the number of persons smoking at home in multilevel models adjusted for age, height, weight, and ozone levels in girls, and of logFVC in boys (Table 3), whereas both were decreased significantly in both genders if lung function was expressed as a Z-score. The average impact of passive smoking on an individual of mean age and height for each person smoking indoors was a decrease in FEV_1 of 0.30% (6.8 ml) for all individuals, 0.41% (9.2 ml) for girls, and 0.17% (4.1 ml) for boys. For FVC, the average drop was of 0.53% (14.1 ml) for all participants, 0.75% (19.4 ml) for girls, and 0.28% (7.8 ml) for boys. We did not observe a worse adverse effect of secondhand smoking in children with a diagnosis of asthma than in the remainder.

In the models, we tested other possible predictors: reported allergies, degree of physical activity, distance from school to home, and exposure to fuel smoke at home, but no significant association was observed with the outcome variables. Exposure to increased levels of ozone was associated with a decrease in lung function, as reported previously[19]. Tables 2 and 3 show that individuals in the higher quartile of ozone exposure had 3.3% (78.9 ml) less FEV_1 and 2.3% (62.8 ml) less FVC than individuals in the lower air pollution quartile (see online table 1 for the full model). Also, a reported physician's diagnosis of asthma was associated with a significant average drop in FEV_1 of 3.5% (82.7 ml), but without a relevant change in FVC. These effects were additive to those due to second-hand smoking. We found no significant impact of active smoking in lung function or symptoms in the 274 acknowledged direct smokers.

DISCUSSION

In this study, we showed that in the cohort of primary- and secondary-level students (aged 8-17 years) residing in metropolitan Mexico City, secondhand smoking

was associated with an increase in respiratory symptoms, in the frequency of self-reported respiratory infections (common cold, sore throat, and bronchitis), and with a reduction of spirometric pulmonary function. Second-hand smoking was homogeneous across different strata of reported income or parents' education.

Exposure to tobacco smoke prenatally and during infancy is associated with a reduction in the growth of pulmonary function and a greater risk for the development of asthma[27,28], as well as for respiratory symptoms and/or respiratory infections[29,30]. The placenta allows the penetration of smoke products into the fetus[31], likely affecting the development and growth of the forming lungs, with a possible drop in the spirometry indices[6,32].

In our study, an adverse effect on lung function was observed in children exposed to passive smoking, proportional to the number of smokers at home. Although this appears small in magnitude (5-15 ml of FEV_1 or FVC per smoker inside the home), it is also associated with respiratory symptoms, implying persistent inflammation, and is additive to other insults such as air pollution[19], as observed in models depicted in tables 2 and 3. For example, if an individual simultaneously reported respiratory symptoms, frequent respiratory infections, asthma, passive smoking, and residing in an area with ozone levels in the upper quartile, the estimated lung function decrease was 7.6% (183.3 ml) in FEV_1 and 2.9% (78.1 ml) in FVC, which may possibly lead to permanent deficits in lung function.

A decrease in FEV_1 associated with passive smoking was reported in another study as being worse if the exposure was through the mother and in individuals with wheezing[15]. A reduction in FEV_1 and FVC was worse in asthmatic children exposed to SHS compared with exposed children without asthma[33]. However, in our study, previous asthma and wheezing did not predict a more important decrease in lung function with passive smoking, although we were unable to analyze whether children with asthma had more symptoms or a more difficult control if exposed to SHS than those without exposure. Passive smoking was not associated with asthma in a cross-sectional study performed in Mexican children[34].

Respiratory symptoms in children of mothers who smoke during pregnancy may also be worse: of 313 schoolchildren between the ages of 5 and 13 years, 18% were exposed to SHS during pregnancy and had an OR of a 2.2-times greater risk of having cough in comparison with children of non-smoking mothers[35]. Additionally, in our study we did not know whether the mothers smoked during pregnancy, but 23% of mothers acknowledged smoking and some of them could have been smokers during pregnancy[36].

Exposure to air pollution including second-hand smoking is of higher concern in children because ventilation per size is higher than in adults[37], an effect more relevant in Mexico City, with altitude-induced hyperventilation. In addition, the infant's immunological system is immature and more susceptible to respiratory infections, and environmental toxins or infections may interfere with lung growth and development during key periods.

Additional study limitations

We based the assessment of passive smoking on the report of the parents, but lacked measurements of cotinine; were unaware of how many cigarettes they smoked inside the home, which was relevant for adverse effects in previous studies[38,39]; and whether the mothers smoked during pregnancy. Children were studied before attaining final growth, and we do not know whether functional deficits in those exposed to SHS were or were not permanent. In addition, the report of respiratory symptoms and infections was obtained from the questionnaires and may be affected by recall. On the other hand, the study represents a well-characterized cohort of children studied repeatedly with high-quality spirometry measurements, with respiratory symptoms (cough, phlegm, and wheezing) assessed by standard questions utilized in respiratory epidemiology. In other studies, as in ours, the simple parental report of passive smoking was a predictor of symptoms and altered pulmonary function, and questions about smoking at home by the mother, the father and other persons correlated with cotinine levels in children[22].

We found more respiratory symptoms, more frequent respiratory infections, and lower spirometry function in schoolchildren exposed to tobacco smoke at home, with the risk increasing if more persons smoked within the home. This information supports international efforts to eliminate passive smoking in children.

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SUPPLEMENTAL MATERIAL

Table 1. Multilevel linear regression models of mixed effects by gender

	GIRLS			BOYS		
Ln (FEV_1)	β	**p value**	**95% CI**	β	**p value**	**95% CI**
Age (years)	0.03499	0.000	(0.02, 0.05)	–0.07287	0.000	(–0.08, –0.06)
Age^2	–0.00011	0.708	(–0.00, 0.00)	0.00432	0.000	(0.00, 0.01)
Height (cm)	0.01354	0.000	(0.01, 0.01)	0.01543	0.000	(0.01, 0.02)
Weight (kg)	0.00350	0.000	(0.00, 0.00)	0.00154	0.000	(0.00, 0.00)
Exposed to passive smoking	–0.00412	0.003	(–0.01, –0.00)	–0.00173	0.197	(–0.00, 0.00)
Ozone	–0.00096	0.000	(–0.00, –0.00)	–0.00110	0.000	(–0.00, –0.00)
Asthma	–0.04563	0.005	(–0.08, –0.01)	–0.02532	0.110	(–0.06, 0.01)
Respiratory symptoms	0.00195	0.179	(–0.00, 0.00)	–0.00120	0.423	(–0.00, 0.00)
Respiratory infections	–0.00408	0.049	(–0.01, –0.00)	–0.00524	0.010	(–0.01, –0.00)
Constant	–1.55347	0.000	(–1.62, –1.48)	–1.06199	0.000	(–1.14, –0.98)
Ln (FVC)	β	**p value**	**95% CI**	β	**p value**	**95% CI**
Age (years)	0.01353	0.036	(0.00, 0.03)	–0.07368	0.000	(–0.08, –0.06)
Age^2	0.00070	0.008	(0.00, 0.00)	0.00423	0.000	(0.00, 0.00)
Height (cm)	0.01204	0.000	(0.01, 0.01)	0.01418	0.000	(0.01, 0.01)
Weight (kg)	0.00446	0.000	(0.00, 0.01)	0.00258	0.000	(0.00, 0.00)
Exposed to passive smoking	–0.00757	0.000	(–0.01, –0.01)	–0.00280	0.024	(–0.01, –0.00)
Ozone	–0.00056	0.000	(–0.00, –0.00)	–0.00065	0.000	(–0.00, –0.00)
Asthma	0.00726	0.646	(–0.02, 0.04)	0.00498	0.740	(–0.02, 0.03)
Respiratory symptoms	0.00346	0.009	(0.00, 0.01)	0.00156	0.256	(–0.00, 0.00)
Respiratory infections	–0.00406	0.033	(–0.01, –0.00)	–0.00466	0.012	(–0.01, –0.00)
Constant	–1.15742	0.000	(–1.22, –1.09)	–0.79747	0.000	(–0.87, –0.73)
Ln (FEV_1/FVC)	β	**p value**	**95% CI**	β	**p value**	**95% CI**
Age (years)	0.02187	0.000	(0.01, 0.03)	0.00075	0.833	(–0.01, 0.01)
Age^2	–0.00080	0.000	(–0.00, –0.00)	0.00009	0.545	(–0.00, 0.00)
Height (cm)	0.00154	0.000	(0.00, 0.00)	0.00125	0.000	(0.00, 0.00)
Weight (kg)	–0.00113	0.000	(–0.00, –0.00)	–0.00106	0.000	(–0.00, –0.00)
Exposed to passive smoking	0.00359	0.000	(0.00, 0.01)	0.00106	0.204	(–0.00, 0.00)
Ozone	–0.00039	0.000	(–0.00, –0.00)	–0.00044	0.000	(–0.00, –0.00)
Asthma	–0.05216	0.000	(–0.07, –0.04)	–0.03005	0.002	(–0.05, –0.01)
Respiratory symptoms	–0.00160	0.066	(–0.00, 0.00)	–0.00285	0.002	(–0.01, –0.00)
Respiratory infections	0.00010	0.935	(–0.00, 0.00)	–0.00056	0.657	(–0.00, 0.00)
Constant	–0.40146	0.000	(–0.44, –0.36)	–0.26367	0.000	(–0.31, –0.22)

Ln: lung: FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity.

Table 2. Multilevel linear regression models of mixed effects by gender

	GIRLS			BOYS		
FEV_1 Z-score	**β**	**p value**	**95% CI**	**β**	**p value**	**95% CI**
Exposed to passive smoking	–0.04174	0.000	(–0.06, –0.02)	–0.04076	0.000	(–0.06, –0.02)
Ozone	–0.01032	0.000	(–0.01, –0.01)	–0.01016	0.000	(–0.01, –0.01)
Asthma	–0.34549	0.005	(–0.59, –0.10)	–0.22725	0.074	(–0.48, 0.02)
Respiratory symptoms	0.03225	0.004	(0.01, 0.05)	–0.00129	0.916	(–0.03, 0.02)
Respiratory infections	–0.08591	0.000	(–0.12, –0.06)	–0.08903	0.000	(–0.12, –0.06)
Constant	0.89761	0.000	(0.78, 1.01)	0.74864	0.000	(0.64, 0.86)
FVC Z-score	**β**	**p value**	**95% CI**	**β**	**p value**	**95% CI**
Exposed to passive smoking	–0.07171	0.000	(–0.09, –0.05)	–0.05017	0.000	(–0.07, –0.03)
Ozone	–0.00662	0.000	(–0.01, –0.01)	–0.00660	0.000	(–0.01, –0.01)
Asthma	0.05933	0.636	(–0.19, 0.31)	0.00212	0.987	(–0.25, 0.25)
Respiratory symptoms	0.04155	0.000	(0.02, 0.06)	0.02060	0.084	(–0.00, 0.04)
Respiratory infections	–0.07791	0.000	(–0.11, –0.05)	–0.08134	0.000	(–0.11, –0.05)
Constant	0.58562	0.000	(0.48, 0.69)	0.43609	0.000	(0.33, 0.55)
FEV_1/FVC Z-score	**β**	**p value**	**95% CI**	**β**	**p value**	**95% CI**
Exposed to passive smoking	0.04488	0.000	(0.02, 0.07)	0.00895	0.401	(–0.01, 0.03)
Ozone	–0.00707	0.000	(–0.01, –0.01)	–0.00593	0.000	(–0.01, –0.00)
Asthma	–0.70136	0.000	(–0.93, –0.47)	–0.38995	0.002	(–0.64, –0.14)
Respiratory symptoms	–0.00957	0.410	(–0.03, 0.01)	–0.03850	0.001	(–0.06, –0.01)
Respiratory infections	–0.03360	0.034	(–0.06, –0.00)	–0.01663	0.286	(–0.05, 0.01)
Constant	0.72584	0.000	(0.61, 0.84)	0.46292	0.000	(0.35, 0.57)

FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity.

Table 3. Multilevel linear logistic models predicting symptoms by gender

	GIRLS			BOYS		
Respiratory symptoms	**OR**	**p value**	**95% CI**	**OR**	**p value**	**95% CI**
One person smokes indoors	1.43	0.013	(1.08, 1.89)	1.25	0.130	(0.94, 1.66)
Two people smoking indoors	1.71	0.001	(1.23, 2.38)	1.70	0.002	(1.22, 2.36)
Three people smoking indoors	1.78	0.019	(1.10, 2.87)	2.26	0.001	(1.41, 3.64)
Age	0.70	0.000	(0.62, 0.80)	0.59	0.000	(0.51, 0.67)
Height (cm)	0.97	0.010	(0.95, 0.99)	0.99	0.472	(0.97, 1.01)
Weight (kg)	1.01	0.428	(0.99, 1.03)	1.01	0.335	(0.99, 1.03)
Ozone	0.99	0.230	(0.98, 1.01)	0.98	0.000	(0.97, 0.99)
Constant	125.93	0.000	(9.90, 1,601.4)	81.06	0.001	(6.78, 969.42)
Respiratory infections	**OR**	**p value**	**95% CI**	**OR**	**p value**	**95% CI**
One person smokes indoors	1.27	0.000	(1.12, 1.44)	1.17	0.018	(1.03, 1.33)
Two people smoking indoors	1.34	0.000	(1.16, 1.55)	1.48	0.000	(1.28, 1.71)
Three people smoking indoors	1.48	0.000	(1.19, 1.83)	1.34	0.012	(1.06, 1.68)
Age	0.61	0.000	(0.58, 0.65)	0.72	0.000	(0.68, 0.76)
Height (cm)	1.04	0.000	(1.03, 1.05)	1.00	0.393	(0.98, 1.00)
Weight (kg)	0.98	0.000	(0.98, 0.99)	1.00	0.215	(0.99, 1.00)
Ozone	1.04	0.000	(1.03, 1.05)	1.04	0.000	(1.04, 1.05)
Constant	0.08	0.000	(0.02, 0.25)	2.52	0.121	(0.78, 8.09)

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HLA Risk Haplotype: Insulin Deficiency in Pediatric Type 1 Diabetes

Rita A. Gómez-Díaz[1,5]*, Elisa Nishimura-Meguro[2], Eulalia Garrido-Magaña[2], Lorena Lizárraga Paulin[3], Blanca E. Aguilar Herrera[3], Carolina Bekker-Méndez[4], Roberto Medina Santillán[5], Rodrigo Barquera[6,7], Rafael Mondragón-González[1] and Niels H. Wacher[1]

[1]Department of Medical Investigation in Clinical Epidemiology, UMAE Hospital de Especialidades; [2]Department of Endocrinology, UMAE Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS; [3]Department of Pediatric Endocrinology, UMAE Hospital General Dr. Gaudencio González Garza; [4]Department of Medical Investigation in Infectology and Immunology, Hospital de Infectología, Centro Médico Nacional La Raza, IMSS; [5]School of Medicine, Instituto Politécnico Nacional; [6]Laboratory of Physiology, Biochemistry and Genetics, Escuela Nacional de Antropología e Historia; [7]Department of Immunogenetics and Human Identification, Laboratorios Diagnomol, Mexico City, Mexico

ABSTRACT

Background: Certain HLA class II haplotypes have long been related with the risk of developing type 1 diabetes. The presence of the HLA haplotype DRB1*04/DQA1*03/DQB1*03:02, together with specific β-cell autoantibodies, contributes to the development and/or severity of insulin deficiency in type 1 diabetes. **Objective**: To evaluate the association of HLA risk haplotype HLA-DRB1/-DQA1/-DQB1 with β-cell function and antibody markers in recent-onset type 1 diabetes patients, their siblings, and controls. **Methods**: We studied recently diagnosed type 1 diabetes pediatric patients, their siblings, and healthy controls, analyzing autoantibodies (anti-glutamic acid decarboxylase, anti-IA-2, and anti-insulin), HLA risk and protector haplotypes, and β-cell function (plasma proinsulin, insulin and C-peptide). X^2, ANOVA or Kruskal-Wallis and multiple logistic regression were used to analyze data. **Results:** We included 46 patients, 72 siblings, and 160 controls. Prevalence of anti-tyrosine phosphatase-related islet antigen 2 and anti-glutamic acid decarboxylase antibodies was higher in patients than siblings and controls. We found risk haplotype DRB1*04/DQA1*03/DQB1*03:02 in 95.7% of patients vs. 51.87% of controls; DRB1*03:01/DQA1*05/DQB1*02 in 47.8% of patients vs. 8.12% of controls; and DRB1*14/DQA1*05/DQB1*03:01 in 2.2% of patients vs. 20.0% of controls. With DRB1*04/DQA1*03/DQB1*03:02, the prevalence of antibodies was significantly higher in patients, although not within any single group. In regression model based on insulin secretion, only anti-tyrosine phosphatase-related islet antigen 2 antibodies and age were associated with the risk haplotype. **Conclusions:** The DRB1*04/DQA1*03/DQB1*03:02 haplotype increased the risk for lower insulin, proinsulin, and C-peptide concentrations, suggesting an association with the severity of insulin deficiency in type 1 diabetes patients. This haplotype, added to antibody positivity, is a predictor of deficient insulin secretion in a Mexican pediatric population. (REV INVES CLIN. 2016;68:128-36)

Key words: HLA. Proinsulin. C-peptide. Mexican. Antibody.

Corresponding author:
*Rita A. Gómez-Díaz
Unidad de Investigación Médica en Epidemiología Clínica
UMAE Hospital de Especialidades, CMN-SXXI, IMSS
Av. Cuauhtémoc, 330
Col. Doctores, Del. Cuauhtémoc
C.P. 06725, Ciudad de México, México
E-mail: ritagomezdiaz@yahoo.com.mx

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INTRODUCTION

Type 1 diabetes (T1D) is a multifactorial and polygenic disease. It has long been recognized that class II alleles and haplotypes of the human leukocyte antigen system (HLA) on chromosome 6p21.3 are related to the risk of developing T1D[1]. There are many non-HLA T1D susceptibility genes, including: insulin gene (*INS*) on chromosome 11p15[2]; polymorphic, cytotoxic T-lymphocyte-associated protein gene (*CTLA4*) on chromosome 2q33[3]; protein tyrosine phosphatase, non-receptor type 22 gene (*PTPN22*) on chromosome 1p13[4]; interleukin-2 receptor alpha (*IL2RA*)[5]; and the interferon induced with helicase C domain (*IFIH1*) gene[6]. However, conditions and associations related with diabetes vary among different populations[7]. In addition, Rodríguez-Ventura, et al. found that risk haplotypes vary between type 1 and type 2 diabetes in the Mexican population[8]. As noted by Gómez-Díaz, et al.[9], various studies have found an association between incidence of T1D and the Latin American gene profile[10-12]. This was in line with the findings of the WHO DiaMond Molecular Epidemiology Sub-project performed in 1996[13]. Both Cruz-Tapias, et al.[14] and Gorodezky, et al.[15] have identified the risk alleles for T1D in the Latin American population, while Santos, et al.[16] and Mimbacas, et al.[17] reported risk alleles in Chilean and Uruguayan populations, respectively. However, Redondo, et al. identified DRB1*1401 and DQA1*0102/DQB1*0602 as protector alleles[18].

It has been accepted that positivity to β-cell autoantibodies is a risk factor for progression to T1D. As found in the European Nicotinamide Diabetes Intervention Trial (ENDIT), positivity for multiple antibodies in young patients is a fairly reliable indicator of later development of this disease[19]. This would be especially true in the case of patients' siblings with an HLA risk haplotype.

The aim of this study was to search for differences in markers of β-cell function (assessed by plasma proinsulin, insulin, C-peptide, HbA1c), β-cell antibodies (AB) (i.e. anti-glutamic acid decarboxylase 65 [GAD], anti-tyrosine phosphatase-related islet antigen 2 [IA-2], and anti-insulin), and lipid profile between individuals stratified according to their HLA Class II haplotypes (HLA-DRB1/-DQA1/-DQB1), in well-defined groups of recent-onset (≤ 3 months) T1D in Mexican pediatric patients, their siblings, and healthy controls.

METHODS

Study population

This was an analytical cross-sectional study of pediatric patients 2-17 years of age (cases), recently diagnosed (within the last three months) with T1D (according to the American Diabetes Association criteria)[20] and their siblings; healthy siblings included were free of acute infection at the time of inclusion. The control group consisted of healthy subjects of the same age and sex and their first-degree relatives (parents and siblings), enrolled among the children of employees of the Mexican Social Security Institute (IMSS) and from students of the elementary school "Escuela Benito Juárez", provided they were insured under IMSS, and were matched for age (± 6 months) and sex. Both they and their families were free of T1D and had no clinically evident infection. Patients with type 2 diabetes, maturity onset diabetes of the young, neonatal, or secondary diabetes, or any other autoimmune disease were excluded from the study. All the participants were of Mexican Mestizo origin, as defined by Gorodezky, et al.[21].

The study was conducted at the Endocrinology Department, Unidad Médica de Alta Especialidad (UMAE)-Hospital de Pediatría; Pediatric Endocrinology, UMAE Hospital General "Dr. Gaudencio González Garza", Centro Médico Nacional "La Raza"; and UMAE Hospital de Especialidades, Centro Médico Nacional (CMN) "Siglo XXI", all in Mexico City, with previous approval by the National Commission for Scientific Research from CMN SXXI, Instituto Mexicano del Seguro Social (Mexican Social Security Institute, IMSS).

All participants or their parents were asked to sign an informed consent. From all study subjects, a complete medical history (diabetes in the family, date of onset of diabetes, if applicable) and anthropometric measurements (age, weight, height, body mass index [BMI], blood pressure) were taken, and a 10 cc peripheral blood sample was obtained eight hours after the last meal for a complete blood count and blood chemistry.

Laboratory procedures

Complete blood count and blood chemistry were performed, including glucose, creatinine, total cholesterol, HDL-cholesterol and LDL-cholesterol, triglycerides, glycated hemoglobin (HbA1c), β-cell function tests

(plasma, proinsulin, insulin and C-peptide), and β-cell antibodies (anti-GAD, anti-IA-2, and anti-insulin). Class II-HLA typing (haplotype) was done. Anti-insulin antibodies in patients were determined only in those with T1D who had not yet started insulin therapy.

Anti-GAD and anti-IA-2 antibodies (AB) were determined by commercially available ELISA kits following the manufacturer's instructions (Genway Biotech, San Diego, CA). Anti-insulin-AB (detection range 0.312-20.0 U/ml) were also determined by commercially available ELISA kit following the manufacturer's instructions (Medipan GMBH, Berlin, Germany). Glucose, creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were measured using the Synchron CX® analyzer (Beckman Systems, Fullerton, CA) according to standard protocols. The coefficient of variation for cholesterol and HDL-cholesterol were 3.3 and 2.5%, respectively. Insulin was determined by a quantitative radioimmunoassay (RIA) kit (Merck Millipore, St. Charles, Missouri), following manufacturer's instructions. Proinsulin and C-peptide concentrations were determined by RIA using reagents from Millipore Corporation (St. Charles, Missouri). The normal fasting proinsulin range was 7.9 ± 1.5 pM; sensitivity of the kit was estimated at 2 pM. The corresponding values for C-peptide were 0.16-4.99 and 0.021 nmol/l, respectively. The HbA1c percentage was determined from whole blood using ion exchange high-performance liquid chromatography (normal range 4-6%).

HLA-DR, -DQA, -DQB typing

Genomic DNA of T1D patients and their siblings was extracted from 1 ml peripheral blood mononuclear cells (PBMC), and was isolated using the QIAamp® DNA mini kit (Qiagen, Valencia, CA). DNA was quantified by spectrophotometry, adjusted to a final concentration of 60 ng/µl, and stored at −20 °C until tested. HLA typing for *HLA-DRB1* and *-DQB1* was performed according to the 11th International Histocompatibility Workshop[22], using a method designed to provide medium-low resolution of Class II type (SSP UniTray® Pel-freez Dynal Biotech, Roche, USA). Briefly, group-specific primer sets were used to amplify genomic DNA using a 96-well thermal tray. Genomic DNA sample was mixed with a reaction buffer and Taq polymerase. Mixture was dispensed to the 96 UniTray® wells for sealing and then thermal cycling. After completing 35 cycles, PCR products were loaded onto a 2% agarose gel for electrophoresis. Finally, the ethidium bromide stained gel was photographed and analyzed with UniMatchTM Plus analysis software program.

Statistical analysis

The Shapiro-Wilk test was used to assess if variables had a normal distribution. The X^2 test was used to compare proportions of haplotype frequencies. To compare characteristics among the groups, continuous variables were analyzed using either analysis of variance (ANOVA) test for normally distributed variables, or Kruskal-Wallis test for non-normally distributed continuous variables.

Multiple logistic regression analyses evaluating the association between the risk haplotype and antibodies and insulin secretion were performed using SPSS Windows v. 17.0 (SPSS, Inc). Haplotypes were obtained by maximum likelihood methods using Arlequin v. 3.0 software[23]. For all tests, $p < 0.05$ was considered significant.

RESULTS

We analyzed 46 patients, 72 siblings, and 160 healthy controls. Table 1 shows their anthropometrical, clinical, genetic, and immunological characteristics. As expected, all the anthropometrical characteristics were statistically different between the groups. Overweight and obesity were more frequent in the control group; in addition, they were older. There were statistically significant differences between groups in HbA1c, glucose, C-peptide, creatinine, total cholesterol, triglycerides, presence of anti-GAD-AB, and anti-IA-2-AB ($p < 0.001$), and HDL-cholesterol ($p = 0.019$).

When comparing positivity for antibodies between the three groups, the presence of one or more antibody was significantly more frequent in the patient group, followed by the siblings group ($p < 0.001$) (Table 1).

The analysis blocks of genetic frequency of HLA in patients and their siblings are shown in tables 2 and 3, respectively. There were significant differences in haplotype frequency between patients and controls: haplotype DRB1*04/DQA1*03/DQB1*03:02 was found in 95.7% of patients (n = 44) vs. 51.87% of controls (n = 83) ($p = 0.007$); and haplotype

Table 1. Anthropometric, clinical, genetic, and immunological characteristics of the study participants

	Patients with T1D	Controls	Siblings	p value
(n)	46	160	72	
Age, years	9.6 (7.8-12.9)	32.4 (24.2-42.0)	15.0 (9.1-23.0)	< **0.001**
Sex F/M (%)	25/21 (54.3)	86/74 (53.7)	36/36 (50.0)	< **0.001**
Weight, kg	36.6 ± 16.3 (10-68)	64.6 ± 20.2 (10-103)	53.6 ± 24.5 (14-112)	< **0.001**
Height, m	1.36 ± 0.21 (0.8-1.7)	1.56 ± 0.17 (0.8-1.8)	1.48 ± 0.21 (1.0-1.8)	< **0.001**
BMI, kg/m^2	18.4 ± 3.6 (11.39-25.69)	25.5 ± 5.3 (13.0-36.2)	22.6 ± 6.1 (12.9-41.9)	< **0.001**
Normal, n (%)	32 (69.6)	74 (46.2)	39 (54.1)	< **0.001**
Overweight, n (%)	13 (28.2)	55 (34.3)	24 (33.3)	< **0.001**
Obesity, n (%)	1 (2.2)	28 (17.5)	8 (11.1)	< **0.001**
SBP, mmHg	90 (80-110)	100 (69-130)	100 (80-130)	< **0.001**
DBP, mmHg	60 (50-70)	70 (60-80)	60 (40-100)	< **0.001**
HbA1c, %; mmol/mol	8.0 (6.6-9.2); 64 (49-77)	5.5 (5.0-5.8); 37 (31-40)	5.3 (4.9-5.6); 34 (30-38)	< **0.001**
Proinsulin, ng/ml	15.9 (7.7-21.4)	9.9 (6.7-16.3)	10.1 (7.3-14.7)	0.098
Insulin, µIU/ml pmol/l	16.2 (10.0-21.2) 97.19 (60-127.19)	15.6 (10.3-23.2) 93.6 (61.80-139.2)	13.9 (9.1-18.3) 83.40 (54.59-109.80)	0.530
C-peptide, ng/ml nmol/l	0.65 (0.23-1.26) 0.21 (0.07-0.41)	2.3 (1.5-3.0) 0.76 (0.49-0.99)	1.73 (1.10-2.52) 0.57 (0.36-0.83)	< **0.001**
Glucose, mg/dl	95 (80.0-127.5)	89 (83-97)	86 (80-90)	< **0.001**
C-peptide/glucose ratio	0.007 (0.002-0.011)	0.021 (0.014-0.031)	0.020 (0.012-0.028)	< **0.001**
Creatinine, mg/dl	0.52 ± 0.15 (0.2-1.0)	0.72 ± 0.18 (0.27-1.18)	0.63 ± 0.21 (0.23-1.1)	< **0.001**
Anti-GAD-AB, cases + (%)	16 (34.8)	20 (12.5)	13 (18.1)	< **0.001**
Anti-IA-2-AB, cases + (%)	20 (43.5)	13 (8.1)	7 (9.7)	< **0.001**
Anti-insulin-AB, cases + (%)	5 (10.9)	16 (10.0)	8(11.1)	0.799
≥ 1 antibody, n (%)	28 (60.9)	43 (26.9)	23 (31.9)	< **0.001**
≥ 2 antibodies, n (%)	12 (26.0)	7 (4.3)	5 (6.9)	< **0.001**
Total cholesterol, mg/dl	158.4 ± 40.0 (82-252)	179.2 ± 37.7 (93-278)	161.5 ± 36.3 (107-281)	< **0.001**
Triglycerides, mg/dl	66.5 (50.7-82.5)	124.0 (86.2-182.5)	91.0 (60.0-143.5)	< **0.001**
HDL-C, mg/dl	53.7 ± 14.0 (25.0-86.0)	47.1 ± 12.3 (13.0-79.0)	50.6 ± 13.4 (18.0-85.0)	**0.019**
LDL-C, mg/dl	141.9±35.3 (70.6-212.0)	145.8±38.6 (11.2-244.0)	138.8±33.1 (83.2-258.2)	0.155

Data are mean ± SD or median (interquartile range) for abnormal distribution. ANOVA or Kruskal Wallis was applied as appropriate. A p value < 0.05 was considered significant. Overweight was defined as BMI > 85 percentile and < 95 percentile in children and BMI > 25 and < 30 in adults; obesity as BMI ≥ 95 percentile in children and BMI ≥ 30 in adults. Note: In the patients with type 1 diabetes, anti-insulin Ab was only determined for those patients not on insulin therapy.
T1D: type 1 diabetes; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; GAD: glutamic acid decarboxylase; IA2: tyrosine-phosphatase antibodies; +: positive.

DRB1*03:01/DQA1*05/DQB1*02 in 47.8% (n = 22) vs. 8.12% (n = 13) (p = 0.002) (Table 4). Haplotype DRB1*14/DQA1*05/DQB1*03:01, thought to be protector, was present in 2.2% of patients (n = 1) vs. 20.0% of controls (n = 32) (p = 0.001) (Table 4).

Haplotypes DRB1*04/DQA1*03/DQB1*03:02 and DRB1*03:01/DQA1*05/DQB1*02, considered to indicate risk, were more frequent in patients than siblings or controls. These were also considerably more frequent in siblings than controls, as expected.

The most frequent risk haplotype (DRB1*04/DQA1*03/DQB1*03:02) was associated with a greater prevalence of positivity for one (p < 0.001) or ≥ two (p = 0.006) antibodies in the whole population. However, our sample size was not large enough to find differences between groups (Table 5).

Regarding β-cell function, the presence of the DRB1*04/DQA1*03/DQB1*03:02 haplotype was associated with a risk for higher proinsulin (p = 0.024) and lower C-peptide (p = 0.043) and C-peptide/glucose ratio

Table 2. Frequency of HLA-DRB1, DQA1, and DQB1 in patients with type 1 diabetes

HLA-DRB1	Patients		HLA-DQA1	Patients		HLA-DQB1	Patients	
Allele	n = 46	GF	Allele	n = 46	GF	Allele	n = 46	GF
DRB1*04	47	0.511	DQA1*03	49	0.533	DQB1*03:02	44	0.478
DRB1*03:01	22	0.239	DQA1*05	29	0.315	DQB1*02	27	0.293
DRB1*08	6	0.065	DQA1*01	6	0.065	DQB1*03:01	7	0.076
DRB1*12	4	0.043	DQA1*04	6	0.065	DQB1*04	7	0.076
DRB1*01	3	0.033	DQA1*02	2	0.022	DQB1*05	5	0.054
DRB1*13	2	0.022				DQB1*06	1	0.011
DRB1*07	2	0.022				DQB1*03:03	1	0.011
DRB1*14	2	0.022						
DRB1*09	2	0.022						
DRB1*01:03	1	0.011						
DRB1*16	1	0.011						

GF: genetic frequency.

Table 3. Frequency of HLA-DRB1, DQA1, and DQB1 in the siblings group

HLA-DRB1	Siblings		HLA-DQA1	Siblings		HLA-DQB1	Siblings	
Allele	n = 72	GF	Allele	n = 72	GF	Allele	n = 72	GF
DRB1*04	63	0.438	DQA1*03	67	0.465	DQB1*03:02	62	0.431
DRB1*03:01	16	0.111	DQA1*05	42	0.292	DQB1*02	28	0.194
DRB1*08	15	0.104	DQA1*04	15	0.104	DQB1*03:01	26	0.181
DRB1*14	9	0.062	DQA1*01	8	0.056	DQB1*04	15	0.104
DRB1*07	8	0.056	DQA1*02	8	0.056	DQB1*06	8	0.056
DRB1*12	8	0.056	DQA1*XX	4	0.028	DQB1*XX	3	0.021
DRB1*16	5	0.035				DQB1*03:03	1	0.007
DRB1*XX	4	0.028				DQB1*05	1	0.007
DRB1*09	4	0.028						
DRB1*11	4	0.028						
DRB1*15	3	0.021						
DRB1*13	3	0.021						
DRB1*01:03	1	0.007						
DRB1*01	1	0.007						

GF: genetic frequency.

($p < 0.001$) in patients (Table 5). No differences in the concentrations of insulin secretion indicators were observed in controls or siblings (Table 5). Siblings who were obese and carried the risk haplotype DRB1*04/DQA1*03/DQB1*03:02 were more likely to have positive antibodies than controls.

Multiple logistic regression models were performed, with proinsulin and C-peptide as dependent variables, to evaluate insulin secretion capacity as predictor of the risk haplotype DRB1*04/DQA1*03/DQB1*03:02 (Table 6). In the model based on proinsulin, only anti-IA-2-AB was associated (OR: 5.0; 95% CI: 1.125-22.691; $p = 0.035$). However, in the C-peptide model, only age was associated with the risk haplotype (OR: 1.0; 95% CI: 1.000-1.062; $p = 0.046$).

DISCUSSION

The presence of the HLA haplotype DRB1*04/DQA1*03/DQB1*03:02 together with antibody positivity contributes to the development and/or severity of insulin deficiency in T1D. The finding of the HLA risk haplotypes DRB1*04/DQA1*03/DQB1*03:02 and DRB1*03:01/DQA1*05/DQB1*02 was replicated in this sample of the Mexican population.

Table 4. Frequency of haplotypes in the study participants

Haplotype	Patients (n = 46)			Controls (n = 160)			Siblings (n = 72)		
	HF	(n)	Δ'	HF	(n)	Δ'	HF	(n)	Δ'
DRB1*04, DQA1*03, DQB1*03:02	**0.4783**	**44**	**1.0000**	**0.2600**	**83**	**1.0000**	**0.4236**	**61**	**0.9713***
DRB1*03:01, DQA1*05, DQB1*02	**0.2391**	**22**	**1.0000**	**0.0410**	**13**	**1.0000**	**0.1111**	**16**	**1.0000***
DRB1*08, DQA1*04, DQB1*04	0.0652	6	1.0000	0.1540	49	1.0000	0.1042	15	1.0000
DRB1*12, DQA1*05, DQB1*03:01	0.0435	4	1.0000	0.0000	0	–	0.0556	8	1.0000
DRB1*01, DQA1*01, DQB1*05	0.0326	3	1.0000	0.0660	21	1.0000	0.0069	1	1.0000
DRB1*04, DQA1*03, DQB1*02	0.0217	2	–0.8550	0.0000	0	–	0.0139	2	–0.8367
DRB1*07, DQA1*02, DQB1*02	0.0217	2	1.0000	0.0590	19	1.0000	0.048/6	7	0.8448
DRB1*01:03, DQA1*05, DQB1*03:01	0.0109	1	1.0000	0.0000	0	–	0.0069	1	1.0000
DRB1*14, DQA1*05, DQB1*03:01	0.0109	1	–0.7204	0.0000	0	–	0.0000	0	–
DRB1*04, DQA1*03, DQB1*04	0.0109	1	0.2923	0.0000	0	–	0.0000	0	0.4814
DRB1*09, DQA1*03, DQB1*02	0.0109	1	1.0000	0.0000	0	–	0.0208	3	0.6897
DRB1*09, DQA1*03, DQB1*03:03	0.0109	1	0.4713	0.0000	0	–	0.0069	1	1.0000
DRB1*13, DQA1*01, DQB1*05	0.0109	1	1.00000	0.0000	0	–	0.0000	0	–
DRB1*13, DQA1*01, DQB1*06	0.0109	1	1.0000	0.0302	10	1.0000	0.0208	3	1.0000
DRB1*14, DQA1*01, DQB1*05	0.0109	1	1.0000	0.0130	4	1.0000	0.0000	0	–
DRB1*14, DQA1*05, DQB1*03:01	**0.0109**	**1**	**1.0000**	**0.1010**	**32**	**1.0000**	**0.0625**	**9**	**1.0000***
DRB1*16, DQA1*05, DQB1*03:01	0.0000	0	–	0.0560	18	1.0000	0.0347	5	1.0000
DRB1*11, DQA1*01, DQB1*06	0.0000	0	–	0.0000	0	–	0.0069	1	1.0000
DRB1*11, DQA1*05, DQB1*03:01	0.0000	0	–	0.0000	0	–	0.0208	3	1.0000
DRB1*14, DQA1*05, DQB1*03:02	0.0000	0	–	0.0000	0	–	0.0070	1	–0.7097
DRB1*15, DQA1*01, DQB1*06	0.0000	0	–	0.0000	0	–	0.0208	3	1.0000

*A p value < 0.05 was considered significant.
HF: Haplotype frequency.

The DRB1*04 and DRB1*03:01 haplotypes were found to be strongly associated with the presence of T1D, and there seemed to be a protective effect of the DRB1*14 haplotype in this population, as found by Redondo, et al.[18] and Zuñiga, et al.[24], and also found in Chilean[25] and, recently, Iranian[26] populations. It is accepted that siblings of patients with diabetes share a greater risk of developing the disease than offspring of patients[27,28]. Aly, et al. indicated similar findings, especially when siblings share both risk haplotypes[29].

The excessive representation of native haplotypes could be the effect of at least one of two possible scenarios: (i) an increase in the presence of risk haplotypes for reasons as yet not understood, reducing the frequencies of the characteristic native haplotypes in the affected groups; (ii) the environment plays a role in the suppression of the autoimmune condition in such a way that native risk haplotypes do not trigger the autoimmune condition, similar to what occurs with other diseases such as lupus[30-32].

Our findings of risk and protector haplotypes differ from the literature, possibly due to the Mestizo combinations of the study population. Redondo, et al.[18] found protector trends among Caucasians in haplotypes (DQA1*0102/DQB1*0602, DRB1*1401/DQA1*0101/DQB1*0503) that never appeared in our population, neither in patients nor in controls. While we found the same most frequent risk haplotype as Gorodezky, et al. (DRB1*04/ DQA1*03/ DQB1*03:02)[15], the present study found this haplotype to be present at a much higher percentage (95.6 vs. 51.4% in patients, and 51.8 vs. 6.3% in controls). With regard to the risk haplotype DRB1*14/DQA1*05/DQB1*03:01, our findings are in agreement with Gorodezky, et al.[21]. However, when we analyzed the most common protector haplotype noted by Gorodezky, et al. (DRB1*0501/DQA1*0102/DQB1*0602) with positivity of antibodies, the protective value they reported was absent in our population. Further studies should target the protector value of these two haplotypes.

Table 5. Effect of the presence of risk haplotype DRB1*04/DQA1*03/DQB1*03:02 on antibody positivity and beta cell function in study participants

	Patients with T1D		Controls		Siblings		All groups
	HLA II risk		HLA II risk		HLA II risk		HLA II risk
	n (% of 44)	OR (95% CI) p	n (% of 83)	OR (95% CI) p	n (% of 61)	OR (95% CI) p	OR (95% CI) p
Anti-GAD-AB (cases +)	15 (34.0)	5.4 (0.6-48.2) 0.096	10 (12.0)	1.1 (0.4-2.9) 0.765	10 (16.3)	1.5 (0.3-6.4) 0.518	2.4 (1.209-5.015) **0.011**
Anti-IA-2-AB (cases +)	18 (40.9)	3.0 (0.5-16.8) 0.199	10 (12.0)	4.1 (1.1-15.8) **0.024**	6 (9.8)	1.3 (0.1-12.3) 0.785	3.8 (1.752-8.594) **0.001**
Anti-Insulin-AB (cases +)	4 (9.0)	1.0 (0.8-11.5) 1.000	5 (6.0)	0.5 (0.1-1.6) 0.259	5 (8.1)	2.4 (0.2-21.0) 0.401	0.9 (0.480-2.040) 0.977
≥ 1 positive antibodies	25 (56.8)	4.1 (0.8-19.5) 0.059	20 (24.0)	0.9 (0.4-1.9) 0.955	16 (26.2)	(0.3-3.2) 0.851	9.0 (2.560-31.638) **0.001**
≥ 2 positive antibodies	11 (25.0)	2.8 (0.3-20.2) 0.254	5 (6.0)	2.9 (0.5-15.7) 0.183	5 (8.1)	1.1 (1.0-1.2) 0.112	5.0 (1.4-17.9) **0.006**
Proinsulin (percentile > 75)	14 (31.8)	2.0 (1.3-2.8) **0.024**	12 (14.4)	1.3 (0.2-8.8) 0.727	7 (11.4)	0.5 (0.1-2.0) 0.356	1.5 (0.809-2.951) 0.186
Insulin (percentile > 75)	6 (13.6)	4.8 (0.5-45.4) 0.143	13 (15.6)	-0- 0.103	9 (14.7)	1.8 (0.440-7.787) 0.396	0.9 (0.478-1.849) 0.858
C-peptide (percentile > 75)	1 (2.2)	0.1 (0.008-1.32) **0.043**	12 (14.4)	0.2 (0.02-2.9) 0.272	7 (11.4)	1.4 (0.384-5.535) 0.578	0.2 (0.134-0.610) **0.001**
C peptide/ glucose ratio	---	---	11 (13.2)	0.8 (0.36-2.0) 0.735	10 (16.3)	2.0 (0.475-8.660) 0.334	0.4 (0.220-1.094) 0.079

NOTE: In the patients with type 1 diabetes, anti-insulin-AB was only determined for those patients not on insulin therapy. Abnormal values for proinsulin, insulin, and C-peptide were those above the 75 percentile. There were no cases of positivity for any antibody in the presence of HLA II protector haplotypes.
The X^2 test was used to compare proportions of haplotype frequencies. A p value <0.05 was considered significant.
T1D: type 1 diabetes; +: positive; IA2: tyrosine-phosphatase antibodies; GAD: glutamic acid decarboxylase.

Table 6. Association between the HLA haplotype DRB1*04/DQA1*03/DQB1*03:02 and antibodies and insulin secretion

Model 1. Proinsulin (percentile > 75)				
Variable	Beta	OR	95% CI	p value
Anti-IA-2	1.620	5.052	1.125-22.691	0.035
Model 2. C-peptide (percentile > 75)				
Variable	Beta	OR	95% CI	p value
Age	0.030	1.031	1.000-1.062	0.046

Models adjusted for age, sex, anti-GAD, anti-IA 2, anti-insulin, LDL-cholesterol and triglycerides, according to risk haplotype DRB1*04/DQA1*03/DQB1*03:02.
IA2: tyrosine-phosphatase antibodies.

Patients carrying at least one risk haplotype were younger at the age of onset than those not carrying any risk haplotype, as reported by Easton[33]. In addition, according to Gillespie, et al.[34], younger age of the patient at onset of the disease increases the risk for siblings.

Patients had significantly higher concentrations of proinsulin and lower concentrations of C-peptide than controls or siblings. These differences in insulin, proinsulin, and C-peptide concentrations may be explained by the fact that genetic load impacts the progression of the disease. The difference in antibody titers among the patient group shows the role of HLA association with an immune condition rather than a metabolic explanation of the disease.

Lower insulin, proinsulin, and C-peptide concentrations in carriers of two risk haplotypes may be taken as evidence of genetic factors underlying the severity of insulin deficiency in T1D. Genetic testing in siblings may be of clinical interest when a child is diagnosed with T1D, and these children should be carefully monitored. Further studies need to be conducted in populations with different ethnicities and distinct ancestral genetic proportions.

Evidence has shown that age at onset of diabetes also affects anti-GAD-AB and anti-IA-2-AB positivity. Howson, et al. found that both were associated with older age at diagnosis, while the same study indicated a

faster decline in antibody positivity with younger age at onset of the disease[35]. Anti-GAD and anti-IA-2 antibodies are among the most reliable markers of autoimmune activity in diabetes in general[36]. Nevertheless, the percentages of positivity relate with several factors, including ethnicity, testing method, and duration of the disease. In the present study, patients with T1D showed a higher percentage of positivity for anti-GAD and anti-IA-2 antibodies than healthy subjects, as expected. In the presence of risk haplotypes, it would be expected that the frequency of autoimmune antibodies would increase in all subjects. However, siblings carrying the risk haplotype DRB1*04/DQA1*03/DQB1*03:02 showed a higher percentage of positivity for antibodies than controls carrying this haplotype, supporting the need for careful monitoring.

In the regression analysis models, only anti-IA-2-AB was significant when using a model based on proinsulin in carriers of haplotype DRB1*04/DQA1*03/DQB1*03:02. Age was significant in the C-peptide model. This is in contrast with the findings of the ENDIT study[19], which showed that antibody positivity and age determined risk, but genotype did not.

The present study has some limitations. First, the sample was relatively small, and further studies with larger samples and follow-up are suggested. In addition, the ages and BMI of the control group were different from those of the cases. These differences were controlled in the multivariate analyses.

In conclusion, the presence of the DRB1*04/DQA1*03/DQB1*03:02 haplotype increased the risk for lower insulin, proinsulin, and C-peptide concentrations, suggesting an association with the severity of insulin deficiency in type 1 diabetes patients. This haplotype, added to β-cell antibody positivity, is a predictor of deficient insulin secretion in the Mexican pediatric population.

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PERMANYER

Rev Inves Clin. 2016;68:137-42 ORIGINAL ARTICLE

Globe Salvage With Intra-Arterial Topotecan-Melphalan Chemotherapy in Children With a Single Eye

Carlos A. Leal-Leal[1]*, Laura Asencio-López[1], Jesús Higuera-Calleja[4], Max Bernal-Moreno[3], Vanessa Bosch-Canto[2], Juan Chávez-Pacheco[1], Gabriela Isaac-Otero[1] and Maja Beck-Popovic[5]

[1]Oncology Service; [2]Ophthalmology Service; [3]Radiology and Imaging Service, Instituto Nacional de Pediatría; [4]Radiology and Imaging Service, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico; [5]Oncology Service, Centre Hospitalier Universitaire Vaudois, Lausanne, Vaud, Switzerland

ABSTRACT

Introduction: Intra-arterial chemotherapy is a novel therapeutic modality for retinoblastoma patients. Intra-arterial chemotherapy involves the administration of a super-selective drug through the ophthalmic artery, resulting in better ocular penetration and low systemic toxicity. **Objective:** The aim of this report was to evaluate the feasibility of intra-arterial chemotherapy in a large referral center in Mexico City. **Methods:** We included patients with bilateral retinoblastoma, one enucleation, and active disease in the other eye after at least two courses of systemic chemotherapy combined with topical treatments. All patients were treated with three courses of a combination of melphalan 4 mg and topotecan 1 mg. Patients were examined under general anesthesia three weeks after each chemotherapy cycle. **Results:** From 14 eligible patients, three could not be treated due to inaccessibility of the ophthalmic artery. A complete response was observed in 5/11 patients, three in Stage C according to the International Classification for Intraocular Retinoblastoma, one in Stage D, and one in Stage B. The eyes of three patients were enucleated as a result of active/progressive disease, one in Stage B and two in Stage D. Eye preservation was 55% after a mean follow-up of 171 days (range 21-336). **Conclusions:** Super-selective intra-arterial chemotherapy is safe and effective for preventing the enucleation of 55% of affected eyes in this group of patients. (REV INVES CLIN. 2016;68:137-42)

Key words: Retinoblastoma. Intra-arterial chemotherapy. Ocular rescue. Melphalan. Topotecan.

Corresponding author:
*Carlos A. Leal-Leal
Servicio de Oncología, Instituto Nacional de Pediatría
Insurgentes Sur 3700-C Insurgentes
Cuicuilco, Del. Coyoacán
C.P. 04530, Ciudad de México, México
E-mail: drcarlosaleal@gmail.com

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INTRODUCTION

Retinoblastoma is the most frequent ocular malignancy in children[1]. In Mexican infants and toddlers it is the second most frequent solid neoplasia after brain tumors[2]. In developed countries, including the USA, retinoblastoma is a curable disease, with ocular survival being a priority[3].

Systemic chemotherapy, either in a neoadjuvant or adjuvant setting after enucleation, is currently the standard treatment. However, since the ocular volume represents less than 1% of the body surface and there is low drug penetration into the vitreous chamber, the actual amount of the chemotherapy agent reaching the eye remains quite low when delivered intravenously and is also systemically toxic[4].

Intra-arterial chemotherapy (IAC) involves super-selective drug administration through the ophthalmic artery, resulting in better penetration of the ocular structures and lower systemic toxicity[5]. After various treatment failures it was first used as rescue therapy in recurrent retinoblastoma, but also proved to be effective as first-line treatment in Group C and D eyes, according to the International Classification of Intraocular Retinoblastoma (ICIRB)[6]. Furthermore, the salvage of Group E eyes has been reported[7]. The ICIRB is the newest staging system. It classifies intraocular retinoblastoma into five groups from A to E, based on the size and location of the tumor, and predicts the possibilities of rescuing the eye(s) (Table 1).

Although IAC has been known since 2004, only recently has it been applied in Mexico. Therefore, the aim of this study was to evaluate the feasibility of IAC in a referral center of a country with a middle-income population.

Table 1. International Classification for Intraocular Retinoblastoma (ICIRB)

Group	Description
A Eyes with small discrete tumors away from critical structures.	Tumors ≤ 3 mm confined to the retina. > 3 mm from the foveola and > 1.5 mm from the optic nerve. Non-vitreous or subretinal seeding.
B Eyes with no vitreous or subretinal seeding and discrete retinal tumor of any size or location.	Tumors > 3 mm, no vitreous or subretinal seeding. Subretinal fluid > 5 mm from the base of the tumor.
C Eyes with only focal vitreous or subretinal seeding and discrete retinal tumors of any size and location.	Seeding local, fine, and limited. Tumors discrete and of any size and location, up to one quadrant of subretinal fluid.
D Eyes with diffuse vitreous or subretinal seeding and/or massive, non-discrete endophytic or exophytic disease. Eyes with more extensive seeding than Group C.	Massive and/or diffuse intraocular disseminated disease. More than one quadrant of retinal detachment. Fine greasy vitreous seeding or avascular masses. Subretinal seeding, plaque-like.
E Eyes that have been destroyed anatomically or functionally by the tumor. Eyes with one or more than the following:	Irreversible neovascular glaucoma. Massive intraocular hemorrhage. Aseptic orbital cellulitis. Tumor anterior to anterior vitreous face. Tumor touching the lens. Diffuse infiltrating; retinoblastoma. Phthisis or pre-phthisis.

MATERIALS AND METHODS

Study design and patients

This was a prospective, longitudinal study. Patient inclusion criteria were: age > 12 months, previously diagnosed bilateral disease, enucleation of one eye, confirmation of active disease in the remaining eye, and having received at least two courses of systemic chemotherapy combined with local treatments (photocoagulation, thermotherapy) six weeks prior to intra-arterial treatment (Table 2). All patients had a complete physical examination to demonstrate active disease; the fundi were examined under anesthesia and RetCam images were obtained. Three patients with extraocular disease, glaucoma, or prior external-beam radiotherapy within six weeks of the study treatment were excluded. The study was conducted following the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the National Pediatrics Institute (Instituto Nacional de Pediatría, INP). Legal representatives of all enrolled patients provided written informed consent for the procedures.

Table 2. Results according to disease group

Patient no.	Previous treatment	ICIRB classification	Status after 3 IAC treatments	Follow-up (days)
1	Systemic chemotherapy + photocoagulation	C	Partial response	133
2	Systemic chemotherapy + photocoagulation	C	No response	91
3	Systemic chemotherapy + photocoagulation	D	Partial response	21
4	Systemic chemotherapy + photocoagulation	B	No response	149
5	Systemic chemotherapy + photocoagulation	D	Complete response	336
6	Systemic chemotherapy + photocoagulation	C	Complete response	289
7	Systemic chemotherapy + photocoagulation	D	No response	330
8	Systemic chemotherapy + photocoagulation	C	Complete response	122
9	Systemic chemotherapy + photocoagulation	C	Complete response	86
10	Systemic chemotherapy + photocoagulation	D	Partial response	282
11	Systemic chemotherapy + photocoagulation	B	Complete response	121

IAC: intra-arterial chemotherapy; ICIRB: International Classification for Intraocular Retinoblastoma.

Intra-arterial chemotherapy

All patients were treated by the same interventional neuroradiologist, following the technique described by Abramson, et al.[4] (Fig. 1). Drugs used were melphalan at a dose of 4 mg diluted in 20 ml of 0.9% sodium chloride, and topotecan at a dose of 1 mg diluted in 20 ml of 0.9% sodium chloride, each administered over 30 minutes for a total of one hour; drug dosages were based on the patient's age as per Gobin, et al.[8]. After treatment an angiography was obtained to detect any complications from the treatment.

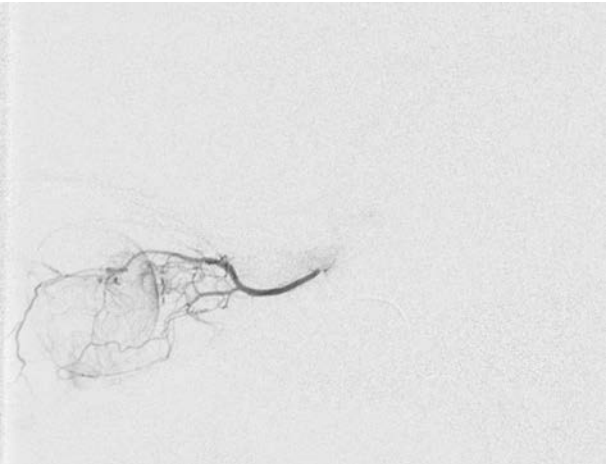
Patients were evaluated under general anesthesia with RetCam images 21 days after each IAC. Treatment consisted of three courses administered at 28-day intervals. Response was defined by our workgroup as follows: no response, if tumor size reduction was < 25% from the original size; partial response, if reduction was 25-50%; good response, if reduction was 50-75%; and complete response, if the reduction was > 75%.

All adverse events relating to the treatment procedure were recorded. The final response was evaluated by RetCam imaging after the third IAC cycle.

Statistical analysis

Analysis was based on the ICIRB stage at the time of diagnosis compared with the last RetCam image. Statistical analysis was performed using the SPSS program (IBM SPSS Statistics v.19, Chicago, IL.). Kaplan-Meier curves were constructed for globe rescue analysis.

Figure 1. Digital subtraction of ophthalmic artery after contrast infusion.



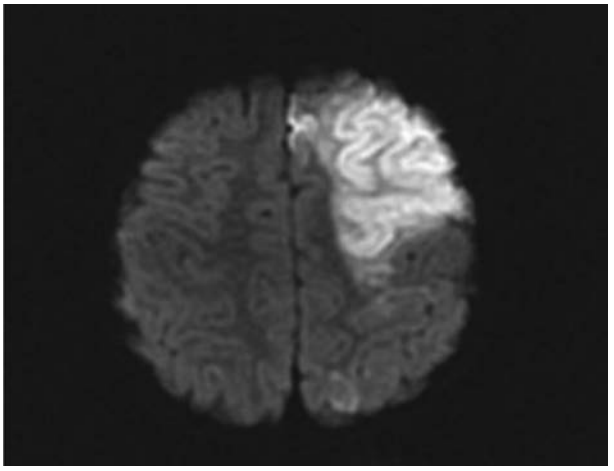
RESULTS

From 14 patients eligible for the study, three could not be treated due to inaccessibility of the ophthalmic artery. In the 11 treated patients, evaluation was performed after three IAC courses.

The mean age at presentation was 22.6 months (range, 12-36); seven patients were male and four were female. The group distribution included: two eyes in Group B, five eyes in Group C, and four eyes in Group D. Results of IAC according to the initial disease stage are shown in table 2.

A complete response was observed in 5/11 patients, one patient in Group B, three in Group C, and one in

Figure 2. Magnetic resonance imaging showing cerebral ischemia in patient no. 8 after the first course of intra-arterial chemotherapy.



Group D. In three patients, a partial response was observed, including one patient in Group C (patient no. 1), and two in Group D (patients nos. 3 and 10). These patients received further treatment (intravenous topotecan) to avoid enucleation. In one of the two Group D patients, disease progressed and the eye had to be enucleated. Of the three remaining patients, one had a stable disease (patient no. 2, Group C), but developed vitreous seeds and was rescued with intravitreal melphalan. The other two patients, one in Group B (patient no. 4) and one in Group D (patient no. 7), had progressive disease and the eyes had to be enucleated.

During the first treatment course, two of 11 patients developed acute complications. Patient no. 7 had nausea, vomiting, and a prolonged 48-hour hospitalization, but recovered completely. Patient no. 8 developed hemiparesis and severe ipsilateral headache, evaluated by neurological examination and magnetic resonance imaging. The latter revealed an ischemic area in the left parietal lobe, ipsilateral to the treated eye (Fig. 2). The angiography performed before melphalan administration showed no anatomical abnormalities, but the access to the ophthalmic artery was prolonged (30 minutes); perhaps this technical complication led to vasospasm and ischemia. The patient recovered completely one month later, after which she received the other two courses. No hematological toxicity or other systemic or local complications were observed.

Figure 3. Event-free (no enucleation) survival of the whole group.

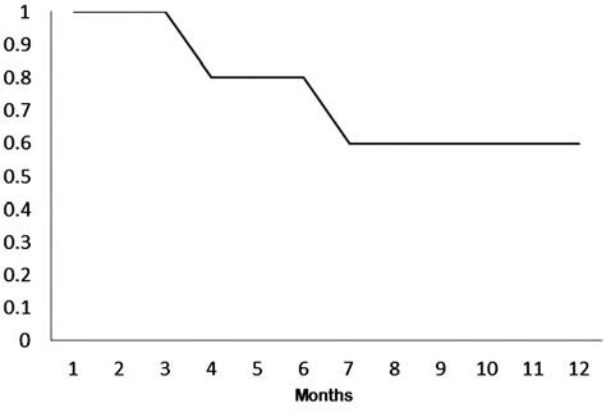
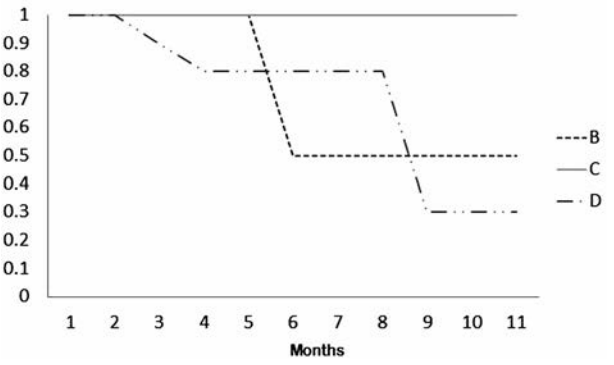


Figure 4. Event-free survival according to the initial ocular stage.



Kaplan-Meier curves showed eye preservation in 55% of cases (n = 6) after a mean follow-up of 171 days (range, 21-336) (Fig. 3). All Group C patients were rescued, as were 50% in Group B and 30% in Group D. The majority of eyes had Group C disease, and only two eyes belonged to Group B; establishing firm conclusions in the latter group was impossible since one of the two Group B eyes progressed and had to be enucleated (Fig. 4).

DISCUSSION

Intra-arterial chemotherapy was first described in the 1960s by Ellsworth[9], then further developed in Japan and reported by Kaneko, et al. in 2003[10]. In 2006, Abramson, et al.[4] modified the technique described by Kaneko, et al. by selectively introducing a microcatheter into the ophthalmic artery to directly administer

melphalan, carboplatin, and topotecan. Results of this therapy were reported in 2010, with 28 eyes initially treated in 23 patients over a three-year period. Only one eye had to be enucleated (3.5%) due to disease progression, and no eyes were enucleated as a result of treatment-related adverse events. Overall ocular survival was 100% at one year and 89% after two years of follow-up[3].

Recently, Absamson, et al. reported their results with IAC from 2006 to 2015; their enucleation rates decreased from over 95% to less than 10% in advanced-stage disease[11].

In middle-income countries such as Argentina, IAC is used as rescue therapy after retinoblastoma relapse and is more effective than periocular and intravenous chemotherapy[12], preserving up to 50% of eyes. Our study has shown that according to published guidelines, IAC can be safely administered in our local setting to patients with resistant or progressive retinoblastoma. Efficacy was satisfactory, with a complete response in 5/11 patients with bilateral disease and a single eye that did not respond to previous treatment with photocoagulation and systemic chemotherapy. Furthermore, 55% of eyes were rescued, thus avoiding enucleation. These results are comparable to those in centers with greater experience and similar patient cohorts, as reported by Gobin, et al.[8]. However, further patient accrual and longer follow-up periods are required to evaluate long-term eye preservation and drug toxicity. One of the major prognostic factors for retinoblastoma is its stage at the time of diagnosis. When IAC is used as the primary treatment modality, 80% of eyes can be saved, mostly in Groups C and D. Although advanced disease (E stage) is rare in developed countries, 50% of cases can also be rescued with IAC[13].

Experienced medical centers (i.e. treating more than 50 eyes each year) have adopted this treatment modality as their primary approach in Groups B, C, and D eyes and as rescue therapy at any stage[14]. In Mexico, the usual advanced stage at diagnosis[15] does not allow this type of treatment as the primary approach, although at our referral institution, an increasing number of patients are being diagnosed in earlier disease groups.

Although there is no established standard in terms of the types of drugs to be used for intra-arterial treatment, animal studies have shown that melphalan and topotecan can achieve therapeutic concentrations in the eye without significant levels in plasma[16,17]. Abramson, et al. have reported the combined use of melphalan (3.0-7.5 mg), topotecan (0.3-0.5 mg), and carboplatin (15-30 mg)[5], whereas Gombos, et al. used exclusively melphalan at a dose of 3-5 mg, increasing it up to 7.5 mg according to tumor response[18]. In addition, Munier, et al.[19] consolidated intra-arterial treatment with local therapies. In patients with a low response to melphalan only, Shields, et al. added 30 mg of carboplatin to 3 mg of melphalan[7] and demonstrated that the simultaneous use of two or more drugs is more effective than a single drug[20]. In our study, a combination of melphalan and topotecan was used. In all cases, we used 4 mg of melphalan and 1 mg of topotecan according to the patient's age[8].

Intra-arterial chemotherapy is a complex procedure that is not exempt from complications. In our series, we report one neurological event with no long-term sequelae. The patient presented this event after the first IAC, but we still completed three IAC sessions, obtaining a full response and rescuing the affected eye. The ocular and extraocular complications of the procedure have been reported by Monroy, et al.[21]. In our center we have not experienced any other complications.

Intra-arterial treatment is not a completely new treatment modality, but has existed for more than 20 years as first- or second-line treatment in certain parts of the world[18]. In the last eight years this treatment modality has been reconsidered, has been modified, and has been increasingly used as a conservative treatment modality, with possibly lesser toxicity when managing retinoblastoma. A recent survey by Grigorovski, et al.[14] reported that 63 centers in 35 countries were familiar with the procedure as an ocular rescue treatment. In Mexico, a country with 10-11 million children under the age of five years[22], this type of therapeutic procedure had never been used, and only four medical centers nationwide have the capacity to perform it.

After comparing the costs of this procedure with the number and duration of hospitalizations and the complications resulting from the usual treatment modalities, IAC is clearly cost-effective.

In conclusion, retinoblastoma is a highly curable cancer when diagnosed early and treated effectively. Thus, rescue of these eyes should become a priority in developing countries. Intra-arterial chemotherapy is an effective and safe therapeutic modality, which fosters the rescue of eyes with intraocular retinoblastoma and precludes the need for external-beam radiotherapy. Over half of pretreated eyes were salvaged, so it should be considered as the first option when rescuing single eyes in patients with retinoblastoma[23]. Although IAC could be more efficient if used as the primary treatment, this possibility needs to be further proven at our institution. This treatment modality requires expensive material resources and the highly specialized expertise of a multidisciplinary team, and should therefore be concentrated in a few centers in developing countries. In the long-term it could result in better cost-effectiveness, thus avoiding the additional costs of hospitalization and development of systemic complications.

Education is pivotal, particularly among healthcare professionals, since early detection of retinoblastoma leads to a higher proportion of intraocular local treatments for disease eradication.

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PERMANYER

Rev Inves Clin. 2016;68:143-6 ORIGINAL ARTICLE

Lethal Keratitis, Ichthyosis, and Deafness Syndrome Due to the A88V Connexin 26 Mutation

Carmen Esmer[1], Julio C. Salas-Alanis[2], Oscar R. Fajardo-Ramirez[2], Brenda Ramírez[3], Rong Hua[4] and Keith Choate[4]*

[1]Department of Genetics, Hospital Central Dr. Ignacio Morones Prieto; [2]Department of Basic Sciences, Universidad de Monterrey; [3]Department of Pediatric Dermatology, Hospital del Niño y la Mujer, Monterrey, N.L., Mexico; [4]Department of Dermatology, Yale University School of Medicine, New Haven, USA

ABSTRACT

Keratitis-ichthyosis-deafness syndrome is a well-characterized disease that has been related to mutations in the *GJB6* gene. Clinical features such as erythrokeratoderma, palmoplantar keratoderma, alopecia, and progressive vascularizing keratitis, among others, are well known in this entity. In this report we describe a newborn female patient diagnosed with keratitis-ichthyosis-deafness syndrome with a lethal outcome due to sepsis. The patient harbored the mutation A88V that has been previously reported in lethal cases. (REV INVES CLIN. 2016;68:143-6)

Key words: KID syndrome. A88V mutation. Keratoderma.

INTRODUCTION

Keratitis-ichthyosis-deafness syndrome (KID, OMIM #148210) is a very rare genodermatosis with less than 100 cases reported in the literature. This syndrome is characterized by erythrokeratoderma, palmoplantar keratoderma, alopecia, progressive vascularizing keratitis, dry eyes, blepharitis, and conjunctivitis. In addition, non-progressive, congenital, sensorineural hearing loss is consistently present[1]. It has been reported that 64% of cases are sporadic, with a small fraction involving a dominant mutation in the *GJB2* gene[2]. This gene encodes a protein called connexin 26 (Cx26), and it has been shown that its mutations disrupt gap junction in intercellular communications through several mechanisms, such as mislocalization of the encoded protein, alteration of ion conductance, and formation of hemichannels with abnormal function[3]. Alterations at the molecular level in the *GJB2* gene have been related to deafness and skin disorders: Bart-Pumphrey syndrome (BPS), palmoplantar keratoderma (PPK), Vohwinkel syndrome (VS), keratitis-ichthyosis-deafness syndrome (KID), and hystrix-like ichthyosis-deafness syndrome (HID)[4]. The mutations reported in protein Cx26 are

Corresponding author:
*Keith Choate
Department of Dermatology
Yale University School of Medicine
333 Cedar Street
PO Box 208059
New Haven, CT 06520-8059, USA
E-mail: keith.choate@yale.edu

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Figure 1. a) Leonine facies with erythroderma, alopecia, and well-demarcated hyperkeratotic plaques associated to the scalp and frontal regions with deep furrows; b) hyperkeratotic plaques with a verrucous surface; c) hyperkeratotic plaques with ichthyosis-hystrix-like scaling on shoulder; d) scattered follicular hyperkeratosis in the trunk and wrinkled skin; e-f) palmoplantar keratoderma with severe constriction and onychodystrophy.

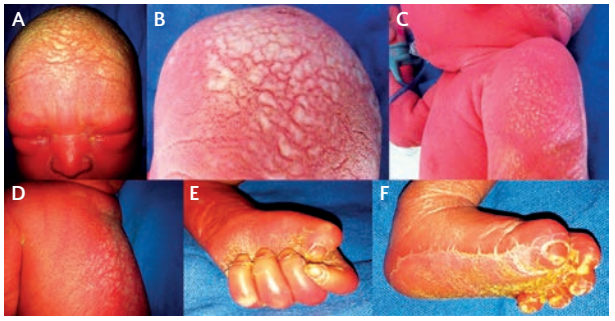
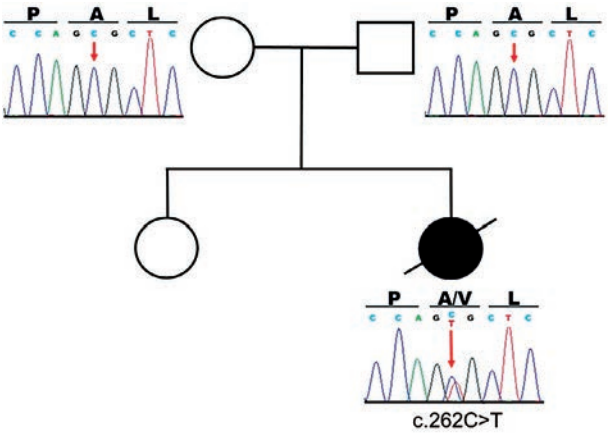


Figure 2. Pedigree of the patient with the *de novo* A88V mutation. The affected child is heterozygous for the c.262C>T (A88V) variant.



G12R, N14K, A40V, G45E, D50N, and A88V[5-7]; all of these increase hemichannel opening. Unfortunately, patients with the mutations G45E and A88V experience skin breakdown and recurrent infection, and eventually die from septicemia during the first year of life[8].

The objective of this study was to report a Mexican female infant with a fatal outcome who harbored the A88V mutation.

MATERIAL AND METHODS

DNA isolation and exome sequencing

After clinical examination and obtaining signed informed consent, blood samples were taken from the patient and both parents in tubes containing EDTA as an anticoagulant (BD Vacutainer®, Franklin Lakes, NJ). DNA was extracted using the Easy-DNA™ kit from Invitrogen (Carlsbad, CA). Samples were sequenced in the MiSeq platform from Illumina Company (San Diego, CA) following the manufacturer's protocols. The procedure was carried out in the Department of Dermatology at Yale University. The mutation detected was validated by Sanger sequencing in the same institution.

RESULTS

The patient was the second child of healthy, unrelated parents. Their first pregnancy was a non-affected child. The patient was born after 35 weeks of an uneventful pregnancy, weighed 2 kg, and measured 48 cm. She presented alopecia totalis, leonine facies, a wizened forehead, a scowl with deep furrows, and well-demarcated hyperkeratotic plaques on the scalp. Erythroderma and verrucous plaques covered her trunk and back. Her limbs showed ichthyosis-hystrix-like scaling and severe palmoplantar keratoderma that caused fixed flexion of the digits, which were tapered and had hyperconvex nails (Fig. 1). The external auditory canals were blocked by scale, and auditory brain stem potentials were not performed. Exams of the heart, brain, and kidney were normal. She developed sepsis at day 3 and was treated with cefotaxime (150 mg/kg/day) followed by fluconazole (3 mg/kg/day) and meropenem (120 mg/kg/day), without improvement; the patient died at nine days of age.

The DNA obtained from the patient was analyzed by exome sequencing; a heterozygous mutation (c.262C>T; p.A88V) located at exon 2 of the *GJB2* gene was found. Neither parent presented any genetic alteration in the *GJB2* sequence (Fig. 2).

DISCUSSION

We present a clinical case of sporadic KID syndrome harboring a *de novo* A88V mutation. Fatal KID syndrome has previously been described in eight families (Table 1)[8-15]. These cases were related to infection and sepsis, which complicated about half of the cases,

Table 1. Findings of the fatal form of keratitis-ichthyosis-deafness syndrome

Cases	Mallory, et al. 1989[13]	Helm, et al. 1990[10]	Berker, et al. 1993[11]	Gilliam, et al. 2002[14]	Janneke, et al. 2005[8]	Sbidian, et al. 2010[12]	Koppelhus, et al. 2011[15]	Haruna, et al. 2010[9]	Present case
Mutation	NE	NE	NE	G45E‡	G45E	G45E§	A88V	A88V	A88V
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Angola/African	Caucasian	Japanese	Mexican
Prematurity	34 weeks	38 weeks*	33 weeks	36 weeks	36 weeks	1/4	33 weeks	–	34 weeks
Alopecia	+	+	+		+	4/4	+	+	+
Ichthyosiform erythroderma	+	+	+	+	+	4/4	+	+	+
Palmoplantar keratoderma	–	–	+	+	+	4/4	+	–	+
Nails	–	–	Small	Thick	–	Dystrophy	Dystrophy	–	Brittle
Deafness	+	+	NE†	+	+†	NE	+	+	NE†
Eyes	–	+	–	+	+	0/4	–	+	–
Additional findings	Inguinal hernia Hirschsprung	–	–	–	–	–	Hydrocephalus	–	Fixed flexion digits
Death due to sepsis	+	+	+	+	+	4/4	+	+	+
Age at death	3 m	3 m	2 m	6 m	12 m	30 d, 5 m, 30 d, 10 d	3 m	3 y 5 m	9 d

*Premature labor at 34 weeks; †External auditory canals blocked by desquamation; ‡Griffit, et al. 2006; §Four siblings are described.
NE: not evaluated; y: years; m: months; d: days.

with bacteria and fungi being the most recognized agents, although a case with disseminated cytomegalovirus infection has been also described[10]. The underlying causes for increased susceptibility to infection remain unclear, but they can be related to extensive skin damage, resulting in the loss of the protector barrier and an increased amount of scaling. It is debatable if early and aggressive antibiotic prophylaxis could modify the lethality of this disorder or improve survival. Other measures, such as antiseptic baths and emollients, are useful at least in the less aggressive variants.

The case reported herein presented a severe palmoplantar keratoderma that produced tight skin in the palms and soles, causing flexion contractures in all digits (hands and feet). Some of these congenital findings resemble those found in Vohwinkel syndrome, which is another entity related to *GJB2*; however, digital involvement in Vohwinkel syndrome is an acquired feature and is always associated to pseudoainhum[16].

Connexins have conserved structural domains that include four transmembrane, two extracellular, and three cytoplasmic domains[17]. To date, 12 mutations in the gene encoding CX26 have been described as causative of KID syndrome, all located in the first transmembrane helix, the first extracellular loop, or the N terminal domain[18]. Interestingly, the mutations G45E (p.Gly45Glu) and A88V (p.Ala88Val) are linked to the fatal phenotype, and cases harboring these mutations suffer recurrent infections, eventual septicemia, and breathing problems[8]. Regarding the mutation found in this neonate female (A88V), it has been previously reported in two other cases with a fatal course and the mutation is located in the second transmembrane helix of CX26[9,15].

It is been reported that the A88V mutation produces enhanced hemichannel activity compared to the wild-type genotype, resulting in accelerated cell death that explains the etiology of the KID syndrome[3,19]. *In vitro* assays have shown that the magnitude of the hemichannel currents produced by the genotype D50A (a mutation present in a non-severe version of KID syndrome) was less than the currents produced by the genotype A88V, suggesting that the severity of the syndrome may correlate with the relative increase in hemichannel activity caused by the respective mutation[3,20].

Fatal cases are related to G45E or A88V mutations and represent about 10% of all KID syndromes, which shows a strong genotype-phenotype correlation in terms of survival. The case reported here harbored the A88V mutation and, consequently, the patient died during the second week of life.

Finally, caregivers must be aware of the lethal nature of some KID syndromes, with sepsis being a key factor of this lethality. Therefore, early and aggressive antibiotic treatment and other isolation measures should be used immediately after birth in order to improve survival.

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PERMANYER

Rev Inves Clin. 2016;68:147-53 ORIGINAL ARTICLE

Clinical Characteristics and Mortality of Influenza A H1N1 and Influenza-Like Illness in Mexico City in the 2013-2014 Winter Season

David Martínez-Briseño, Luis Torre-Bouscoulet, Julio de Jesús Herrera-Zamora, Julián Díaz-Rico, Gabriel Sandoval-Macías, Rogelio Pérez-Padilla, Carmen Hernández-Cárdenas, Justino Regalado-Pineda, Jorge Salas-Hernández and Patricio Santillán-Doherty*

Instituto Nacional de Enfermedades Respiratorias (INER), Mexico City, Mexico

ABSTRACT

Background: The 2013-2014 influenza season in Mexico City was severe and mainly due to influenza A H1N1, as was the 2009 pandemic. **Objective:** To describe features of the outbreak and to compare the characteristics of patients with and without viral identification. **Methods:** We reviewed the medical charts of all individuals with influenza or influenza-like illness admitted to a referral hospital for respiratory diseases in Mexico City from January 2013 to March 2014, whether influenza virus was identified or not. **Results**: We included 233 patients with influenza-like illness, 99 of whom had laboratory confirmed influenza; one-half of all patients required mechanical ventilation and 25% were admitted to the intensive care unit. Patients with confirmed influenza had a more severe disease than those without confirmation. A total of 52 (22.3%) patients died in hospital; survival was greater among patients hospitalized in the intensive care unit compared with those who remained in regular wards. **Conclusions:** Influenza A H1N1 continues to cause significant outbreaks in Mexico City. Patients with influenza-like illness had a similar clinical course regardless of laboratory confirmation of influenza, suggesting that their illness likely belonged to the same outbreak. Mechanical ventilation in regular hospital wards may be lifesaving, although the outcome is worse than at an intensive care unit. (REV INVES CLIN. 2016;68:147-53)

Key words: Influenza. Mechanical ventilation. Intensive care unit. Mortality.

INTRODUCTION

In March 2009, the novel influenza A (H1N1) was first reported in the southwestern USA and in Mexico, becoming the first pandemic of the 21st century. The population and healthcare system in Mexico City experienced the first and greatest early burden of the critical illness[1]. The 2009 influenza A H1N1 pandemic caused the deaths of 18,449 individuals with laboratory confirmed infection worldwide[2]. However, recent data increase the estimates to 200,000, including persons who died from influenza-like illness (ILI) or

Corresponding author:
*Patricio Santillán-Doherty
Instituto Nacional de Enfermedades Respiratorias (INER)
Calzada de Tlalpan, 4502
Col. Sección XVI, Del. Tlalpan
C.P. 14080, Ciudad de México, México
E-mail: patricio.santilland@gmail.com

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from diverse complications and who had no viral testing in countries or regions without access to the standard influenza virus real-time polymerase chain reaction (RT-PCR)[3]. In December 2013 we identified a rapidly growing number of persons with ILI, including severe cases, many of whom had laboratory confirmed influenza A H1N1. In 2011 Mexico experienced another winter outbreak of influenza A H1N1[4]. This report describes the clinical characteristics of patients hospitalized in our center from December 1, 2013 to February 28, 2014 with ILI as well as confirmed cases of influenza A H1N1 virus infection. In addition, we explored potential cofactors involved with mortality.

MATERIAL AND METHODS

Design and population study

We carried out a cross-sectional study collecting, in a standardized questionnaire, information from the clinical records of patients who were admitted from January 1, 2013 to March 3, 2014 to the National Institute of Respiratory Diseases of Mexico (INER). We included patients 18 years of age or older with confirmed influenza A H1N1 or ILI. Influenza-like illness was defined according to the World Health Organization (WHO) as sudden-onset fever (> 38°C) with cough or sore throat, in the absence of other diagnoses. A case of confirmed influenza A H1N1 is usually defined as the presence of ILI plus a positive RT-PCR or viral culture[5], but for the present work, influenza confirmation was obtained by RT-PCR. Sociodemographic variables comprised age, gender, and place of residence. We measured weight and height and estimated the body mass index (BMI) of patients. As clinical variables, we included respiratory signs and symptoms as well as smoking status, days of hospitalization, the presence and duration of mechanical ventilation, admission to the intensive care unit (ICU), vaccine against influenza, and in-hospital death. Laboratory measurements included hemoglobin (g/dl), platelet count ($\times 10^3$/µl), leukocytes ($\times 10^3$/µl), lymphocytes ($\times 10^3$/µl), creatinine (mg/dl), blood urea nitrogen (BUN, mg/dl), albumin (g/dl), bilirubin (mg/dl), creatine phosphokinase (CPK, U/ml), and lactate dehydrogenase (LDH, U/l).

We recorded the presence of comorbidities for each patient as the following dichotomous variables (yes/no): overweight/obesity; systemic arterial hypertension; asthma; type 2 diabetes mellitus (DM2); heart disease (most common was chronic heart failure [CHF]); chronic obstructive pulmonary disease (COPD); interstitial lung disease (ILD); human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS), and pulmonary tuberculosis (TB). Data on the number of medical emergency consultations, hospital admissions, and patients on mechanical ventilation due to ILI were obtained from the Department of Epidemiological Surveillance of the INER.

Statistical analysis

We compared all the variables, stratifying by condition of influenza (ILI vs. laboratory confirmed influenza A H1N1) and death to identify risk factors. Continuous variables were compared using Student's *t* test and categorical variables, with Pearson's chi-square test. Risk of in-hospital death was estimated by fitting logistic regression models and survival function, employing Kaplan-Meier models and Cox proportional hazard models as function of influenza confirmation, mechanical ventilation, place of care, presence of exposure, and comorbidities. A p value of < 0.05 was considered as statistically significant for all tests. Analysis was conducted using STATA v.12 statistical software (Stata Corp., College Station, TX, USA).

RESULTS

A total of 233 patients were included; mean age was 46.9 years (standard deviation [SD]: 13.8 years), and 137 (58.8%) were male. Patients were hospitalized for 19 days on average (SD: 18 days). Influenza was confirmed in 99 patients (42.5%), whereas 134 (57.5%) had ILI with negative RT-PCR tests, missing tests, or insufficient samples. Most of the patients (68.2%) resided in Mexico City, while the rest came from another state. Less than 5% of the included patients were vaccinated for the season when the illness occurred. A comorbid condition was identified in 92.3% of all patients. One-half of the patients (50.6%) received mechanical ventilation and 25.3% were admitted to the ICU. Sixty-one patients had worsening respiratory failure and required mechanical ventilation while in a general hospital ward since there were no beds available in the ICU. Fifty-two patients (22.3%) died in hospital (Table 1).

Table 1. Sociodemographic and clinical characteristics of the entire group (n = 233)

Variable	Value*
Age, years	46.9 (13.8)
Male, n (%)	137.0 (58.8)
Residence in Mexico City, n (%)	159.0 (68.2)
Influenza A H1N1, n (%)	99.0 (42.5)
BMI, kg/m^2	30.9 (6.9)
Influenza vaccine, n (%)	10.0 (4.3)
Hospitalization, days	18.8 (17.9)
Active smoker, n (%)	69.0 (29.6)
Mechanical ventilation, n (%)	118.0 (50.6)
Admitted to ICU, n (%)	59.0 (25.3)
Platelet count, × 10^3/µl	223.9 (107.3)
Creatinine, mg/dl	1.2 (0.91)
BUN, mg/dl	19.1 (16.1)
Albumin, g/dl	3.0 (0.7)
Bilirubin, mg/dl	0.8 (0.5)
CPK, U/ml†	157.0 (67.0, 411.0)
LDH, U/l	482.7 (419.5)
Hemoptysis, n (%)	39.0 (16.7)
Diabetes mellitus, n (%)	20.0 (8.6)
COPD, n (%)	12.0 (5.2)
Any comorbidity‡, n (%)	215.0 (92.3)
Death, n (%)	52.0 (22.3)

*Mean and standard deviation (SD) or number and percentage when indicated; †Represent median, 25th and 75th percentile. ‡Obesity, hypertension, asthma, heart disease (most common, chronic heart failure), interstitial lung disease, human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), tuberculosis. BMI: body mass index; ICU: intensive care unit; BUN: blood urea nitrogen; CPK: creatinine phosphokinase; LDH: lactic dehydrogenase; COPD: chronic obstructive pulmonary disease.

The clinical characteristics and laboratory results of the patients on hospital admission stratified by the condition of influenza are presented in table 2. No difference was found between the two groups in the proportion of patients who lived in Mexico City. Individuals with confirmed influenza had more severe disease compared with those with ILI. Patients with confirmed influenza required more mechanical ventilation and ICU admission and had higher levels of LDH and CPK, but the difference in mortality, although higher in confirmed cases, was not statistically significant. Confirmed cases also had a higher BMI than those with ILI.

Patients who died were often older ($p < 0.01$), male ($p < 0.05$), on mechanical ventilation ($p < 0.01$), and had hemoptysis ($p < 0.01$) or diabetes mellitus ($p < 0.05$). In addition, deceased patients had more abnormalities in several laboratory tests: higher than normal BUN, bilirubin, CPK and LDH, or lower albumin. The BMI was not different between groups (Table 3).

Of 118 patients who received mechanical ventilation, 61 (51.7%) were cared for outside the ICU (regular hospital wards, emergency room), and 57 (48.3%) at the ICU. Of the 61 patients treated outside the ICU, 35 (57.4%) died, compared with 14 of 57 (24.6%) admitted to the ICU ($p < 0.01$) (Table 4).

Results of logistic regression suggest no differences between patients with confirmed influenza or ILI in most measurements, except for a lower platelet count in individuals with confirmed influenza (odds ratio [OR]: 0.99; $p < 0.01$) (Table 5).

A Cox proportional hazard model confirmed that ICU admission reduced the risk for death (hazard ratio [HR]: 0.27; $p < 0.01$), while hemoptysis (HR: 2.24; $p < 0.01$), mechanical ventilation (HR: 6.23; $p < 0.05$), age (HR: 1.04; $p < 0.05$), and being male (HR: 4.72; $p < 0.01$) were associated with death. No statistical difference in mortality was found between patients with confirmed influenza and those with ILI (HR: 0.99; $p = 0.99$) (Table 6).

Figure 1 depicts Kaplan-Meier survival curves for patients who were ventilated, stratifying by place of care (ICU vs. ward). Survival was higher among patients admitted to the ICU than among those treated in wards ($p < 0.01$). Mean 95% confidence interval [95% CI] of survivor days were 89.8 days (range, 75.0-104.5 days) for patients in the ICU and 33.4 days (range, 25.0-41.8 days) for those cared for in wards. Data were confirmed in multivariate Cox proportional hazard models (HR: 0.27; $p < 0.01$) (Table 6), also demonstrating an increased risk of death in patients receiving mechanical ventilation (HR: 6.23; $p < 0.05$). Figure 2 illustrates the survival functions obtained from the model in table 6 for patient care at ICU and in wards. The functions were evaluated using the average values reported in table 1 for continuous variables and considering a male patient with hemoptysis, diabetes mellitus, and mechanical ventilation. The figure presents higher survival for patients admitted to the ICU compared with those cared for in regular wards under similar conditions. Figure 3 describes the number of medical consultations, hospital admissions, and patients on mechanical ventilation in the 2013-2014 winter season compared to previous outbreaks since the 2009 H1N1 pandemic.

Table 2. Sociodemographic and clinical characteristics by influenza condition

Variable*	ILI n = 134	Influenza A H1N1 n = 99	P value
Age, years	46.9 (14.5)	46.9 (12.8)	0.994
Male, n (%)	74.0 (55.2)	63.0 (63.6)	0.197
Residence in Mexico City, n (%)	98.0 (73.1)	61.0 (61.6)	0.062
BMI, kg/m^2	29.6 (6.6)	32.5 (6.9)	**< 0.01**
Hospitalization, days	16.8 (15.4)	21.4 (20.7)	0.062
Active smoker, n (%)	40.0 (29.9)	29.0 (29.3)	0.475
Mechanical ventilation, n (%)	60.0 (44.8)	58.0 (58.7)	**< 0.05**
Admitted to ICU, n (%)	27.0 (20.2)	32.0 (32.3)	**< 0.05**
Platelet count, × 10^3/µl	241.4 (115.6)	200.1 (89.9)	**< 0.01**
Creatinine, mg/dl	1.1 (1.0)	1.2 (0.8)	0.177
BUN, mg/dl	17.7 (16.6)	21.1 (15.1)	0.114
Albumin, g/dl	3.9 (0.7)	2.9 (0.6)	0.076
Bilirubin, mg/dl	0.7 (0.5)	0.8 (0.6)	0.827
CPK, U/ml†	122.0 (58, 361)	195.0 (99, 575)	**< 0.01**
LDH, U/l	396.3 (312.5)	599.6 (510.1)	**< 0.01**
Hemoptysis, n (%)	22.0 (16.4)	17.0 (17.2)	0.879
Diabetes mellitus, n (%)	12.0 (8.9)	8.0 (8.1)	0.814
COPD, n (%)	6.0 (4.5)	6.0 (6.1)	0.589
Any comorbidity, n (%)	123.0 (91.8)	92.0 (92.9)	0.748
Death, n (%)	27.0 (20.2)	25.0 (25.3)	0.355

*Data are mean and standard deviation (SD) or number and percentage when indicated; †Represent median, 25th and 75th percentile; medians compared by Wilcoxon rank-sum test.
ILI: influenza-like illness; BMI: body mass index; ICU: intensive care unit; BUN: blood urea nitrogen; CPK: creatine phosphokinase; LDH: lactate dehydrogenase; COPD: chronic obstructive pulmonary disease.

Table 3. Sociodemographic and clinical characteristics by mortality

Variable*	Surviving patients n = 181	Deceased patients n = 52	P value
Age, years	44.9 (13.6)	53.7(12.4)	**< 0.01**
Male, n (%)	99.0 (54.7)	38.0 (73.1)	**0.018**
Residence in Mexico City, n (%)	128.0 (70.7)	31.0 (59.6)	0.130
BMI, kg/m^2	30.7 (7.2)	31.3 (5.5)	0.570
Hospitalization, days	18.5 (16.8)	19.6 (21.7)	0.756
Active smoker, n (%)	58.0 (32.0)	11.0 (21.2)	0.450
Influenza A H1N1, n (%)	74.0 (40.9)	25.0 (48.1)	0.355
Mechanical ventilation, n (%)	69.0 (38.1)	9.0 (94.2)	**< 0.01**
Admitted to ICU, n (%)	45.0 (24.9)	14.0 (26.9)	0.763
Platelet count, × 10^3/µl	235.1 (109.1)	184.4 (91.1)	0.136
Creatinine, mg/dl	1.1 (0.9)	1.5 (1.0)	0.140
BUN, mg/dl	15.9 (12.2)	30.6 (22.1)	**< 0.01**
Albumin, g/dl	3.1 (0.7)	2.6 (0.5)	**< 0.01**
Bilirubin, mg/dl	0.7 (0.4)	0.9 (0.7)	**< 0.01**
CPK, U/ml †	130.0 (63, 341)	251.0 (102, 662)	**0.011**
LDH, U/l	386.5 (281.1)	821.1 (613.0)	**< 0.01**
Hemoptysis, n (%)	21.0 (11.6)	18.0 (34.6)	**< 0.01**
Diabetes mellitus, n (%)	11.0 (6.1)	9.0 (17.3)	**0.011**
COPD, n (%)	10.0 (5.5)	2.0 (3.9)	0.629
Any comorbidity, n (%)	165.0 (91.2)	50.0 (96.2)	0.235

*Data are mean and standard deviation (SD) or number and percentage when indicated; †Represent median, 25th and 75th percentile; medians compared by Wilcoxon rank-sum test.
BMI: body mass index; ICU: intensive care unit; BUN: blood urea nitrogen; CPK: creatine phosphokinase; LDH: lactate dehydrogenase; COPD: chronic obstructive pulmonary disease.

Table 4. Sociodemographic and clinical characteristics by place of care

Variable*	Hospital ward n = 174	ICU n = 59	P value
Age, years	47.54 (14.1)	44.9 (12.7)	0.204
Male, n (%)	99.0 (56.9)	38.0 (64.4)	0.311
Residence in Mexico City, n (%)	115.0 (66.1)	44.0 (74.5)	0.226
BMI, kg/m^2	30.7 (6.9)	31.3 (6.8)	0.599
Inpatient days	13.2 (12.7)	34.9 (21.2)	< **0.01**
Active smoker, n (%)	45.0 (25.9)	24.0 (40.7)	< **0.01**
Influenza A H1N1, n (%)	67.0 (38.5)	32.0 (54.2)	< **0.05**
Mechanical ventilation, n (%)	61.0 (35.1)	57.0 (96.6)	< **0.01**
Platelet count, × 10^3/µl	223.80 (104.9)	224.4 (114.9)	0.970
Creatinine, mg/dl	1.1 (0.8)	1.3 (1.1)	0.212
BUN, mg/dl	17.8 (15.1)	23.1 (18.3)	< **0.05**
Albumin, g/dl	3.2 (0.7)	2.6 (0.5)	< **0.01**
Bilirubin, mg/dl	0.8 (0.6)	0.7 (0.3)	0.051
CPK, U/ml†	122.0 (64, 305)	328.0 (130, 753)	< **0.01**
LDH, U/l	425.2 (420.0)	649.1 (374.2)	< **0.01**
Hemoptysis, n (%)	28.0 (16.1)	11.0 (18.6)	0.650
Diabetes mellitus, n (%)	15.0 (8.6)	5.0 (8.5)	0.972
COPD, n (%)	12.0 (6.9)	0 (0)	< **0.05**
Any comorbidity, n (%)	164.0 (94.3)	51.0 (86.4)	0.052
Death, n (%)	38.0 (21.8)	14.0 (23.7)	0.763

*Data are mean and standard deviation (SD) or number and percentage when indicated. †Represent median and percentile 25th and 75th; medians compared with the Wilcoxon rank-sum test.
BMI: body mass index; BUN: blood urea nitrogen; CPK: creatinine phosphokinase; LDH: lactic dehydrogenase; COPD: chronic obstructive pulmonary disease.

Table 5. Logistic models for influenza A H1N1 (vs. influenza-like illness without viral confirmation)*

Variables	OR (95% CI)	P value
Death	0.84 (0.34-2.03)	0.69
Age, years	0.99 (0.97-1.01)	0.28
Masculine gender	1.42 (0.80-2.54)	0.23
BMI, kg/m^2	1.01 (0.98-1.04)	0.46
Inpatient days	1.01 (0.99-0.03)	0.51
Platelet count, × 10^3/µl	0.99 (0.994-0.999)	< **0.01**
CPK, U/ml	1 (1.00-1.001)	0.58
LDH, U/l	1 (1.00-1.00)	0.10
Mechanical ventilation	0.76 (0.32-1.79)	0.53
Cared for at ICU	1.36 (0.57-3.25)	0.48
Observations	217*	

*Models use patients with complete information in all variables. In this case, 93% (217/233) of patients had complete information in all the variables included in model.
OR: odds ratio; 95% CI: 95% confidence interval; BMI: body mass index; CPK: creatinine phosphokinase; LDH: lactic dehydrogenase; ICU: intensive care unit.

Table 6. Cox proportional hazard models of factors associated with death for all patients*

Variables	HR (95% CI)	P value
Cared for at ICU	0.27 (0.21-0.59)	< **0.01**
Hemoptysis	2.24 (1.08-4.64)	< **0.05**
Mechanical ventilation	6.23 (1.39-27.87)	< **0.05**
Influenza A H1N1	0.99 (0.50-1.08)	0.99
Age, years	1.04 (1.00-1.08)	< **0.05**
Masculine gender	4.72 (1.73-12.87)	< **0.01**
BMI, kg/m^2	1.04 (0.97-1.11)	0.231
Observations	210†	

*Model included lactic dehydrogenase, bilirubin levels, albumin, blood urea nitrogen, creatinine phosphokinase, platelet count, and diabetes mellitus, but no significant association was observed. †Models (n = 210/233 or 90%) include patients with complete information in all variables.
HR: hazard ratio; 95% CI: 95% confidence interval; ICU: intensive care unit; BMI: body mass index.

Figure 1. Crude survival function for ventilated patients stratifying by place of care.

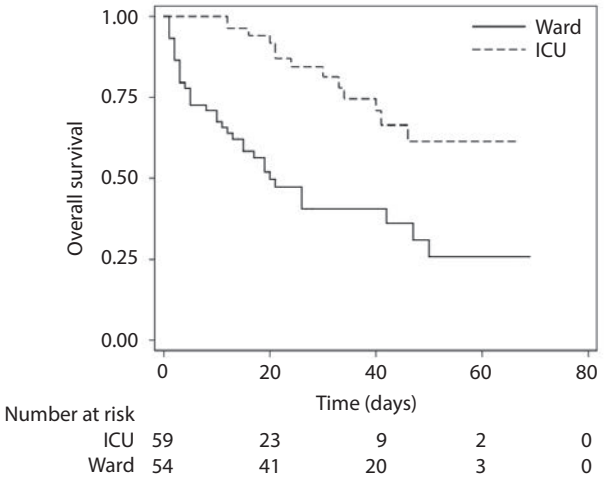


Figure 2. Adjusted survival function for all patients stratifying by place of care.
*Survival curves were estimated using coefficients from table 5, and mean values are reported in table 1 for continuous variables and considering a male patient with hemoptysis, diabetes mellitus, and mechanical ventilation

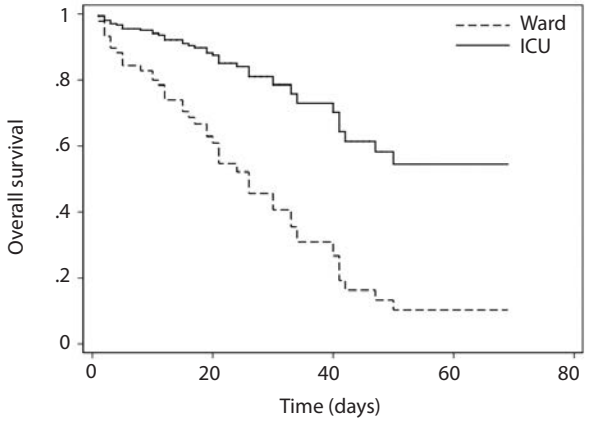
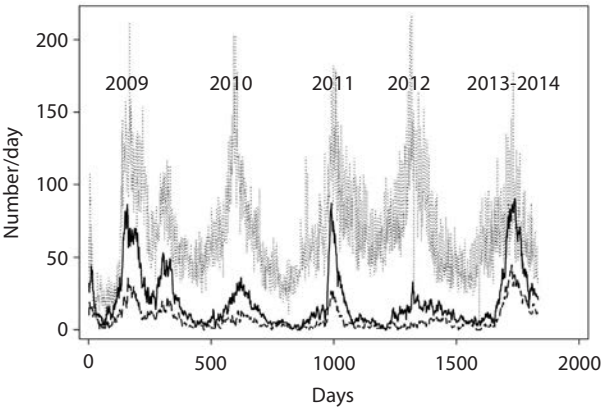


Figure 3. Historical number of emergency room consultations (upper graph), hospital admissions (middle continuous line), and patients on mechanical ventilation (lower line) with influenza-like illness from INER as a function of time from April 23, 2009, during the influenza pandemic when surveillance started.
We observed an annual outbreak of influenza-like illness, although years with severe cases alternated with milder years. The incidence in the 2013-2014 season was as high as that in the second pandemic peak.



DISCUSSION

The influenza outbreak in Mexico City in the winter of 2013-2014 was predominantly due to influenza A H1N1, as it was in 2009 and 2011, and tended to be more severe than the outbreaks of 2012 and 2010, when influenza virus subtype H3N2 variants predominated. During 2013 we observed a severe outbreak with a significant number of individuals requiring hospitalization (with a maximum of 90 out of 180 hospital beds available) and mechanical ventilation (maximum of 45 patients simultaneously), and with a number of hospital deaths similar to what we had seen during the 2009 pandemic (Fig. 3). Nearly all hospitalized individuals (96%) lacked the seasonal influenza vaccine; unfortunately, vaccination rates in Mexico are low, even in individuals with special risk factors for influenza complications. Again, individuals with ILI, similar to patients with confirmed influenza in most of the features studied although with a slightly milder disease, comprised about 50% of patients cared for[6]. A negative RT-PCR may have been due to several factors, such as delay in sampling (although with lung damage already in course), an inadequate sample, or upper airway sampling instead of samples obtained from lung secretions. In general, during an influenza outbreak, individuals with ILI should be considered as having influenza and be treated without delay with oseltamivir (Tamiflu®) or equivalent drugs accordingly, as we did in our hospital. This is important since estimates of influenza severity are often lower because counts are based mostly on confirmed cases. Recently, however, official reports from the Ministry of Health in Mexico included several indicators based on the clinical definition of cases without requiring viral confirmation.

Hemoptysis has only been described as a predictor of hospitalization or care at the ICU[7,8]; however, in our patients its presence increased the risk of death, even though we had a lower prevalence (16%) than in other studies (30%)[7].

Nearly all patients were obese and had a high number of comorbidities, both risk factors for hospitalization and death from influenza[7-9]. The latter suggest that patients with chronic diseases were particularly vulnerable in this outbreak. Patients with the described characteristics should be immunized. By focusing on populations with chronic diseases, we may be able to prevent cases, hospitalization, and mortality from influenza.

Patients admitted to the ICU had a better outcome compared with those cared for in regular hospital wards, even after adjusting for mechanical ventilation and disease severity. In our hospital, during severe outbreaks, mechanical ventilation in patients with respiratory failure is initiated in regular wards when the ICU is saturated, which is life-saving in the short term but, as seen in this outbreak, prognosis may be worse for those not admitted to the ICU. This was likely due to a shortage of trained personnel for the surveillance of critical patients 24 hours a day and during weekends, as important as the lack of sufficient mechanical ventilators outside the ICU. In reference hospitals for respiratory diseases, an increase in requirements for ICU beds is observed during the influenza season, and having additional personnel trained in ICU is very important for improving care in the regular wards or for increasing the permanent ICU facilities.

Influenza remains a significant health risk and is preventable; however, immunization must cover a higher proportion of the population and the vaccine should be administered prior to the winter season. Influenza H1N1 has become a seasonal strain and continues to cause severe outbreaks, possibly because small variants of the virus may still be present in several outbreaks, such as those reported[10], alternating in Mexico in winter with a predominance of H3N2, the variant causing more severe outbreaks in the USA.

Our study has limitations derived from the information available in clinical charts obtained during an important influenza outbreak in a referral hospital for respiratory diseases. Outcomes were worse than in population or regional hospitals outbreaks, representing the most severe portion of cases. However, this does not imply that case mortality was similar to that in general hospitals or even less in outpatients.

In conclusion, Mexico continues to experience winter outbreaks with influenza A H1N1, likely due to incomplete vaccine coverage and small viral variants. During these outbreaks, the requirement for ICU beds and appropriate personnel increases. An alternative would be to open, for influenza, fully equipped and staffed ICU beds in other hospitals.

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PERMANYER

Rev Inves Clin. 2016;68:154-62 ORIGINAL ARTICLE

G80A Single Nucleotide Polymorphism in Reduced Folate Carrier-1 Gene in a Mexican Population and its Impact on Survival in Patients with Acute Lymphoblastic Leukemia

Myrna Candelaria[1,2]*, Juan Ojeda[2], Olga Gutiérrez-Hernández[1], Lucia Taja-Chayeb[1], Silvia Vidal-Milián[1] and Alfonso Dueñas-González[3]

[1]Pharmacogenetics Laboratory; [2]Hematology Department; [3]Instituto de Investigaciones Biomédicas, UNAM and Instituto Nacional de Cancerología, Mexico City, Mexico

ABSTRACT

Background: Hyper-CVAD is the treatment for patients with acute lymphoblastic leukemia in our institution. **Objective:** To evaluate the impact of single nucleotide polymorphisms at genes associated with methotrexate metabolism on survival. **Methods:** The presence of the single nucleotide polymorphisms G80A at *reduced folate carrier-1* gene and C677T in the *methylenetetrahydrofolate reductase* gene was determined by denaturing high performance liquid chromatography and validated by sequencing. Both single nucleotide polymorphisms were evaluated in 71 healthy donors and in an exploratory pilot trial with acute lymphoblastic leukemia patients to determine the influence of these single nucleotide polymorphisms on clinical outcome. Clinical characteristics, response, and outcome were registered. A Cox regression analysis was done to evaluate factors influencing response and overall survival. **Results:** There were no differences in the frequency of single nucleotide polymorphisms between volunteers and acute lymphoblastic leukemia patients according to the Hardy-Weinberg test. Sensitivity and specificity were 72 and 91% for the G80A, and 64 and 75% for the C677T, respectively. The multivariate analysis showed that the T-immunophenotype and the presence of single nucleotide polymorphism G80A *reduced folate carrier-1* were associated with a shorter relapse-free survival and overall survival. **Conclusions:** The presence of G80A single nucleotide polymorphism at reduced folate carrier-1 gene in acute lymphoblastic leukemia patients was associated with a poorer prognosis. (REV INVES CLIN. 2016;68:154-62)

Key words: RFC1 gene. MTHFR4 gene. SNP. Leukemia. Methotrexate.

Corresponding author:
*Myrna Candelaria
Pharmacogenetics Laboratory
Instituto Nacional de Cancerología
Av. San Fernando, 22
Col. Sección XVI
C.P. 14080, Ciudad de México, Mexico
E-mail: candelariamyrna@gmail.com

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) in adults remains a significant treatment challenge, in contrast with pediatric ALL where considerable improvements in long-term survival and even cure have been achieved over the last 30 years. Long-term overall survival for this group remains relatively poor, between 20 and 40%. Current research in adult ALL has mainly focused in optimizing the use of cytotoxic drugs. In this regard, pharmacogenetics is of considerable interest, particularly for methotrexate (MTX), a folate analog drug that is essential in regimens for ALL[1-5].

The influx of MTX into cells depends on the reduced folate carrier protein (RFC), which efficiently transports folates and MTX into cells and, once inside, is converted into methotrexate polyglutamates (MTXPG) by the enzyme folyl-polyglutamate synthetase[6,7]. The MTX and its polyglutamates affect intracellular folate metabolism by inhibiting dihydrofolate reductase and thymidylate synthase. Therefore, the inhibition of DNA biosynthesis induced by MTX is multifactorial, including both partial depletion of reduced folates and direct inhibition of folate-dependent enzymes. The effectiveness of MTX depends on its concentration and retention in cells.

The *RFC1* gene is located on the long arm of chromosome 21 (21q22.2-22.3) and encodes a membrane protein called reduced folate carrier[8]. The RFC1 single nucleotide polymorphism (SNP) 80G>A (rs1051266) leads to the substitution of guanine for adenine in the 80th position, which results in the substitution of arginine for histidine at the residue 27 in the structure of the protein[9]. Chan, et al. showed that this change results in decreased receptor affinity and variations in the transmembrane transport of folic acid antimetabolites. In *ex vivo* studies, the folate concentrations in serum were higher in the 80AA genotype than the allele G variant: 19 vs. 15 mmol/l, respectively[10]. Banerjee, et al. analyzed the relationship between the *RFC1* G80A polymorphism and the risk of relapse of ALL in children. They found that in 204 ALL patients studied, the *RFC1* 80AA variant was associated with higher serum concentrations of MTX[11], which has also been observed by other authors[12].

On the other hand, the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folic acid cycle[12]. A common genetic polymorphism in the *MTHFR* gene results from a C®T substitution. Individuals with the T/T genotype commonly have elevations in plasmatic homocysteine and differences in response to folic acid supplementation compared with normal (C/C) or heterozygous (C/T) genotypes. This polymorphism is highly prevalent in the Mexican population[13-15], particularly among Nahua and Mixtec groups. However, in other regions of the country, such as the north, the prevalence is similar to Caucasian regions[14]. In addition, this SNP may influence the therapeutic response to antifolate drugs such as MTX. The frequencies of the C677T allelic variants differ according to ethnicity. In Europe, 8-20% of the Caucasian population is homozygous for the 677T allele and almost 40% is heterozygous[2,16]. Although some authors[12,17] have described the influence of this SNP on survival and toxicity in patients with ALL whose treatment includes MTX, its role is still unclear. Thus, in this work we evaluated the feasibility of using denaturing high-performance liquid chromatography (DHPLC) to determine *RFC1* and *MTHFR* gene polymorphisms as well as to correlate these SNPs with the toxicity and outcome in adults with ALL receiving MTX as part of the hyper-CVAD regimen.

MATERIAL AND METHODS

Study population

Patients

A total of 31 adult patients with ALL were included from January 2011 to December 2012 at the National Cancer Institute (INCan) in Mexico in this prospective, exploratory pilot trial to assess the influence of the *RFC1 G80A and MTHFR C677T* SNPs on response and overall survival.

Healthy individuals

Blood samples from 71 consecutive healthy, volunteer blood donors were obtained by venous puncture from the arm; none of the donors were related to our ALL patients.

Inclusion criteria

The patients were untreated, Mexican, older than 15 years of age, and with normal renal and hepatic function. After inclusion and blood sample collection, patients began treatment with hyper-CVAD. Patients with a history of hypersensitivity to MTX or to any of the other drugs included in the hyper-CVAD regimen were excluded. This protocol was approved by the IRB Committee and registered at www.clinicaltrials.gov (Identifier # NCT01307241). Healthy individuals and patients signed an informed consent.

Baseline clinical and pathological characteristics were recorded. Patients were assessed for response using the International Working Group Criteria for acute leukemia[18]. Overall survival (OS) was defined as the time since diagnosis until death or date of the last visit. Relapse-free survival (RFS) was defined as the time since remission was achieved until relapse was documented.

Laboratory procedures

DNA extraction

Genomic DNA was obtained using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA was quantified in a NanoDrop (Applied Biosystems) and stored at −20 °C.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was performed using the following oligonucleotides: Forward: 5'-AGT GTCACCTTCGTCCC-3' Reverse: 5'-TCCCGCGTGAAG TTCTTG-3' for the *RFC1* gene, and Forward: 5'- GG AGCTTTGAGGCTGACCTGAA-3' Reverse: 5'-AGGAC GGTGCGGTGAGAGTG-3' for the *MTHFR* gene. The PCR was performed in a total volume of 25 µl containing 100 ng genomic DNA, 1 µmol/l oligonucleotides (forward and reverse), 200 µM dNTPs (Fermentas Life Sciences, USA), 0.25 U optimase polymerase enzyme (Applied Biosystems), and PCR 1 x buffer (15 mM MgCl2, Perkin Elmer, Foster City, CA). The PCR was performed on a GeneAmp® 9700 thermal cycler (Applied Biosystems) using an initial denaturation step at 94 °C for five minutes, followed by 40 cycles at 94 °C for 30 seconds, annealing (60 °C for *RFC1* and 55 °C for *MTHFR*) for 30 seconds, and 72 °C for 30 seconds; a final extension was performed at 72 °C for seven minutes. Products were electrophoresed in 2% agarose gels.

Denaturing high-performance liquid chromatography analysis

The PCR products were denatured at 95 °C during 10 minutes and were cooled until 25 °C, decreasing 2 °C/minute to allow for homo- or heteroduplex formation (Transgenomics, Inc; San José, CA) according to conditions determined by DHPLC software. Heterozygous chromatograms were identified by visual analysis and compared with the wild type. Homoduplex cases, which have a single peak chromatogram as wild type, were mixed with known wild type DNA (previously sequenced), to allow heteroduplex formation. Results were reported as wild type (wt) or polymorphic.

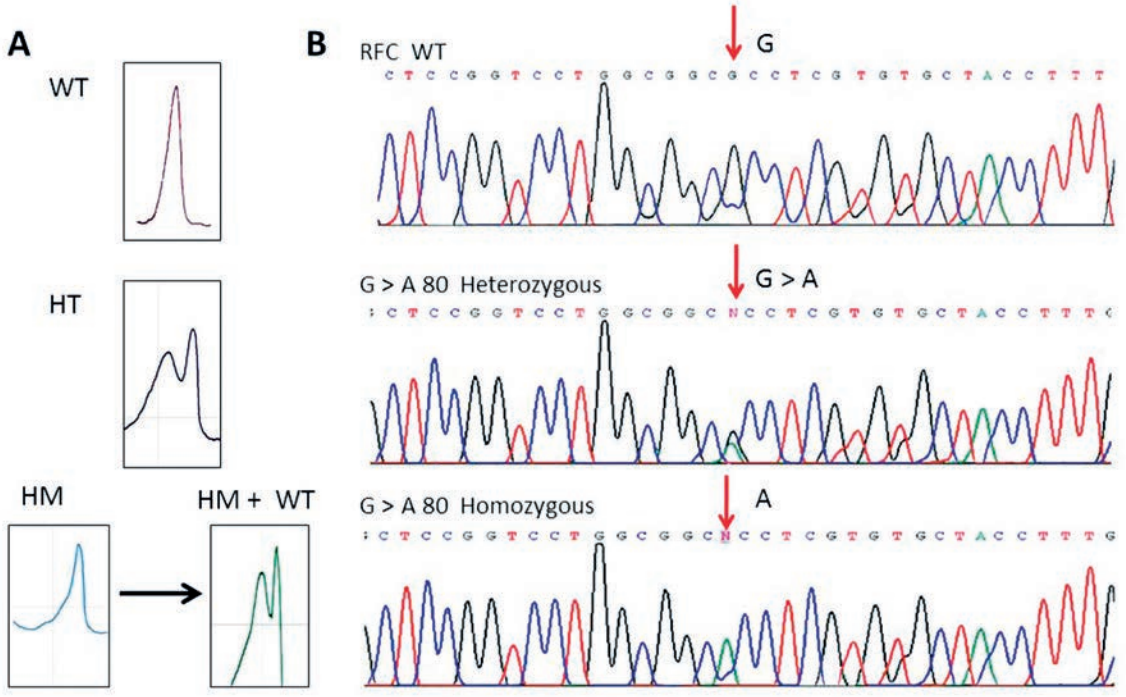
Sequencing

Seventy-one samples from healthy individuals were sequenced (gold standard), regardless of the results of DHPLC analysis from at least two independent amplification products. The ALL samples were amplified and analyzed by DHPLC. The PCR amplicons were purified using isopropanol precipitation, diluted and cycle-sequenced using a BigDye® Terminator kit v3.1 (ABI, Foster City, CA) according to manufacturer instructions in an ABI Prism® 3100 genetic analyzer. Electropherograms were analyzed in both sense and antisense directions.

Statistical analysis

The SNP frequencies were compared between healthy individuals and patients using the Hardy-Weinberg test. The results of DHPLC and Sanger sequencing (gold standard) were analyzed to determine sensitivity: (true positive/ [true positive + false negative]), and specificity: (true negative/ [false positive + true negative]). Survival curves were done by Kaplan-Meier method and compared by log rank test. Cox regression analysis was done to evaluate factors influencing response and overall survival.

Figure 1. RFC gene analysis by **A:** DHPLC showing a single peak for the homozygous wildtype or mutant, whereas multiple peaks were indicative of heteroduplexes containing G>A 80 SNP. When an homozygous sample mixed with wildtype DNA showed a multiple peak profile, indicated the presence of the SNP homozygously. **B:** Sanger sequence confirming DHPLC finding.



RESULTS

Healthy individuals

Blood samples were collected from 71 healthy donors. The mean age was 30.6 years (range 19-57) and 48% were male.

Sensitivity and specificity of DHPLC in healthy individuals: PCR fragments of *MTHFR* and *RFC*1 genes were subjected to DHPLC, and the conditions for analysis (gradient and temperature) were optimized for each fragment to yield characteristic and reproducible profiles. As shown in figure 1, samples with *wt RFC1* sequence eluted as a single peak, whereas multiple peaks were indicative of heteroduplex containing 80G>A SNP. Homozygous polymorphic also showed a single peak, and an elution mixed with a *wt* sample was required to demonstrate this status. These results were confirmed by Sanger sequencing. Likewise, *MTHFR wt* showed a single peak and C>T 677 SNP was documented with multiple peaks (Fig. 2). As shown in table 1, regarding the *MTHFR* SNP from the 71 samples from healthy individuals, 38 showed a polymorphic chromatogram, which was confirmed by sequencing in 33 (true positive); five cases were recorded as false positive (polymorphic chromatogram by DHPLC, but the SNP discarded by sequencing). Eighteen cases had a *wt* chromatogram, but sequencing showed the SNP (false negative), whereas 15 were true negative (*wt* chromatogram and no SNP by sequencing). The calculated sensitivity and specificity were 64 and 75%, respectively, for *MTHFR*, and 72 and 91% for the *RFC1* gene, respectively (Table 1).

Patients

The clinical and pathological characteristics of ALL patients are shown in table 2. The mean age was 26 years (range 16-64); male:female distribution was 17:14. Most (74%) had the common B subtype and six (20%) were positive for the Philadelphia chromosome.

After analyzing DHPLC chromatograms for these polymorphisms, the frequency of the *RFC1* G80A was 50.7% in healthy individuals and 40.7% among leukemia patients. All were heterozygous. Regarding the *MTHFR* C677T SNP, the frequency was 52.7 and

Figure 2. MTHFR gene analysis by **A:** DHPLC showing a single peak for the homozygous wildtype or mutant, whereas multiple peaks were indicative of heteroduplexes containing C > T677 SNP. When an homozygous sample mixed with wildtype DNA showed a multiple peak profile, indicated the presence of the SNP homozygously. **B:** Sanger sequence confirming DHPLC finding.

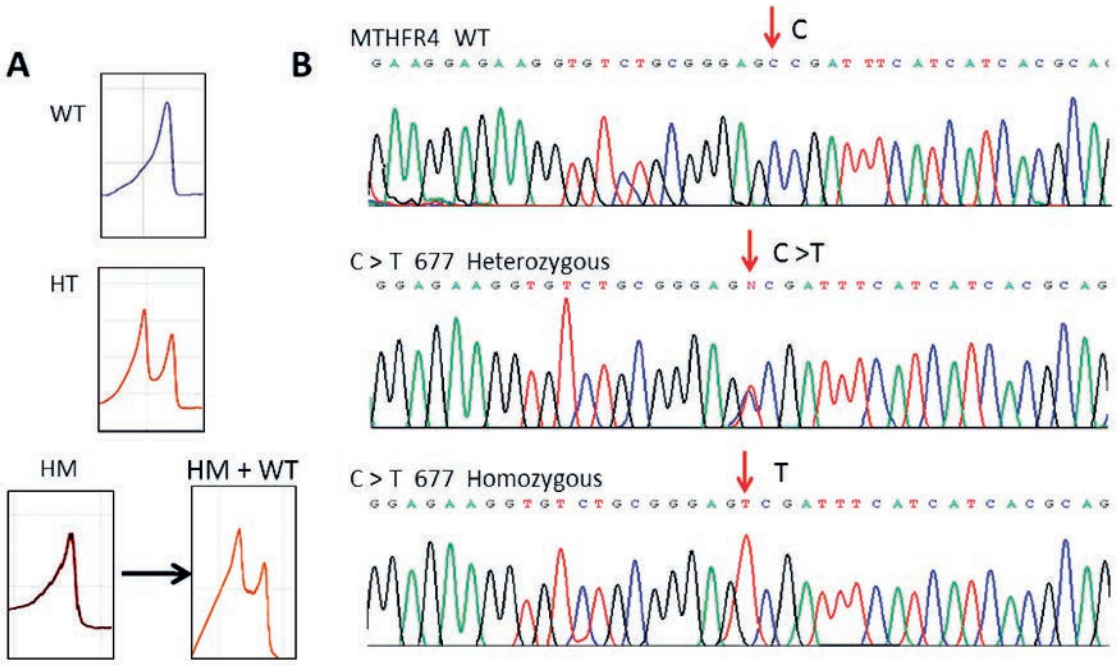


Table 1. Sensitivity and specificity of the denaturing high performance liquid chromatography technique for *methylenetetrahydrofolate reductase* and *reduced folate carrier-1* genes in 71 healthy blood donors

	MTHFR	*RFC1*
True positive (n)	33	34
False positive (n)	5	2
False negative (n)	18	13
True negative (n)	15	22
Total (n)	71	71
Sensitivity (%)	64	72
Specificity (%)	75	91

MTHFR: methylenetetrahydrofolate reductase; RFC: reduced folate carrier.

Table 2. Clinical characteristics of acute lymphoblastic leukemia patients

	Patients
	n = 31
Mean age, years (range)	26 (16-64)
Male:female	17:14
ALL classification (n)	
Pre-B	2
Pro-B	4
Common B	23
Mature B	1
T	1
Cytogenetic analysis (n)	
Philadelphia positive	6
Normal	25
CNS infiltration at diagnosis, n (%)	3 (9.6)

ALL: acute lymphoblastic leukemia; CNS: central nervous system.

72.4%, respectively. There was only one homozygous patient in the leukemia population. After using Hardy-Weinberg test, no statistically significant difference was found with that expected for the analyzed population.

Toxicity

As expected, all patients had grade 4 myelosuppression. No grade 3-4 liver toxicities were documented, and there were no differences in toxicity rates between patients having wild type or any of the SNPs.

In accordance with the international standard criteria, complete response was achieved with the hyper-CVAD regimen in 25 cases (80.6%), and partial response in the remaining six cases. The three-year RFS and OS were 42 and 22%, respectively. Median RFS was

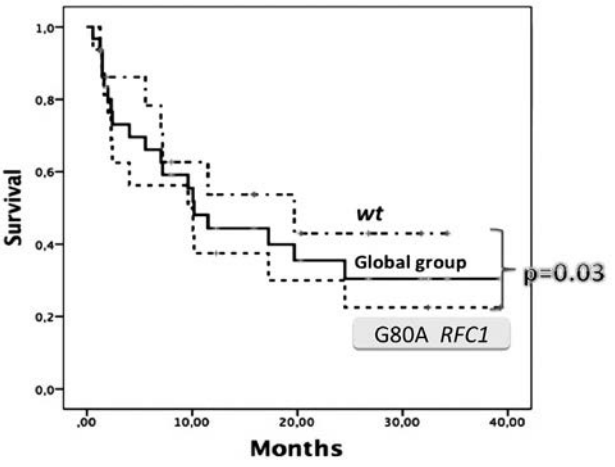
Table 3. Cox regression analysis of factors influencing overall survival and relapse-free survival in acute lymphoblastic leukemia patients

Variable	Overall survival			Relapse-free survival		
	HR	95% confidence interval	p value	HR	95% confidence interval	p value
Gender	1.43	0.88-7.30	0.083	2.6	1.1-205.8	0.930
Age	1.1	1.06-1.94	0.100	1.5	0.56-1.69	0.940
MTHFR C677T SNP	0.68	0.42-1.40	0.183	0.74	0.52-1.10	0.379
RFC1 G80A SNP	1.5	1.2-1.7	0.039	3.8	1.39-10.00	0.0463
Philadelphia chromosome	1.8	0.4-7.0	0.483	0.9	0.11-7.20	0.426
Immunophenotype	1.9	1.2-3.7	0.008	1.1	0.32-2.70	0.039

MTHFR: methylenetetrahydrofolate reductase; RFC: reduced folate carrier; SNP: single nucleotide polymorphism.

1.3 years (95% CI: 0.92-1.8). Median OS was 1.7 years (95% CI: 1.4-2.6). Cox regression analysis was done to evaluate prognostic factors, including age, cytogenetic analysis, immunophenotype of ALL, and presence or absence of any of the SNPs. The presence of 80*G>A RFC* SNP and T-cell immunophenotype was associated with decreased RFS and OS (Table 3 and Fig. 3).

Figure 3. Overall Surviva.



DISCUSSION

The pharmacogenetic approach in chemotherapy constitutes a research field developed to optimize drug doses and schedules for either increasing response rate or reducing toxicity. Hyper-CVAD is considered a standard of care for patients with ALL in many centers worldwide, including our institution[19]. Within this schema, methotrexate constitutes a key drug for ALL treatment. The results of this work add information on the field of pharmacogenetic regarding the impact of the *RFC1* 80G>A SNP on the efficacy of MTX when used in the context of hyper-CVAD for adult ALL patients. Although many other factors related to clinicopathological characteristics of ALL patients were not taken into account, as well as other pharmacogenetic factors that may modulate the effect of the drugs other than MTX in the hyper-CVAD regimen, Cox regression analysis clearly suggest that the *RFC1* 80G>A SNP may help to optimize the dose of MTX when used within this regimen, although this should be confirmed in further studies that include a larger sample of patients.

The *RFC* 80G>A polymorphism has been widely studied in diverse populations searching for associations with increased leukemia risk and with the clinical course (toxicity and prognosis) in leukemia patients receiving MTX. Data from the 71 healthy individuals in our study suggest that our populations have an increased frequency of this SNP (50.7%), similar to that reported among healthy French individuals (36%), whereas in a British population the frequency was 41%[5]. A higher frequency (71%) has been found in a Jordanian female population with rheumatoid arthritis[20]. In this respect, some authors[5] have suggested a relationship between the *RFC1* 80G>A polymorphism and a 2.1-fold increased risk of ALL. Furthermore, Huang, et al.[21], after a stratified analysis by ethnicity, recently demonstrated that the association became more prominent among Caucasians (GA vs. GG: OR: 1.28; 95% CI: 1.12-1.45; $p < 0.001$). In contrast with these results, our findings do not support this association, since there was a trend for a higher frequency of the *RFC1* 80G>A polymorphism in the healthy population compared with ALL patients (50.7 vs. 40.7%, respectively).

Regarding the association between this polymorphism and shorter RFS and OS, there are experimental data supporting it, although there is also contradictory information.

In contrast to our results, in a study including 70 children with ALL, an association was found between the polymorphism and a lower risk of relapse ($p < 0.05$): in patients with the G/A genotype it was 3.97 (95% CI: 1.12-14.06) compared with carriers of the A/A genotype (*wt*) who had a higher probability of relapse (7.84; 95% CI: 1.66-37.10)[22]. In our patients, those with the G/A genotype had a 3.8 HR for relapse.

Other authors[23,24] found that extra copies of chromosome 21, where the *RFC1* gene is located, were associated with increased expression of mRNA and reflect elevated capacities for MTX transport. Our findings are supported by a decreased functional status of this SNP, showing a lower intracellular folate concentration in patients with the *RFC* 80G>A polymorphism, as has been confirmed by Yates and Lucock[25], although other manifestations of dysfunction of this 80G>A polymorphism have not been documented, such as a higher risk of neural tube defects or colorectal cancer[25-29]. In this regard, Chan, et al. showed that this change results in decreased receptor affinity and variations in the transmembrane transport of folic acid antimetabolites. In *ex vivo* studies, the folate concentrations in serum were higher in the 80AA genotype than the allele G variant: 19 vs. 15 mmol/l, respectively[10]. Banerjee, et al. analyzed the relationship between the *RFC1* G80A polymorphism and the risk of relapse in 204 children with ALL and also found that the *RFC1* 80AA variant was associated with a higher serum concentration of MTX[11], which has also been observed by other authors[12]. However, Whetstine, et al.[30] reported that the change of the strongly basic amino acid arginine to histidine, which is a weak base, in a region of the carrier documented to influence folate substrate binding and rates of uptake, might be expected to alter *RFC* transport properties. Nevertheless, by directly comparing the transport properties of Arg27 and His27-hRFC in stable K562 transfectants, no significant differences in the uptake rates of MTX were observed, and only minor differences were calculated in the relative affinities for an assortment of transport substrates. Collectively, these data strongly argue for a lack of major functional differences between the Arg27- and His27-RFCs for reduced folate cofactors and for various antifolates used in cancer chemotherapy, including MTX. On the other hand, researchers in Japan have shown increased liver toxicity in homozygous 80AA, and increased side effects in the form of severe vomiting in the 80GG group homozygotes[31,32]. We found no differences regarding toxicity dependent on this SNP, which may be explained because we had only one homozygous patient (80GG).

In contrast with the *RFC1* 80G>A polymorphism, in our study, the SNP 677C>T at the *MTHFR* gene did not influence RFS or OS. Similar results have been obtained by other authors. A multicenter study from Poland published in 2006[32], in which *MTHFR, TPMT, GSTT1, GSTM1, GSTP1,* and *TS* polymorphisms were determined, showed a significant relationship between genotype 677C>T and an increased death rate (OR: 4.09; 95% CI; $p = 0.028$). Eight of the 31 (26%) patients whose death occurred during treatment had homozygous 677TT, but there was no association with the genotype 677C>T. In line with our results, Deus, et al.[33] also reported that *G80A* polymorphism influenced the survival of pediatric patients with ALL, but neither *G677T* nor *A1298C* in *MTHFR* gene had an effect on survival. In Mexico, the presence of *MTHFR* has been evaluated in different regions of our country[13-15,34] and strong differences in frequencies of C677T polymorphism were documented, being higher among Nahua and Mixtec groups compared with Mestizos[13,14]. Additionally, Ruiz-Argüelles, et al.[34] evaluated the risk of mucosal damage in 28 patients with ALL treated with MTX and, in accord with our findings, they concluded that there was no significant association with mucositis at the gene or at the genotype level. They also postulated that the risk of higher MTX toxicity in patients with decreased MTHFR activity could be neutralized by the normally folate-rich diet in Mexico.

Results of our study demonstrate by multivariate analysis that the G80A SNP is associated with shorter RFS and OS. However, because of the small sample size and unknown clinical data, it is possible that results were due to random effects. In addition, there are more pharmacogenetic variations of genes implicated not only in the metabolism of MTX, but of the other drugs in the hyper-CVAD regimen that were not studied. Therefore, it is possible that their interactions could explain the results observed.

Nevertheless, our results regarding the influence of the G80A polymorphism cannot be underestimated since other studies[1,5,7] have also found this association in leukemia patients receiving MTX. Further studies are needed to establish the value of this pharmacogenetic marker in the optimization of leukemia treatment with MTX.

Finally, our study to determine the feasibility of using DHPLC as a routine method to determine the SNPs here studied and others[35-37], suggest that it can be of value, despite the fact that the sensitivity and specificity we obtained were not so high. However, a number of papers have documented the excellent sensitivity and specificity of DHPLC in detecting mutations[38]. For instance, O'Donovan, et al. have reported a sensitivity and specificity of 100% for detecting mutations in exon H of the Factor IX and exon 16 of the neurofibromatosis type 1 gene[39]. However, under a single hybridization condition, some probes do not have optimal hybridization kinetics and therefore markers located near such sequence contexts cannot be detected. In addition, it is difficult to identify markers that are present as heterozygotes, as well as markers located close to other polymorphisms. As a result, most studies reach a sensitivity of 85-95%, with specificity in some cases as low as 55%[40]. In this regard, the relatively lower sensitivity and specificity here found can potentially be increased by testing different conditions. Regarding the polymorphisms studied, these are preliminary results and require confirmation. However, we could suggest that patients with G80A may be treated with other regimens without methotrexate, or within clinical trials.

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