UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

BIOMEDICINA

IMPLICACIONES CLÍNICAS Y FUNCIONALES DE LA EXPRESIÓN DE miRNAs CIRCULANTES EN PACIENTES CON FORMAS GRAVES DE LA INFECCIÓN POR EL VIRUS A/H1N1

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

MORÁN HERNÁNDEZ JUAN

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UNAM
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Ciencias Biológicas

COORDINACIÓN

Dr. Isidro Ávila Martínez
Director General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que el Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día 11 de enero de 2016, aprobó el jurado para la presentación del examen para obtener el grado de **DOCTOR EN CIENCIAS** del alumno **MORÁN HERNÁNDEZ JUAN** con número de cuenta **510007898** con la tesis titulada **"IMPLICACIONES CLÍNICAS Y FUNCIONALES DE LA EXPRESIÓN DE MIRNAS CIRCULANTES EN PACIENTES CON FORMAS GRAVES DE LA INFECCIÓN POR EL VIRUS A/H1N1"**, realizada bajo la dirección del **DR. JOAQUIN ALEJANDRO ZÚÑIGA RAMOS:**

Presidente:	DR. GILBERTO VARGAS ALARCÓN
Vocal:	DR. FEDERICO ÁVILA MORENO
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Suplente:	DR. LUIS PADILLA NORIEGA

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, D.F., a 24 de febrero de 2016

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

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Quien no conoce nada, no ama nada. Quien no puede hacer nada, no comprende nada. Quien nada comprende, nada vale. Pero quien comprende también ama, observa, ve... Cuanto mayor es el conocimiento inherente a una cosa, más grande es el amor … Quien cree que todas las frutas maduran al mismo tiempo que las frutillas nada sabe acerca de las uvas."
Paracelso

INDICE

LISTA DE TABLAS Y FIGURAS

LISTA DE SIGLAS Y ABREVIATURAS EN INGLÉS

pdm A/H1N1: pandemic A/H1N1 virus.
miRNAs: micro-RNAs
IL-1RA: **I**nter**l**eukin-**1** **R**eceptor **A**ntagonist
IL-2: **I**nter**l**eukin-**2**
IL-6: **I**nte**l**eukin-**6**
CXCL8: Chemokine (**C**-**X**-**C** motif) **L**igand 8
IFN-γ: **I**nter**f**ero**n** gamma
CXCL-10: Chemokine (**C**-**X**-**C** motif) **L**igand 10
G-CSF: **G**ranulocyte **C**olony **S**timulating **F**actor
ISAV: **I**nfectious **S**almon **A**nemia **V**irus
HA: **H**em**a**gglutinin
NA: **N**eur**a**minidase
M2: **M**atrix **2**
vRNP: **v**iral **R**ibo**n**ucleo**p**rotein complex
HEF: **H**emagglutinin-**E**sterase-**F**usion
CM2: **M**inor Envelope Protein
PB1: **P**olymerase **B**asic **1**
NjM: **N**eighbor-**j**oining **M**ethod
PB2: **P**olymerase **B**asic **2**
PA: **P**olymerase **A**cid
NP: **N**ucleo**p**rotein
HE: **H**emagglutinin **E**sterasa
Neu4,5Ac2: **4**-*O*-**5** **N** - **ac**etylneuroaminic acid
F: Viral **F**usion Protein
M: **M**atrix protein
ORF: **O**pen **R**eading **F**rame
M1: **M**atrix **1**
NS1: **N**on**s**tructural protein **1** (interferon-antagonist)
NS2: **N**on**s**tructural protein **2**
NEP: **N**uclear **E**xport **P**rotein
HEF: **H**emagglutinin-**E**sterase-**F**usion
ML: Non-essential Accessory Protein
HRP: **H**eterotrimeric **R**NA-dependent RNA **P**olymerase
Gln: **Gl**utami**n**e
Gly: **Gly**cine
Leu: **Leu**cine
Ser: **Ser**ine
Asp: **Asp**artic Acid or **asp**artate

Arg: **Arg**inine
PC6: **P**roprotein **C**onvertase
FURIN: **furin**, paired basic amino acid cleaving enzyme
MDCK: **M**adin-**D**arby **C**anine **K**idney cells
pre-miRNAs: miRNA **pre**cursor
mRNA: **m**essenger **RNA**
AGO: **A**r**go**naute
RISC: **R**NA-**I**nduced **S**ilencing **C**omplex
pri-miRNA: **pri**mary miRNA
DGCR8: **D**i**G**eorge Syndrome **C**ritical **R**egion **G**ene **8**
TRBP: **T**ransactivation-**R**esponse RNA-**B**inding **P**rotein
HSC-70-HSP-90: **H**eat **S**hock **C**ognate**-70** – **H**eat **S**hock **P**rotein **90**
siRNAs: **s**mall **i**nterfering **RNAs**
3'UTR: 3' **Unt**ranslated **Región**
ARDS: **A**cute **R**espiratory **D**istress **S**yndrome
HAI: **H**em**a**gglutination **I**nhibition test
r-RT-PCR: **r**eal time **R**everse **T**ranscriptase **P**olymerase **C**hain **R**eaction
CDC: **C**enters for **D**iseases **C**ontrol and Prevention
PBS: **P**hosphate-**B**uffered **S**alines
TLDAs: Taqman Low Density Arrays
qRT-PCR: **q**uantitative **R**everse **T**ranscriptase **P**olymerase **C**hain **R**eaction
cDNA: complementary DNA
$TCID_{50}$: **T**issue **C**ulture **I**nfection **D**ose 50%
KEGG**: K**yoto **E**ncyclopedia of **G**enes and **G**enomes
C_T: **C**ycle **T**hreshold
IL-1b: **I**nterleukin **1** beta
IL-4: **I**nterleukin 4
IL-5: **I**nterleukin 5
IL-7: **I**nterleukin 7
IL-10: **I**nterleukin 10
IL-12: **I**nterleukin 12
IL-13: **I**nterleukin 13
IL-15: **I**nterleukin 15
IL-17: **I**nterleukin 17
TNF-a: **T**umor **N**ecrosis **F**actor **a**lpha
CCL2: **C**-**C** motif Chemokine **L**igand 2
CCL3: **C**-**C** motif Chemokine **Ligand** 3
CCL5: **C**-**C** motif Chemokine **L**igand 5
GM-CSF: **G**ranulocyte-**M**acrophage **C**olony-**S**timulating **F**actor
PDGF-bb: **P**latelet-**D**erived **G**rowth **F**actor bb
FGF: **F**ibroblast **G**rowth **F**actor
VEGF: **V**ascular **E**ndothelial **G**rowth **F**actor
ANOVA: **A**nalysis of **V**ariance
BMI: **B**ody **M**ass **I**ndex
PaO2/FiO2: Arterial Oxygen **Pa**rtial Pressure/**F**ractional **i**nspired **O**xygen
TLRs: **T**oll **L**ike **R**eceptors
SIRS: **S**ystemic **I**nflammatory **R**esponse **S**yndrome
SOFA: **S**equential **O**rgan **F**ailure **A**ssessment
DENV: **Den**gue **V**irus
DHF: **D**engue **H**emorrhagic **F**ever
DF: **D**engue **F**ever
SOCS1: **S**uppressors **o**f **C**ytokine **S**ignaling-1
JAK/STAT: **J**anus **K**inases/ **S**ignal **T**ransducer and **A**ctivator of **T**ranscription
GAS: γ–**A**ctivated **S**equence
Egfl7: **EGF**-**l**ike domain multiple **7**
pDCs: **p**lasmacytoid **D**endritic **C**ell**s**

RESUMEN.

Introducción. En los pacientes infectados con el virus de influenza pandémica (pdm A/H1N1) la sobreproducción de citocinas pro-inflamatorias y de quimiocinas esta frecuentemente asociada con las manifestaciones clínicas severas de la enfermedad. Los micro-RNAs (miRNAs) son RNAs pequeños no codificantes que regulan la expresión génica a nivel post-transcripcional y que en distintas condiciones pro-inflamatorias son potenciales biomarcadores y blancos terapéuticos. **Materiales y Métodos.** El presente trabajo estudió el perfil de 756 miRNAs circulantes en pacientes graves y no graves infectados con el virus pdm A/H1N1, en contraste de individuos clasificados como contactos intradomiciliarios no-relacionados de los pacientes infectados por el mismo virus pero que no desarrollaron enfermedad aguda y un grupo de controles como individuos clínicamente sanos no expuestos al virus pdm A/H1N1. Posteriormente, se evaluaron los niveles de citocinas, quimiocinas y factores de crecimiento que potencialmente pudiesen estar asociados como blancos potenciales de los miRNAs que se encontraron diferencialmente expresados en el mapeo inicial; al termino se realizó un análisis bioinformático para la predicción de los blancos moleculares de los miRNAs así como de redes de interacción entre vías de señalización celular y de los miRNAs expresados diferencialmente. **Resultados.** Los pacientes con infección grave mostraron una sobre expresión de miR-150 circulante ($p<0.05$) con respecto de los pacientes con enfermedad no grave. miR-29c, miR-145, miR-22, miR-210, miR-126 y miR-222 presentaron un patrón de expresión diferencial entre los pacientes con infección por el virus pdm H1N1 y los individuos sanos. Se detectaron correlaciones significativas ($p<0.05$) entre los niveles de miR-150 e IL-1RA, IL-2, IL-6, CXCL8, IFN-γ, CXCL-10 y G-CSF (por sus siglas en inglés), particularmente en los pacientes con enfermedad grave. **Conclusión.** La sobre expresión de miR-150 se encuentra asociada con formas graves de neumonía asociada con la infección por el virus pdm A/H1N1. La expresión diferencial de miRNAs circulantes asociados a los niveles de mediadores inmunológicos en suero en sujetos con infección grave, asociada al virus de pdm A/H1N1 sustenta el potencial de los miRNAs como biomarcadores de progresión y mal pronóstico en infecciones por virus respiratorios.

ABSTRACT.

Introduction. The overproduction of pro-inflammatory cytokines and chemokines is frequently associated with severe clinical manifestations in patients with influenza pdm A/H1N1 virus infection. Micro-RNAs (miRNAs) are highly conserved small non-coding RNA molecules that post-transcriptionally regulate gene expression and are potential biomarkers and therapeutic targets in different inflammatory conditions. **Materials and Methods.** In this study we analyzed the profile of 756 circulating miRNAs in critically ill pdm A/H1N1 patients, pdm A/H1N1 patients with milder disease, asymptomatic housemates exposed to the pdm A/H1N1 virus but that did not developed respiratory symptoms of the disease and unexposed healthy controls. Cytokine, chemokine and growth factors that were potential targets of differentially expressed miRNAs were also assessed. Cellular pathways enrichment and interactome analysis of these miRNAs were also performed. **Results**. Critically ill patients exhibited a significant over-expression of circulating miR-150 ($p<0.05$) when compared to patients with milder disease. miR-29c, miR-145, miR-22, miR-210, miR-126 and miRT-222 were differentially expressed between patients with the infection of the pdm A/H1N1 virus and healthy people. Significant correlations ($p<0.05$) between circulating levels of miR-150 with IL-1RA, IL-2, IL-6, CXCL8, IFN-γ, CXCL10 and G-CSF were detected, particularly in critically ill patients. **Conclusion.** The up-regulation of miR-150 is associated with poorer outcomes of A/H1N1 infection. The differential expression of miRNAs related with immune processes in severe pdm A/H1N1 disease support the potential role of these miRNAs as biomarkers of disease progression.

INTRODUCCIÓN.

La pandemia de influenza del 2009 causada por el virus de influenza pdm A/H1N1 provocó el fallecimiento de más de 12200 personas en más de 208 países en un periodo de tiempo comprendido entre el mes de mayo y diciembre del 2009 (http://www.who.int/csr/don/2009_12_30/en/) [1], este suceso alertó y recordó la potencialidad – característica de los virus de influenza del género A – para provocar brotes epidémicos y pandémicos. Los datos oficiales de morbilidad por virus de influenza pandémico suman 78868 casos en México del año 2009 al 2013 (http://www.epidemiologia.salud.gob.mx/anuario/html/anuarios.html) [2]. El número de fallecimientos durante el 2009-2010 en México llegó a 1743 individuos [3, 4] y tan solo durante el año 2014 se reportaron 67094 casos nuevos de influenza sin distinguir entre el virus correspondiente a la nueva variante del 2009 y las cepas virales estacionales que circulan en la población humana durante la época invernal [2], estos datos demuestran que las cepas virales causantes de la influenza tipo A, continúan siendo de importancia clínica para la salud pública en México.

Diversos estudios han mostrado tanto en modelos experimentales como en pacientes infectados con el virus de influenza pdm A/H1N1 la sobreproducción de citocinas pro-inflamatorias y de quimiocinas [5-7]. Este incremento de mediadores inflamatorios esta frecuentemente asociado con las manifestaciones clínicas severas de la enfermedad [7-10].

Los micro-RNAs son moléculas de RNA pequeños no codificantes que regulan la expresión génica y que en distintas condiciones inflamatorias son reguladores del sistema inmune [11] así como blancos terapéuticos en potencia [12-14].

En este trabajo se ha estudiado el perfil de miRNAs circulantes en pacientes graves y no graves infectados con el virus de influenza pdm A/H1N1, así como de individuos que fueron contactos intradomiciliarios no relacionados de los pacientes infectados por el mismo virus y un grupo control de individuos sanos con el fin de obtener datos que permitieran asociar a estos reguladores de la expresión genética con el perfil inflamatorio observado en los pacientes estudiados, por lo cual se

evaluaron los niveles de citocinas, quimiocinas y factores de crecimiento que potencialmente fueran blancos moleculares de los miRNAs que se encontraron diferencialmente expresados. Adicionalmente se realizó un análisis bioinformático para la predicción de los blancos moleculares de los miRNAs expresados diferencialmente. El conocimiento de los blancos moleculares de los miRNAs permitió la construcción de redes de interacción entre vías de señalización celular (los blancos moleculares son los componentes de cada vía de señalización celular mostrada en las redes de interacción) y los miRNAs expresados diferencialmente.

Los pacientes con infección grave mostraron una sobre expresión de miR-150 circulante ($p<0.05$) con respecto de los pacientes con enfermedad no severa. miR29c, miR-145, miR-22, miR-210, miR-126 y miR-222 presentaron un patrón de expresión diferencial entre los pacientes con infección por el virus de influenza pdm A/H1N1 y los individuos sanos. Se detectaron correlaciones significativas ($p<0.05$) entre los niveles de miR-150 e IL-1RA, IL-2, IL-6, CXCL8, IFN-γ, CXCL-10 y G-CSF, particularmente en los pacientes con enfermedad severa. La sobre expresión de miR-150 esta asociada a la forma severa de la infección por el virus pdm A/H1N1 [9]. La expresión diferencial de miRNAs circulantes asociados a los niveles de mediadores inmunológicos en suero en sujetos con infección severa asociada al virus de influenza pdm A/H1N1 soporta el potencial que tienen los miRNAs como biomarcadores de progresión hacia formas graves de la enfermedad.

OBJETIVOS

OBJETIVO GENERAL.

Evaluar el perfil de expresión de miRNAs circulantes y su asociación con el desarrollo de una respuesta inflamatoria exacerbada en pacientes con diferentes formas clínicas de la infección por el virus pdm A/H1N1.

OBJETIVOS ESPECÍFICOS.

1. Conocer el perfil de expresión de miRNAs circulantes en 4 grupos de individuos: Controles sanos, contactos intra-domiciliarios no relacionados de sujetos con la infección por el virus pdm A/H1N1, pacientes graves y pacientes no graves infectados con el virus pdm A/H1N1.

2. Identificar ***in silicio*** los blancos moleculares de los miRNAs diferencialmente expresados y las vías de señalización celular en que participan.

3. Medir los niveles de mediadores inflamatorios (citocinas y quimiocinas) en suero de los grupos de estudio y asociarlos al perfil de expresión de miRNAs.

4. Realizar un modelo ***in vitro*** de infección con las cepas pdm H1N1 y estacional H3N2 del virus de influenza A y medir la expresión de los miRNAs diferencialmente expresados en la "**forma grave"** de la infección por el virus A/H1N1.

ANTECEDENTES

ORTOMIXOVIRUS

La familia *Orthomyxoviridae* contiene seis géneros: tres son denominados *Influenza virus A, B y C*. (cada género integra una sola especie denominadas: *Virus de Influenza A, B y C* respectivamente), el género *Thogotovirus* que contiene dos especies: *Virus Dhori y Virus Thogoto*, el género *Isavirus* que integra una sola especie denominada Virus Infeccioso de la Anemia de Salmón (ISAV, *por sus siglas en inglés*) y el género *Quaranjavirus*, el cual engloba dos especies: el *Virus Johnston Atoll virus y Virus Quaranfil* [15]. Los segmentos genómicos y sus proteínas codificadas de la familia orthomyxoviridae están enlistados en la Tabla 1 [16].

Los virus de influenza A, B y C, se caracterizan por sus genomas segmentados tipo RNA de cadena negativa. Los datos de secuenciación han confirmado que estos virus tienen un ancestro común (Figura 1) [17, 18], sin embargo también han mostrado divergencia genética a través de re-arreglos de sus segmentos genómicos, lo cual puede ocurrir dentro de cada género o tipo pero no entre distintos géneros. Por microscopia electrónica, el virus de influenza A y B son indistinguibles. Ambos tienen una forma esférica o filamentosa, las variantes esféricas miden aproximadamente 100 nm de diametro y los de forma filamentosa frecuentemente exceden los 300 nm de longitud. Los virus de influenza A se caracterizan por el subtipo de glicoproteínas de superficie, la hemaglutinina (HA, por sus siglas en inglés) y la neuraminidasa (NA, por sus siglas en inglés), (Tabla 1). La organización del virus de influenza B es similar al del virus de influenza A, tiene cuatro proteínas en su envoltura: HA, NA, NB y BM2, las dos últimas en sustitución de M2 (por sus siglas en inglés) característica del virus de influenza A. Los virus de influenza C son diferentes estructuralmente a los virus de influenza A y B; en la superficie de las células infectadas pueden formar estructuras largas con una forma de cuerda de tamaño aproximado de 500 µm. Sin embargo, los virus de influenza C tienen una envoltura lipídica tachonada de glicoproteínas que esta por encima de una proteína de matriz y un complejo ribonucleico viral (vRNP, por sus siglas en inglés) similar a los correspondientes en los virus de influenza A y B. Los virus de influenza C tienen una glicoproteína de superficie principal llamada proteína de fusión hemaglutinina-esterasa (HEF, por sus siglas en inglés) la cual corresponde funcionalmente con la HA y la NA de los virus de influenza A y B así como una proteína de envoltura (CM2, por sus siglas en inglés) [17].

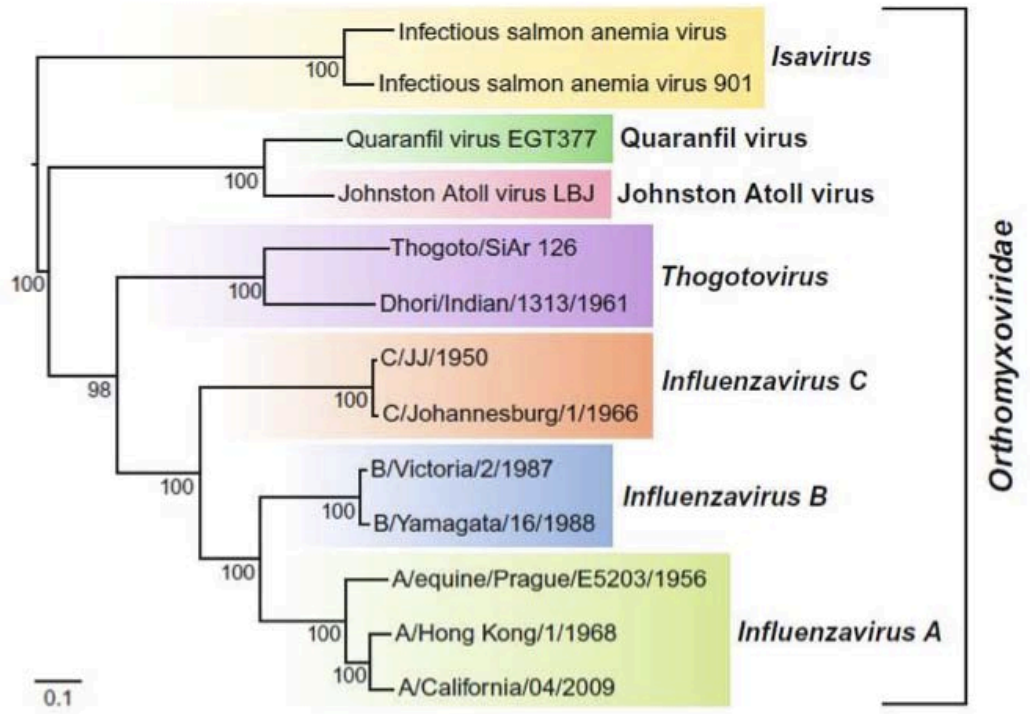

Figura 1. Árbol filogenético de los virus de la familia *Orthomixoviridae*. Las secuencias de nucleótidos de las proteínas Polimerasa básica 1 (PB1,por sus siglas en inglés) se alinearon usando el software trasnAlign and CLUSTAL W y sus relaciones filogenéticas se determinaron con el método de unión de vecinos (NjM, por sus siglas en inglés) usando el software PAUP v 4.0b. (Figura tomada desde McCauley, ICTV, 2011 [18])

Tabla 1 Segmentos Genómicos de la familia *Orthomyxovirus*.

Proteínas y Función	Segmentos Genómicos de la familia Orthomyxovirus					
	ISAV	Influenza A	Influenza B	Influenza C	Thogotovirus	Quaranjavirus
Subunidades de la Polimerasa	PB2 (1) PB1 (2) PA (4)	PB2 (1) PB1 (2) PA (3)	PB2 (1) PB1 (2) PA (3)	PB2 (1) PB1 (2) PA (3)	PB2 (1) PB1 (2) PA (3)	ORF (1) * ORF (2) * ORF (3) *
Nucleoproteína	NP (3)	NP (5)	NP (5)	NP (5)	NP (5)	
Unión a receptor	HE (6)	HA (4)	HA (4)	HEF (4)	Nd	−
Proteína de Corte	HE (6)	NA (6)	NB (6)	HEF (4)	Nd	−
Fusión	F (5)	HA (4)	HA (4)	HEF (4)	gp75 (4)	−
Proteína de Matriz	M (8)	M1 (7)	M1 (7)	M1 (6)	M (6)[Ç]	−
Canal iónico	Nd	M2 (7)	BM2 (7)	CM2 (6)	Nd	−
Antagonistas de IFN tipo I	ORF1 (7), ORF2 (8)	NS1 (8)	NS1 (8)	NS1 (7)	ML (6)[Ç]	−
Exporte Nuclear	Nd	NS2 (8) (NEP)	NS2 (8) (NEP)	NS2 (7) (NEP)	Nd	−
Pro-apoptosis	Nd	PB1-F2 (2)	−	nd	Nd	−
Desconocida	−	−	NB (6)	−	−	ORF (4, 5, 6)

Tabla 1. Segmentos Genómicos de la familia *Orthomyxovirus*. PB2: Polimerasa Básica 2; PB1: Polimerasa Básica 1; PA: Polimerasa Ácida; NP: Nucleoproteína; HE: Hemaglutinina Esterasa [19]; HE hidroliza el ácido siálico, Neu4,5Ac2: Ácido 4-*O*-5 N Acetilneuramínico [19]; F: Proteína de Fusión Viral; M: Proteína de Matriz; ORF: Marco Abierto de Lectura. M1: Proteína de Matriz 1 de Envoltura Nuclear; M2: Canales Iónicos de Matriz 2. NS1: Proteína no Estructural 1, antagonista de interferón; NS2 ó NEP: Proteína no Estructural 2 ó Proteína de Exporte Nuclear; PB1-F2: es una segunda proteína que es expresada por el gen PB1 en otro marco de lectura: +1 y que induce apoptosis[20]; NB: Proteína Integral de Membrana del virus de influenza B, es codificada por el mismo mRNA bicistrónico que codifica para la proteína NA (Análoga a la proteína M2 de influenza) [21]. BM2: Proteína del virus de influenza B que es codificada por un mRNA biscistrónico que codifica también para la proteína M1 [22]; HEF: Glicoproteína de envoltura, Hemaglutinina-Esterasa de Fusión; Gp75: Glicoproteína 75 . ML: Proteína accesoria no esencial. *Los Segmentos de RNA del virus Quaranfill 1-3 (2421 nt, 2404 nt, and 2386 nt) [18] codifican un marco abierto de lectura individual que exhiben homología con las respectivas polimerasas PB2, PA and PB1 de influenza virus. Nd: No detectable. Cada número entre paréntesis indica el número del segmento genómico correspondiente. [Ç]Las proteínas M y ML de Thogotovirus están codificadas por el mismo segmento genómico [23].

VIRUS DE INFLUENZA TIPO A

El virus de influenza A esta tachonado de las glicoproteínas HA y NA en una proporción aproximada de 4:1 las cuales se proyectan a partir de la membrana celular del hospedero (Figura 2) [17]. Estás glicoproteínas permiten la clasificación de las diferentes cepas del virus de influenza A en 18 hemaglutininas (H 1-18) y 11 neuraminidasas (N 1-11) [24]. Una cantidad más pequeña de proteínas M2 atraviesan la envoltura viral en una proporción M2:HA en el orden de 1:10 ó 1:100 [25]. La envoltura viral y sus tres proteínas integrales de membrana HA, NA y M2 recubren la proteína de matriz M1, que encierra al núcleo viral. Al interior de la proteína M1 se encuentran la proteína NEP ó NS2 y el vRNP que esta constituido por los segmentos de RNA viral recubiertos de la proteína NP y de una RNA polimerasa heterotrimerica (HRP, por sus siglas en inglés) compuesta por dos subunidades básicas: PB1 y PB2 y una subunidad PA (Figura 2) [17].

El genoma de los virus de influenza A contienen ocho segmentos de vRNP (Figura 2), los cuales están enumerados del 1 al 8 con respecto a su longitud decreciente (Tabla 1). Los segmentos 1, 3, 4 y 5 codifican solo una proteína por segmento: Las proteínas PB2, PA, HA y NP respectivamente. Todos los virus de influenza A codifican la subunidad polimerasa PB1 en el segmento 2, en algunas cepas este último segmento codifica también – en un marco abierto de lectura alterno – a la proteína accesoria PB1-F2, una proteína pequeña de 87 aminoácidos con actividad pro-apoptótica [26]. El segmento 6 codifica para la proteína NA; el segmento 7 codifica para las dos proteínas M1 y M2, ésta última es codificada a través del proceso de corte y empalme del segmento de RNA [27]. Finalmente, el segmento 8 expresa a la proteína NS1 y además por corte y empalme a la proteína NEP/NS2, la cual participa en el exporte de los complejos vRNPs proveniente del núcleo celular de la célula hospedera [17].

En los extremos de cada vRNP se forma un bucle helicoidal que esta unido a una HRP y el resto esta recubierto de NP rica en arginina, la cual presenta una carga positiva neta que permite la unión del RNA viral fosfatado de carga negativa [27-29]. Todos los segmentos contienen regiones no codificantes 3' y 5' terminal de tamaño variable, cuya secuencia es altamente conservada entre todos los segmentos del virus de influenza. Estas regiones funcionan también como promotores para la replicación y la transcripción del RNA viral a través del complejo RNA polimerasa. Las regiones no codificantes también incluyen la secuencia señal de poliadenilación del mRNA y parte de las señales de empaquetamiento para el ensamble viral [17].

Un genoma segmentado permite cambios antigénicos (antigenic shift) en los virus de influenza tipo A que ocurren cuando una cepa de influenza A adquiere de un virus de influenza de un subtipo distinto el segmento HA y posiblemente el de NA también. Este re-arreglo puede suceder en células infectadas con diferentes virus de animales y humanos; el virus resultante quizá codifique para proteínas antigénicas completamente nuevas para las cuales la población humana no tendría inmunidad preexistente. Una pandemia causada por el virus de influenza A surge cuando un cambio antigénico genera un virus competente en replicación y eficaz para ser transmitido en humanos, dejándolos susceptibles e inmunológicamente naïve. El cambio antigénico ha producido las variantes de los virus responsables de la última epidemia del año 2009, así como la gripe española de principios del siglo XX cuya letalidad no tiene precedentes en la actualidad, dejando alrededor de 50 millones de muertes [30].

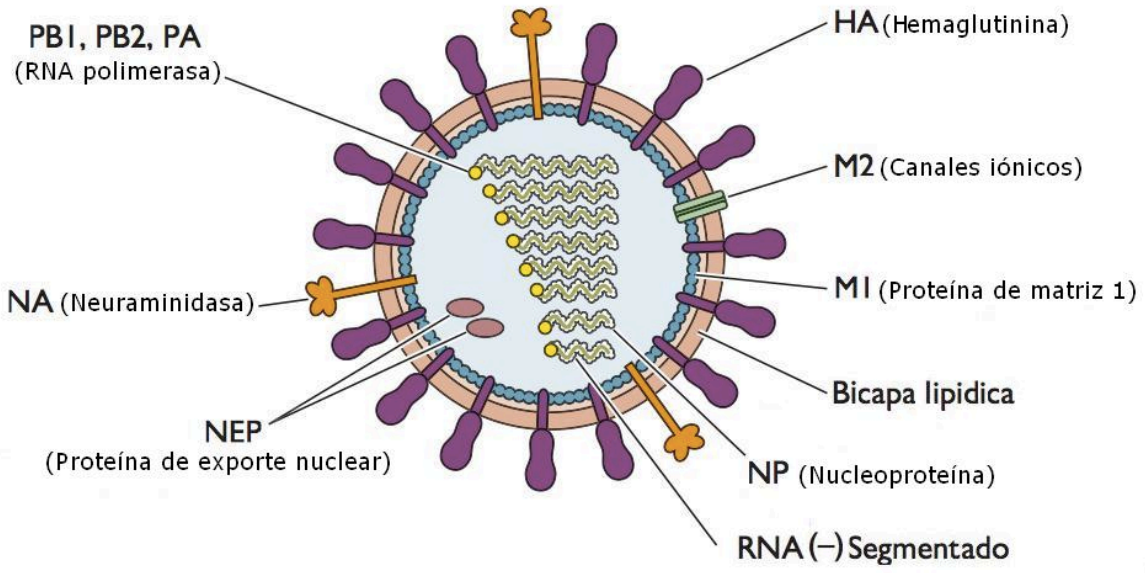

Figura 2. Estructura del virus de influenza A. [Imagen modificada desde: Flint, S. 2009. Principles of virology] [31]

CARACTERÍSTICAS MOLECULARES DEL VIRUS pdm A/H1N1

El virus pdm A/H1N1 que emergió en el año 2009 es resultado de un re-arreglo viral que combina material genético del virus de influenza aviar H1N1 (segmentos genéticos: PB2 y PA), del virus de influenza clásico porcino H1N1 (segmentos genéticos: H1, NP y NS), del virus de influenza humano H3N2 (segmento genético PB1) y del virus de influenza porcino H1N1 Euroasiático (segmentos genéticos: N1 y M) (Figura 3). En total el genoma contiene cerca de 13600 nucleótidos y codifica las 11 proteínas: HA, NA, M1, M2 NP, PB1, PB1-F2, PB2, PA, NS1, NS2 (NEP) [32].

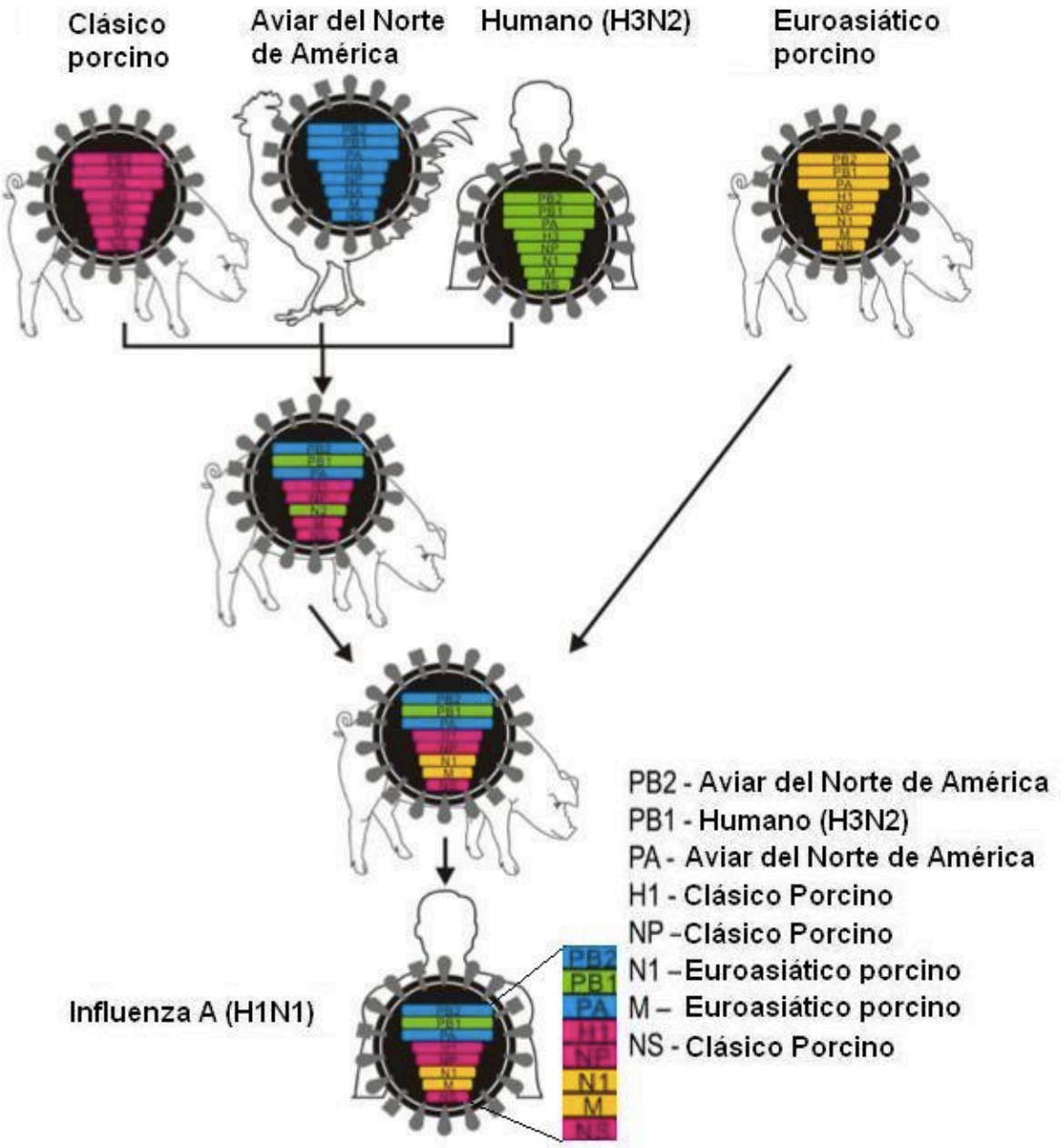

Figura 3. Origen del virus de influenza pdm A/H1N1. Re-arreglo genómico entre las cepas de virus de influenza tipo A que circulan en humanos, aves y cerdos [Imagen modificada desde: Neumann, G. Nature, 2009] [20].

Los análisis filogenéticos indican que el arreglo genómico viral que generó a los re-arreglos triples H1N2 y H3N2 porcinos tuvo lugar en el Norte de América en 1998 los cuales circularon en la población de cerdos de la región; esto incluyó a los virus de influenza H1N1 de origen porcino clásico, el virus H3N2 humano y el aviar H1N1. Posteriormente el genoma del virus H1N2 porcino presentó un nuevo re-arreglo así como la variante porcina Euroasiática H1N1 desde las cuales se dio origen a la cepa porcina H1N1 (S-OIV H1N1) que actualmente circula en humanos [20, 33].

HA EN LA PATOGENICIDAD VIRAL.

La patogenicidad del virus de influenza A es multigénica y los determinantes de la misma difieren entre especies animales. Sin embargo, la proteína HA juega un papel importante en el incremento de la patogenicidad en muchas especies. La HA media la unión del virus a las células del hospedero y la fusión subsecuente de las membranas endosomales necesaria para la liberación del complejo ribonucleico viral en el citoplasma[20].

DISTRIBUCIÓN DE RECEPTOR EN LAS CÉLULAS DE HOSPEDERO.

La especificidad del virus puede ser explicada en parte por la diferencia en la especificidad de los virus de humanos y de aves. Los virus de influenza humanos preferentemente se unen al ácido siálico enlazado a la galactosa a través de enlaces tipo α2,6 (SAα2,6Gal) [34]. Esta preferencia ocurre en las células epiteliales de la tráquea de humanos en contraste con los virus de aves que preferencialmente reconocen ácido siálico unido por enlaces tipo α2,3 a la galactosa (SAα2,3Gal) los cuales son reconocidos preferencialmente en las células epiteliales del tracto intestinal de aves acuáticas (principal sitio de replicación viral de virus de influenza en aves) [20]. Rogers, G. N. Y Paulson, J. C., en 1983 reportaron que diversos aislados humanos del tipo H1 que circularon antes de 1956 - a diferencia de los aislados humanos de tipo H3 que son específicos para los enlaces SAα2,6Gal - aparentemente reconocían también enlaces SAα2,3Gal en adición a los tipo SAα2,6Gal [34].

La especificidad de unión a receptor característica de los virus de humanos y de aves, sugiere que los virus de influenza aviares necesitan adquirir la habilidad de reconocer receptores con enlaces SAα2,6Gal que con más frecuencia son utilizados por los virus de influenza de humanos lo cual podría causar una pandemia. Además, los aislados de las pandemias de 1918, 1957 y 1968 poseen HA que aunque son de origen aviar, reconocen ambos tipos de receptores en humanos (SAα2,3Gal y SAα2,6Gal). A la luz de estos datos, es sorprendente que la infección en humanos por el virus de influenza H5N1 aislado de individuos infectados en Hong Kong en 1997, reconocen preferencialmente receptores tipo SAα2,3Gal [35]. En el año 2006 Van Riel y colaboradores, así como Shinya en el mismo año [36, 37] reportaron que los receptores de humanos presentes en el epitelio que recubre a los bronquiolos y a las paredes alveolares son del tipo SAα2,3Gal - receptor característico de los virus de aves - y los receptores presentes en las células epiteliales de la mucosa nasal, senos paranasales, faringe, tráquea y bronquios son del tipo SAα2,6Gal [20, 36, 37]. Sin embargo Nicholls y colaboradores

demostraron en un modelo ex–vivo que el virus aviar H5N1 puede infectar órganos de las vías respiratorias altas [38], pero el hallazgo de que el pulmón de humanos contiene receptores tipo SAα2,3Gal explica la severidad de la neumonía observada en humanos infectados con la cepa patogénica H5N1 característica de aves [20].

ESPECIFICIDAD DEL RECEPTOR DE HA.

Las diferencias en la especificidad de unión al receptor de los virus de influenza en humanos y de aves están determinadas por los residuos de aminoácidos en un surco del receptor de la HA. Una Gln en la posición 226 y una Gly en la posición 228 de las HAs: H2 y H3 confieren afinidad a receptores de aves, mientras que los aminoácidos Leu y Ser en las mismas posiciones determinan la unión a los receptores de humanos. Para las HAs “H1”, los aminoácidos en la posición 190 y 225 determinan la especificidad de unión al receptor. En HAs tipo H1 característica de virus de humanos, el aminoácido Asp en la posición 190 y otra Asp en la posición 225, permiten la unión a receptores de aves [20, 39]. Durante la pandemia de 1918 circularon dos cepas virales que diferían en su especificidad de unión al receptor: una que reconocía solo receptores de humanos y que se transmitía eficientemente en hurones y otra cepa que reconocía tanto receptores de humanos como de aves y que se transmitía ineficientemente en hurones, dicha especificidad se confería gracias al tipo de aminoácido presente en posiciones 190 y 225 de la HA [40]. La cepa pandémica del virus A/H1N1 que emergió en el año 2009 posee los aminoácidos Asp en la posición 190 y 225, que permite su transmisibilidad de estos virus en humanos [20].

CORTE DE LA HA.

El corte de la HA es esencial para la infectividad viral ya que la región N-terminal de la HA2 (a la que se le denomina: “péptido de fusión”) media la fusión entre la envoltura viral y la membrana endosomal, un paso esencial para la liberación del vRNP al citoplasma. El corte de la HA esta determinado la secuencia de aminoácidos en el sitio de corte. Los virus de influenza de aves, moderadamente patogénicos poseen un residuo de Arg único, que es cortado por proteasas de los órganos respiratorios e intestinales restringiendo localmente la replicación viral. De manera contraria, los virus H5 y H7 que son altamente patógenos poseen múltiples aminoácidos que son reconocidos por proteasas (tales como PC6, FURIN) causando infecciones a nivel sistémico [41].

PAPEL DE PB2 IN LA PATOGENICIDAD Y ESPECIFICIDAD DE HOSPEDERO.

El complejo de replicación viral se ha reconocido como un factor importante de la patogenicidad asociado al virus por su implicación en su proliferación. Subbarao y colaboradores fueron los primeros en demostrar que el aminoácido en la posición 627 de la proteína PB2 del virus de influenza A/Mallard/NY/78 determina el rango de interacción entre el huésped y el hospedero, limitando así la capacidad infectiva de la misma en células MDCK (por sus siglas en inglés) [42]. Diversos estudios se han enfocado en la relevancia de la posición del aminoácido 627 como factor determinante de la virulencia de los virus de influenza tipo A, específicamente PB2-627Lys. Hatta y colaboradores reportaron que la presencia de Lys en la PB2 del virus de influenza H5N1 en la posición 627 le confiere patogenicidad en el ratón, mientras que la presencia de ácido glutámico lo hace no patógeno en estos animales [43].

BIOGENESIS Y FUNCIÓN DE miRNAs

En el año 1993 se reportó en la literatura el descubrimiento de Lin-4, el primer microRNA identificado en el organismo *Caenorhabditis elegans* [44]. A la fecha existen 28645 entradas que corresponden a los precursores de los miRNAs maduros (pre-miRNAs, por sus siglas en inglés) registrados en el repositorio principal de la Web: miRBase database (www.mirbase.org) pertenecientes a 223 especies diferentes y en humanos están reportados 2845 miRNAs de acuerdo a la versión número 21 del año 2016. La primera versión de miRBase [45], inició con 506 entradas pertenecientes a 6 especies, dato que muestra el interés de la comunidad científica en la identificación de nuevos miRNAs y su relevancia en los sistemas biológicos.

Los miRNAs son RNAs pequeños no codificantes de aproximadamente 22 nucleótidos que regulan la expresión genética a nivel post-transcripcional al inhibir la traducción de RNAs mensajeros (mRNAs). El mecanismo estándar para llevar a cabo su función involucra la acción de un complejo de silenciamiento inducido por ARN (RISC, por sus siglas en inglés) compuesto fundamentalmente por la proteína llamada Argonauta (AGO) la cual carga a un miRNA maduro (Figura 4) [46].

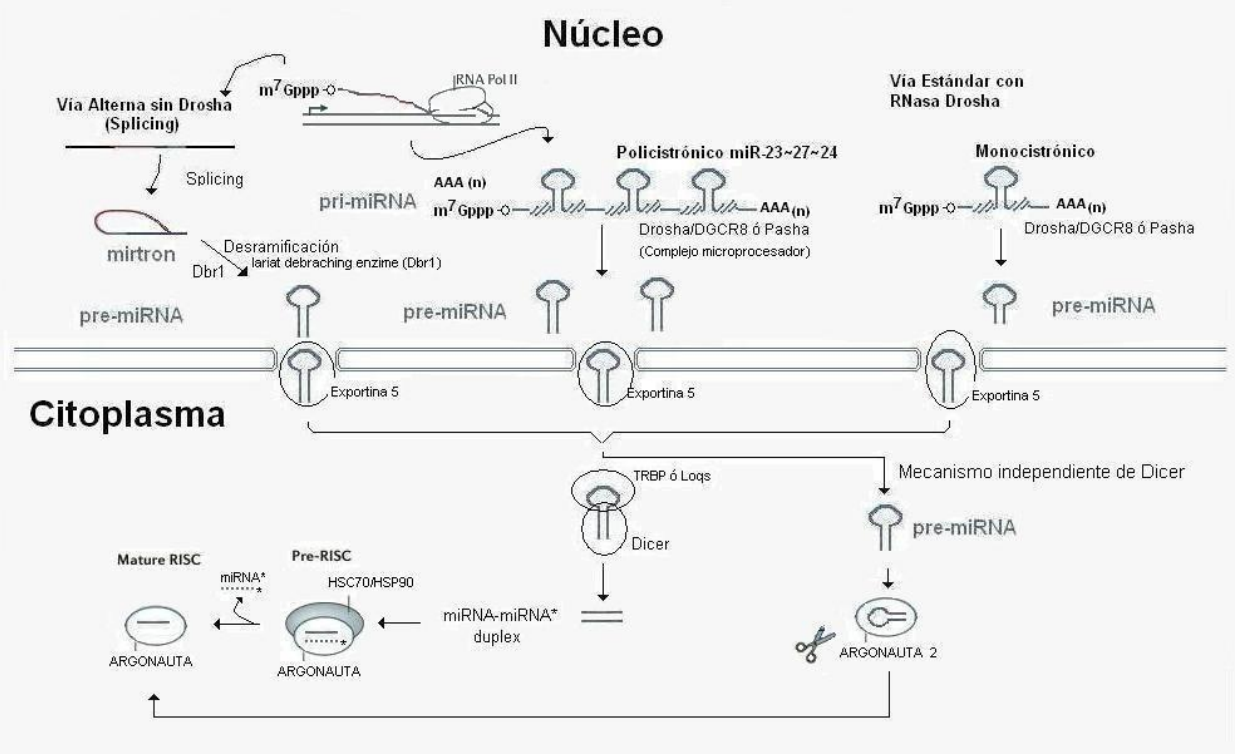

Figura 4. Biogénesis de miRNAs. El proceso de biogénesis inicia en el núcleo en donde la RNA polimerasa II transcribe el gen que codifica para un miRNA que puede ser monocistrónico o policistrónico. La secuencia primaria de un miRNA se denomina pri-miRNA, la cual tiene un cap 7-metilguanosina (m^7Gppp) y termina con una cola de poli-A. El pri-miRNA es reconocido por un complejo denominado microprocesador compuesto por una RNasa llamada Drosha asistida por una proteína que en mamíferos se denomina DGCR8 ó Pasha en otros animales. Una vía alterna de biogénesis de miRNAs que no involucra al complejo microprocesador, es la que se vale del "proceso de corte y empalme" (Splicing) en la cual, una secuencia intrónica que proviene de un gen codificante corresponde a la secuencia de un miRNA y es convertido en un pre-miRNA por una enzima llamada Dbr1 que desramifica el lazo intrónico. El pre-miRNA es exportado hacia el citoplasma por exportina-5 utilizando un poro nuclear. El pre-miRNA puede ser reconocido por otra RNasa llamada Dicer asistida por TRBP ó Loquacius en *D. melanogaster*, el corte enzimatico por Dicer sobre el pre-miRNA forma un duplex miRNA-miRNA* que se carga sobre la proteína Argonauta, asistida por HSC70/HSP90 formando un pre-RISC, el cual después de eliminar la secuencia RNA* complementaria, da origen a un complejo RISC maduro. Existe un mecanismo de biogénesis independiente de Dicer que carga al pre-miRNA directamente sobre Argonauta la cual forma la estructura madura del miRNA.

El proceso de formación y maduración de un miRNA inicia en el núcleo celular, en donde el gen es transcrito a través de un RNA polimerasa II [47, 48]; la secuencia genética que codifica para cualquier miRNA puede ser un gen exclusivo para el mismo miRNA o puede ser una secuencia intrónica proveniente de un gen diferente del miRNA [48]. La primer secuencia que se origina, es una molécula llamada miRNA primario (pri-miRNA, por sus siglas en inglés), la cual estructuralmente es una cadena doble en forma de bucle y tallo debido al plegamiento del transcrito que inicia con un cap 7-metilguanosina (m^7Gppp) y termina una cola de poli-A en su extremo 3'. Esta estructura permite entender la nomenclatura con la cual se puede designar a un miRNA maduro: 3p ó 5p, ya que dicho miRNA maduro que proviene de un pri-miRNA, posteriormente de un pre-miRNA y de una estructura

dúplex llamada miRNA-miRNA*, – de los cuales los dos primeros son sujetos a cortes enzimáticos por las endonucleasas Drosha y Dicer – forzosamente tiene que corresponder a uno de los dos brazos del pre-miRNA, ya sea el brazo 5' ó el brazo 3' (Figura 5) [45]. Drosha actúa como componente de un complejo grande llamado microprocesador (Figura 4). Este complejo nuclear existe en al menos dos formas, un complejo de ~600 kDa de función desconocida y un heterodímero más pequeño que está compuesto por Drosha y su proteína de unión a RNA, codificada por el gen asociado al Sindrome de DiGeorge (DGCR8, por sus siglas en ingles) en mamíferos, y Pasha (*D. melanogaster*) en otros animales [49-52]. El pri-miRNA puede ser transcrito en el núcleo como un gen único o como una unidad transcripcional policistrónica que puede contener más de dos genes [53] (Figura 4). Está estructura primaria es cortada desde el tallo por la RNasa Drosha en sus extremos 5' y 3' lo cual elimina el *cap* y a la cola de *poli-A* y genera al pre-miRNA de ~70 nucleótidos que es exportado al citoplasma a través de un poro nuclear asistido por el receptor de transporte nuclear llamado exportina 5 (Ranbp21) que reconoce los extremos del pre-miRNA y la cual es dependiente de GTP [54-58]. Una vez que el pre-miRNA arriba al citoplasma, es reconocido y cortado en su región llamada horquilla o bucle, lejos del tallo, por la RNasa III *Dicer,* que en mamíferos es asistida por una proteína de unión a RNA (TRBP, por sus siglas en ingles) y en *D. melanogaster* es llamada Loquacious. A partir de un pre-miRNA Dicer libera un RNA de ~22 nucleótidos de doble cadena que en inglés es conocido como miRNA-miRNA*duplex (Figura 4) [59-62].

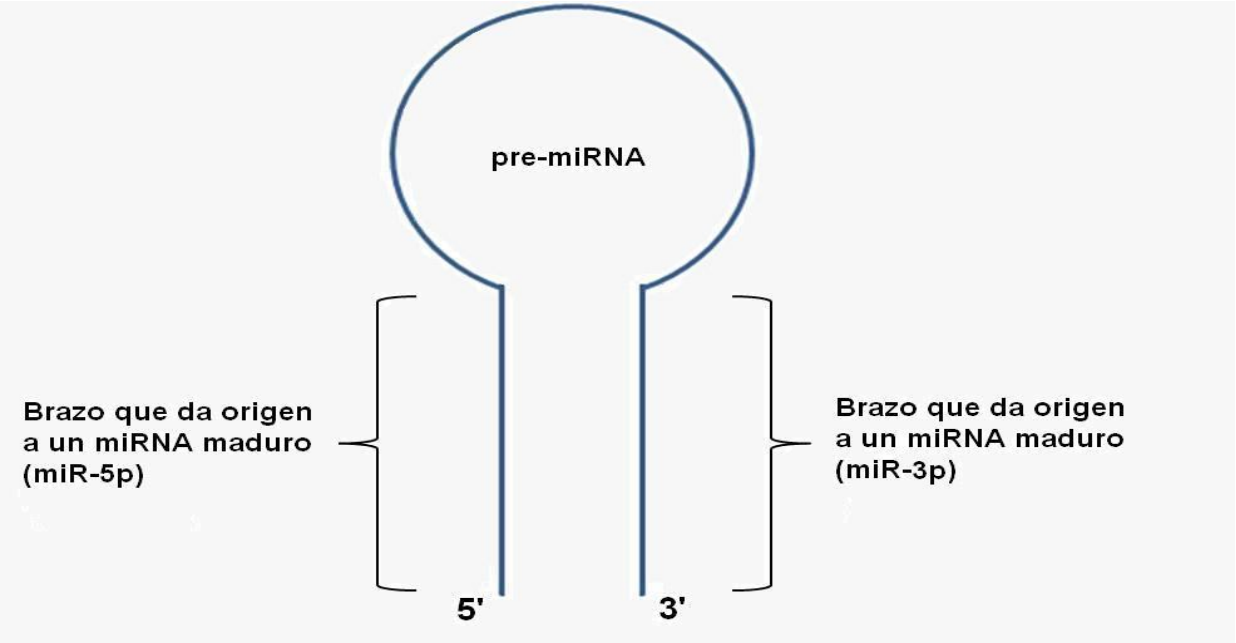

Figura 5. Nomenclatura de miRNAs. Los microRNAs maduros pueden ser designados como 5p ó 3p de acuerdo al brazo del pre-miRNA del cual provienen.

Además del mecanismo de biogénesis de miRNAs descrito anteriormente, existe un mecanismo alternativo en el que algunos miRNAs no necesitan la actividad de Drosha o de Dicer, por ejemplo los miRNAs que provienen de secuencias intrónicas (Mirtrons) [63, 64]. Una vez que el gen codificante para una proteína se transcribe en el núcleo, es sujeto al proceso de corte y empalme (splicing) y la secuencia intrónica que corresponde al Mirtron, es transportada hacia el citoplasma para su maduración como pre-miRNA sin necesidad de Drosha [63]. Así mismo existen pre-miRNAs que no requieren la acción de Dicer y son cargados directamente sobre argonauta para su maduración posterior (Figura 4). Por ejemplo en el pez cebra y en ratón el pre-miR-451 es demasiado corto para ser reconocido por Dicer por lo que el miRNA de doble cadena (miRNA-miRNA*dúplex) se forma una vez que el pre-miR-451 esta unido a argonauta [65-68].

Una vez que Argonauta – que es asistida por proteínas chaperonas (HSC70-HSP90, por sus siglas en ingles) [69] a las cuales se une previamente – carga a un miRNA de doble cadena, formado ya sea por la vía estándar o por la vía alterna mediada por AGO, se expele una de las dos cadenas (el miRNA*)[70, 71] lo cual da lugar al RISC maduro (Figura 4) [72]. En humanos Argonauta 2 (AGO2) es una proteína que compone a RISC y que tiene capacidad catalítica de corte [73, 74], mientras que AGO1, AGO3 y AGO4 carecen de actividad de corte. En otros animales como D. melanogaster están presentes AGO1 y AGO2, la primera es responsable de formar el complejo de silenciamiento para miRNAs y la última para RNAs pequeños interferentes (siRNA, por sus siglas en inglés) [72].

El mecanismo de biogénesis de miRNAs y el de formación del complejo de silenciamiento se han estudiado como eventos independientes, sin embargo, existe evidencia de que el proceso de maduración de un pre-miRNA en el citoplasma a través de Dicer puede acoplarse al complejo de silenciamiento dependiente de RNA de humanos y al silenciamiento postranscripcional de un gen [73, 74].

MECANISMOS DE SUPRESIÓN DE miRNAs

El miRNA maduro cargado en el RISC, guía a AGO hacia secuencias complementarias presentes en el mRNA lo que provoca su represión a nivel de traducción a proteína (Figura 6) [46]. El determinante principal para la unión de AGO a su mRNA blanco es un dominio en el sitio 5' terminal de 6-8

nucleótidos. AGO se asocia a esta región para crear la región semilla (nucleótidos 2 a 8 del miRNA) [75]. Las secuencias que son complementarias a la región semilla (en inglés: seed matches), que están presentes en los mRNAs son responsables de disminuir su expresión al unirse por complementariedad a un miRNA. Estos sitios complementarios pueden estar presentes en cualquier región del mRNA, sin embargo, la región más frecuente es en la región 3' no traducible (3' UTR, por sus siglas en inglés) [76-79]. Debido a que la región usada para crear la región semilla es muy pequeña, más de la mitad de todos los genes codificantes de proteínas en mamíferos son regulados por miRNAs y miles de otros mRNAs que tienen secuencias no conservadas en la región 3'UTR, pueden ser regulados a la baja, sin embargo, existe un fenómeno en el cual se pueden evitar ser blanco de miRNAs que están presentes en las mismas células donde estos genes son expresados. Esto, en inglés es llamado "selective avoidance" y explica la especificidad espacial y temporal de los miRNAs [80-82].

Algunas proteínas AGO cortan mRNAs blancos con una alta complementariedad y el proceso es catalizado por su dominio llamado "PIWI RNasa", el cual posiciona un par de iones Mg^{2+} en el fosfato a escindir de la cadena del RNA. Este mecanismo endonucleolítico proviene de vías de RNA interferentes ancestrales, en el cual el corte del blanco provee de una defensa anti-viral y anti-transposón [83].

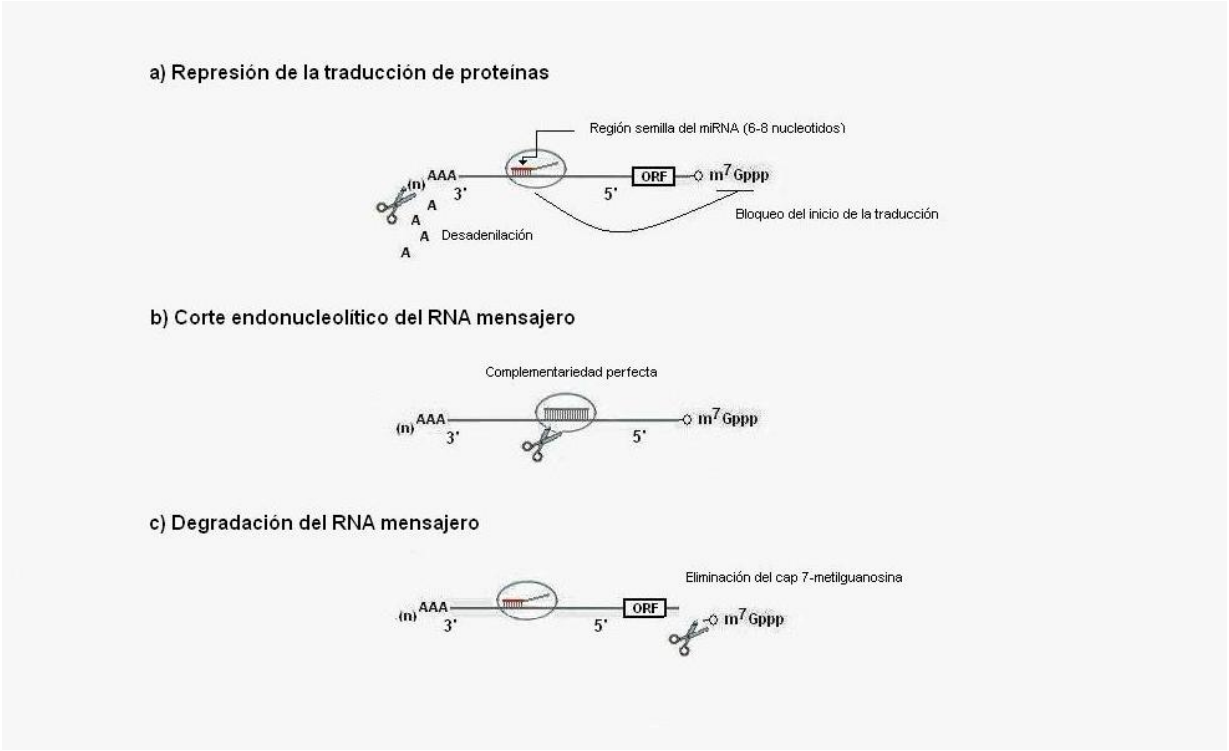

Figura 6. Mecanismos de supresión de microRNAs. a) Represión de la traducción de proteínas a través de la desadenilación del RNA mensajero o por el bloqueo del marco ORF. b) La unión perfecta por complementariedad de bases entre el microRNAs y el

RNA mensajero causa el corte endonucleolítico de RNA mensajero. c) La degradación de RNA mensajero por la eliminación del cap 7-metilguanosina.

En plantas, la complementariedad perfecta entre miRNAs y mRNAs desencadena el corte del mRNA [84-88] y solo una cantidad pequeña de miRNAs no cortan al mRNA blanco, bloqueando solo su traducción a proteína [89, 90]. Por el contrario, en mamíferos, pocas veces la formación del par miRNA-mRNA tiene la complementariedad suficiente para que AGO2 pueda escindir el RNA mensajero blanco [91-93].

En animales, se ha propuesto que los miRNAs bloquean la traducción de los ORF a través de mecanismos que involucran el acortamiento de la cola de poli-A afectando su estabilidad o reclutando cofactores que bloquean el inicio de la traducción. Otro mecanismo reportado es la destrucción de RNAs mensajeros es a través de la vía de eliminación del m^7Gppp (Figura 6) [83].

MATERIALES Y MÉTODOS

SUJETOS EN ESTUDIO.

Durante el brote de Influenza del 2009 en México, en el Instituto Nacional de Enfermedades Respiratorias “Ismael Cosio Villegas” (INER), se colecto suero de pacientes infectados con el virus pdm A/H1N1, de contactos intradomiciliarios y de controles sanos. Se analizó el perfil de miRNAs circulantes en 29 individuos (8 con diagnóstico de neumonía severa y 8 con neumonía no severa asociada al virus A/H1N1, 8 contactos intradomiciliarios y 5 individuos sanos). El diagnóstico de el síndrome de dificultad respiratoria (ARDS, por sus siglas en inglés) fue basado en la definición estándar [94, 95] Los contactos intradomiciliarios fueron considerados como aquellos individuos que estuvieron en contacto directo con pacientes infectados por el virus de influenza pdm A/H1N1, dichos individuos no desarrollaron enfermedad respiratoria aguda. El grupo de individuos sanos incluidos en el estudio no tuvieron historia de infección respiratoria en al menos los últimos seis meses. Sé detectaron anticuerpos específicos para el virus pdm A/H1N1 en 76% de los contactos intradomiciliarios (Inhibición de la hemaglutinación (HAI, por sus siglas en inglés) HAI>1:16), mientras que en el grupo de sujetos sanos no se detectados títulos significativos de anticuerpos.

El Comité de Ética institucional del INER revisó y aprobó el protocolo de este proyecto. Todos los individuos incluidos en el estudio o sus familiares responsables legalmente, particularmente en el caso

de los pacientes graves, firmaron un consentimiento informado. Todos los sujetos fueron individuos mayores de 18 años.

CRITERIOS DE INCLUSIÓN Y EXCLUSIÓN

1. Sujetos Sanos. Se incluyeron en el grupo de sujetos sanos a individuos que firmaron un consentimiento informado institucional, mayores de 18 años, que no hubieran estado en contacto con personas infectadas por el virus pdm A/H1N1, confirmándolo a través de la determinación de títulos de anticuerpos por una prueba HAI [título HAI<1:16] y por último cada sujeto debió tener una confirmación negativa por rRT-PCR que demostrara no haber padecido la infección por influenza pdm A/H1N1.
2. Contactos Intradomiciliarios. Se incluyeron en el grupo de contactos intradomiciliarios a individuos que firmaron un consentimiento informado institucional, mayores de 18 años, que estuvieron en contacto directo con personas infectadas por el virus pdm A/H1N1, confirmándolo a través de la determinación de títulos de anticuerpos por una prueba HAI [titulo HAI>1:16] y por último cada sujeto debió tener una confirmación negativa por rRT-PCR que demostrara no haber padecido la infección por influenza por influenza pdm A/H1N1.
3. Pacientes infectados con el virus de influenza pdm A/H1N1 no graves. Se incluyeron en el grupo de sujetos no graves a individuos que firmaron un consentimiento informado institucional, mayores de 18 años y cada sujeto debió tener una confirmación positiva por rRT-PCR como lo establece el centro para el control y prevención de enfermedades (CDC, por sus siglas en inglés). En adición, estos individuos no debieron requerir de atención en la unidad de cuidados intensivos ni recibir apoyo por ventilación mecánica [96].
4. Pacientes infectados con el virus de influenza pdm A/H1N1 graves. y cada sujeto debió tener una confirmación positiva por rRT-PCR como lo establece el centro para el control y prevención de enfermedades (CDC, por sus siglas en inglés) [96]. Estos individuos debieron pertenecer a la unidad de cuidados intensivos y de recibir ventilación mecánica a causa de un insuficiencia respiratoria grave.

Se excluyeron todos aquellos individuos que no cumplieran con los criterios de inclusión descritos anteriormente para cada grupo de estudio.

DETECCIÓN DEL VIRUS pdm A/H1N1.

Las muestras de hisopados nasales fueron obtenidas de los pacientes hospitalizados en el INER y enviadas al laboratorio de microbiología para el aislamiento viral (RNA mini kit: *Qiagen Westburg, Leusden, The Netherlands*). La detección del virus de influenza se realizó a través de rRT-PCR [96].

DETECCIÓN DE ANTICUERPOS ANTI-pdm A/H1N1.

Los títulos de anticuerpos anti-A/H1N1 en suero fueron evaluados usando la técnica HAI. En resumen, alícuotas de 25 µl de las muestras de suero diluidas en serie con solución salina tamponada con fosfato (PBS, por sus siglas en inglés) se mezclaron con alícuotas de 25 µl de aislados del virus de influenza pdm A/H1N1 aislado en el INER (correspondientes a 4 unidades hemaglutinantes). Las alícuotas de suero-virus diluidas se incubaron por 30 minutos a temperatura ambiente. Cincuenta µl de una alícuota de eritrocitos de pollo al 0.5% fueron adicionados y después de 30 minutos se avaluó la actividad de la inhibición de la hemaglutinación. El título de anticuerpos en suero que inhibieron la hemaglutinación se estableció como la dilución última en la que no hay actividad de la hemaglutinación. Aquellos individuos con títulos de anticuerpos mayores a 1:16 se consideraron como positivos para la exposición/infección por el virus A/H1N1.

MICROARREGLOS DE EXPRESIÓN TIEMPO REAL.

A partir de 250 µL de suero de pacientes y controles sanos, se extrajo RNA total usando Trizol LS (*Invitrogen, Carlsbad, CA*). Una vez obtenido el RNA total se realizó una retrotranscripción usando el sistema Megaplex™ RT Primers, Human Pool A v2.1 y Pool B v3.0 (*Applied Biosystems, Foster City, CA*). El cDNA producto de la retrotranscripción se enriqueció con el sistema Megaplex Pre Amp Primer Human Pool A v2.1 y Pool B v3.0. Los productos amplificados se cargaron directamente en las placas de microarreglos de expresión tiempo real (TLDAs, por sus siglas en inglés [TaqMan Human MicroRNA Array v2.1 A and B v3.0 *Applied Biosystems, Foster City, CA*]) los cuales permiten la cuantificación de 756 miRNAs distintos. Las placas de TLDAs permiten evaluar la expresión de tres genes de referencia distintos: U6, U44 y U48. El gen de referencia U6 fue seleccionado para la normalización y análisis de datos debido a que su expresión fue la más estable (Figura 7). La rRT-PCR de TLDAs se llevó a cabo en el equipo 7900HT Fast Real Time PCR System (*Applied Biosystems, Foster City, CA*).

VALIDACIÓN DE LA EXPRESIÓN DE miRNAS.

Para la cuantificación individual (validación) de los miRNAs diferencialmente expresados y cuantificados a través de TLDAs, se usó el sistema: Mir-X™ miRNA First-Strand Synthesis and SYBR® (qRT-PCR, por sus siglas en inglés) que convierte el RNA total en DNA complementario (cDNA, por sus siglas en inglés) para después ser cuantificado usando SYBR qRT-PCR®. Brevemente: En un tubo individual, el RNA es poliadenilado y retrotranscrito usando una Poly (A) polimerasa y una transcriptasa reversa (SMART™ MMLV). Una vez obtenido el cDNA se procede a la cuantificación por qRT-PCR en otro tubo individual. Para la qRT-PCR, el sistema incluye un oligonucleótido antisentido universal (mRQ 3' Primer™). Los oligonucleótidos sentido, específicos para los miRNAs a validar fueron los siguientes: hsa-miR-210: 5'-CTGTGCGTGTGACAGCGGCTGA; hsa-miR-145: 5'-GTCCAGTTTTCCCAGGAATCCCT; hsa-miR-22: 5'- AAGCTGCCAGTTGAAGAACTGT; hsa-miR29c: 5'- TAGCACCATTTGAAATCGGTTA; hsa-miR-126: 5'- TCGTACCGTGAGTAATAATGCG; hsa-miR-222: 5'- AGCTACATCTGGCTACTGGGT; hsa-miR-150: 5'- TCTCCCAACCCTTGTACCAGTG y U6 Sentido: 5'-GCTTCGGCAGCACATATACTAAAAT U6 Antisentido: 5'-CGCTTCACGAATTTGCGTGTCAT. Las qRT-PCRs se realizaron en el equipo: QuantStudio™ 12K Flex Real-Time PCR (Life Technologies). Las condiciones de la reacción fueron las siguientes: Desnaturalización: 95 º C 10 segundos, qRT-PCR x 40 ciclos: 95 ºC 5 segundos, 60 ºC 20 segundos y la curva de disociación: 95 ºC 60 segundos, 55 ºC 30 segundos, 95 ºC 30 segundos. Se determinaron los niveles de U6 como gen de referencia y el nivel de expresión de los miRNAs se normalizó usando dicho gen a través del método ΔΔCT.

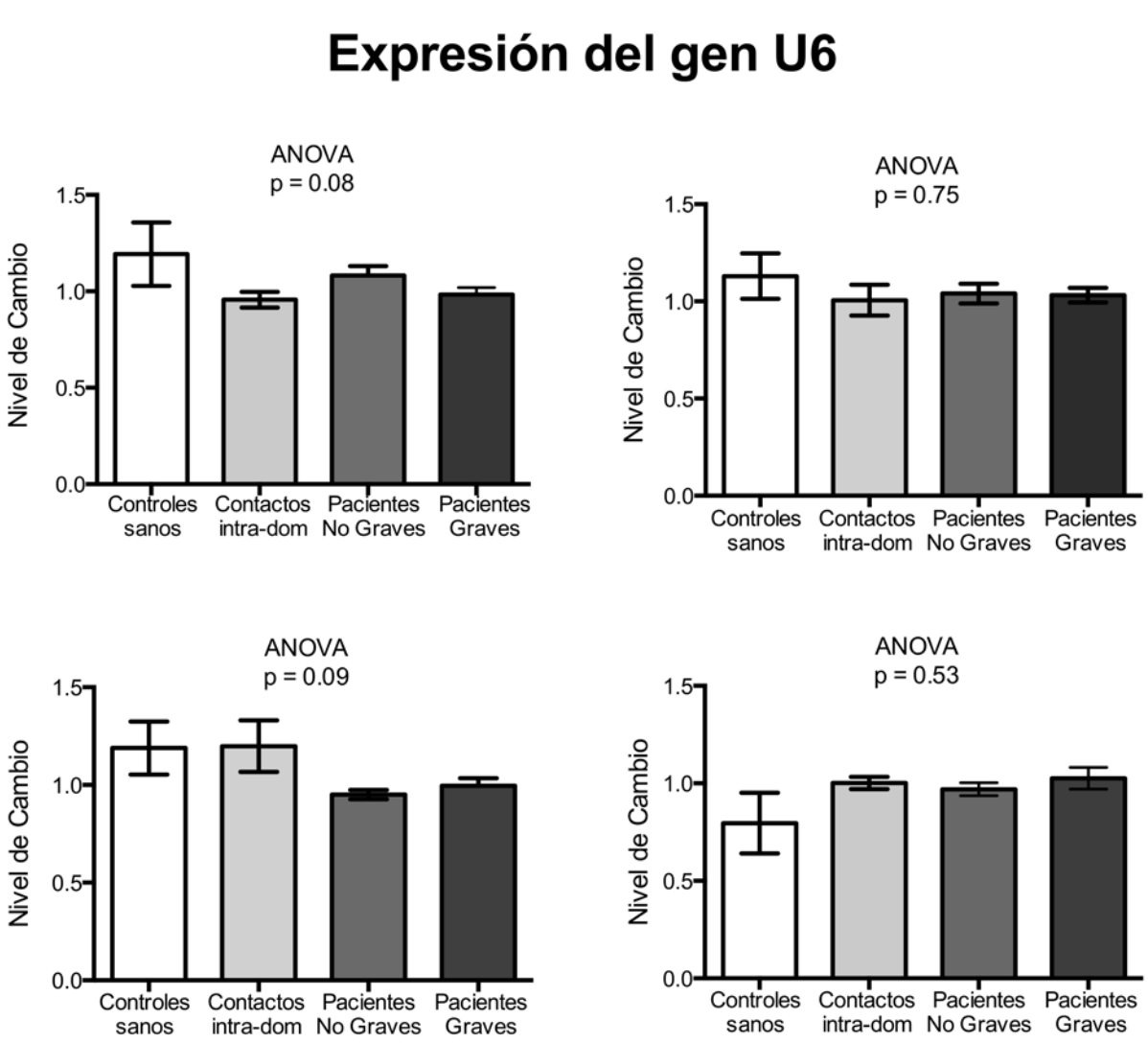

Figura 7. Expresión del gen de referencia U6. El análisis por ANOVA de la expresión del gen U6 – tomado como gen de referencia para la normalización de la expresión de miRNAs – muestra una homogeneidad en su expresión entre los distintos grupos estudiados. Lo anterior permite validar su uso como gen de referencia.

DETERMINACIÓN DE LOS NIVELES DE CITOCINAS, QUIMIOCINAS Y FACTORES DE CRECIMIENTO.

Se determinaron los niveles de citocinas, quimiocinas y factores de crecimiento en suero de todos los individuos incluidos en el estudio (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, CCL2, CCL3, CCL5, CXCL10, G-CSF, GM-CSF, PDGF-bb, FGF and VEGF) a través del instrumento BioPlex-200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) previamente descrito [7].

AISLAMIENTO, IDENTIFICACIÓN Y PROPAGACIÓN DEL VIRUS DE INFLUENZA ESTACIONAL A/H3N2 Y pdm A/H1N1.

Los aislados de los virus estacional H3N2 y pdm A/H1N1 se obtuvieron a partir de pacientes con neumonía asociada al virus de influenza tipo A, los cuales firmaron la carta de consentimiento informado durante su hospitalización en el INER. Se utilizaron células MDCK para el aislado y la propagación viral. Para cada cepa de virus, se evaluó la dosis infectiva al 50% en células MDCK ($TCID_{50}$, por sus siglas en inglés) a través del método de dilución punto final. El titulo del stock se ajustó a 1×10^6 $TCID_{50}$/ml.

INFECCIÓN IN VITRO DE CÉLULAS EPITELIALES A549 CON EL VIRUS DE INFLUENZA pdm A/H1N1 Y EL VIRUS DE INFLUENZA ESTACIONAL H3N2.

Se cultivaron e infectaron por 10 y 24 horas $5x10^5$ células epiteliales A/549 con $5x10^5$ $TCID_{50}$ del virus pdm A/H1N1 ó estacional H3N2. Células A/549 recibieron medio de cultivo sin virus (control negativo). Después de los tiempos indicados, las células fueron recolectadas para el aislamiento de RNA total y la cuantificación de miR-150 por qRT-PCR. Todos los ensayos se realizaron por duplicado.

ANÁLISIS BIOINFORMÁTICO.

Para el análisis de los resultados obtenidos por la tecnología TLDAs se desarrollo un algoritmo en el lenguaje de programación Perl, el cual evaluó para cada miRNA el porcentaje de individuos con valores definidos del ciclo umbral (C_T, por sus siglas en inglés). Aquellos miRNAs que tuvieron determinaciones definidas de C_T en al menos el 50 % de los individuos incluidos en el estudio fueron seleccionados para el análisis de expresión relativa normalizando con el gen de referencia U6 usando la ecuación $\Delta\Delta$Ct. Posteriormente se realizó el análisis estadístico para conocer las diferencias entre los distintos grupos de pacientes estudiados.

Se realizó un enriquecimiento de genes y vías de señalización celular usando el software de análisis DIANA LAB TOOLS (http://diana.imis.athena-innovation.gr). DIANA TOOLS usa el algoritmo microT-CDS v5 para la predicción de los blancos moleculares de miRNAs así como el algoritmo microT v4 para analizar sus interacciones las cuales son originadas a través del algoritmo llamado DIANA-TarBase v6.0. Para el registro de las funciones biológicas de los blancos moleculares y vías de señalización celular de las que forman parte, se utilizó la Enciclopedia Kioto de Genes y Genomas (KEGG, por sus

siglas en inglés). Para el análisis de redes de interacción y modelaje topológico fueron seleccionaron aquellas vías de señalización enriquecidas significativamente (valor de p <0.05) a partir de la predicción de blancos para cada miRNA diferencialmente expresados en el estudio. Las redes de interacción entre miRNAs y blancos moleculares o vías de señalización de las que forman parte, se construyeron usando el software Cytoscape v3.1.1. Para el análisis topológico se usaron gráficos unidireccionales. La centralidad de las redes y tamaños de nodo se calcularon utilizando la herramienta CentiScaPe 1.0 desde el software Cytoscape.

ANÁLISIS ESTADÍSTICO.

Las características demográficas, signos vitales, diagnósticos clínicos, los parámetros de intercambio de gases, pruebas de laboratorio y la función pulmonar se determinaron en la unidad de cuidados intensivos del INER. Las variables continuas se compararon usando la prueba T de student y prueba de ANOVA (por sus siglas en inglés), la variables categóricas se analizaron a través de la prueba chi cuadrada. Para el análisis de la normalidad de los datos se uso la prueba de Kolmogorov-Smirnov. Para el análisis de las diferencias entre dos grupos se usó la prueba de T de student y la prueba U de Mann-Whitney, el criterio de selección fue considerando la normalidad de los datos. La correlación entre los niveles de citocinas/quimiocinas/factores de crecimiento y miRNAs se evaluó usando el coeficiente de correlación de Pearson. Todos los análisis estadísticos se desarrollaron a través del software Graph Pad v5.0.

RESULTADOS

Las características clínicas y demográficas de los grupos de individuos incluidos en el presente estudio se resumen en la Tabla 2. Al realizar una prueba estadística de ANOVA entre los distintos grupos de individuos, no se detectaron diferencias significativas en cuanto a edad e índice de masa corporal promedio (BMI, por sus siglas en inglés). Todos los pacientes graves presentaron ARDS, requirieron ventilación mecánica por lo que fueron admitidos a la Unidad de Cuidados Intensivos del INER en la ciudad de México durante el primer brote epidémico de influenza pandémica por la nueva variante del virus A/H1N1 en el año 2009. Como se esperaba, los pacientes con diagnóstico de ARDS, mostraron un índice de Kirby significativamente más bajo que los pacientes con enfermedad no grave asociada al virus pdm A/H1N1 (Media PaO2/FiO2: 104.7 vs. 227). El índice de Kirby, refleja el estado de

oxigenación de la sangre de un paciente midiendo el intercambio gaseoso y la gravedad de una insuficiencia respiratoria y calculándose a través de la fórmula: presión arterial de oxigeno entre la fracción inspirada de oxígeno (PaO2/FiO2, por sus siglas en inglés)[97]. Los signos y síntomas principales al inicio de la infección tanto en los pacientes graves como aquellos no graves incluyeron tos, fiebre, mialgia, dolor de cabeza y al tercer día de la admisión hospitalaria se presentaron dolor de pecho, disnea y cianosis. Los pacientes con enfermedad severa recibieron Oseltamivir durante el tiempo que permanecieron asistidos por ventilación mecánica mientras que el resto de pacientes permanecieron tratados durante 5 días posteriores al ingreso hospitalario.

Tabla 2. Datos clínicos y demográficos de los individuos en estudio.

	Controles Sanos (n=5)	**Contactos Intra-domiciliarios (n=8)**	**A/H1N1 No Graves (n=8)**	**A/H1N1 Graves (n=8)**	**Valor p**
Edad. Años (media ±SD)	41.1 (±9.7)	41.6 (±6.6)	40.5 (±13.6)	49.5 (±12.9)	0.47[a]
Hombres (%)	3/5 (60%)	2/8 (25%)	4/8 (50.0%)	5/8 (62.5%)	-
Ventilación mecánica (%)	0/5 (0%)	0/8 (0%)	0/9 (0%)	7/7(100%)	-
Días en UCI* (media ±SD)	0	0	0	15(± 10)	-
Índice de Masa C (kg/m^2)	28.3 (±2.1)	29.6 (6.4)	32 (7.0)	30.8 (3.3)	0.75[a]
Índice de Kirby (media ±SD)	-	-	227 (±20.8)	104.7 (±45)	0.006[b]
Diabetes tipo 2 (%)	0/5 (0%)	1/8 (12.5%)*	2/9 (22.2%)*	1/7(14.2%)*	-

Tabla 2. Los datos están expresados como medias ± desviación estándar SD, número o porcentaje. [a] ANOVA, [b] Prueba t de Student. *Unidad de Cuidados Intensivos.

EL VIRUS pdm A/H1N1 INDUCE UNA ALTA EXPRESIÓN DE miR-150 *IN VIVO* E *IN VITRO*.

Mediante la cuantificación por micro-arreglos de expresión rRT-PCR: TLDAs, se detectó un aumento significativo en el nivel de expresión de miR-150 en el suero de pacientes con infección severa por el virus pdm A/H1N1 al compararlo con los pacientes con infección no grave asociada al virus pdm A/H1N1 ($p = 0.0087$) y con respecto de los controles sanos ($p = 0.0235$) (Figura 8A). Este incremento significativo en los niveles circulantes de miR-150 en pacientes graves, se confirmó mediante análisis de expresión por qRT-PCR individual como método de validación sobre los resultados previamente obtenidos a través de la tecnología de microarreglos (Figura 8B).

Para corroborar si los niveles en suero de miR-150 en los pacientes con neumonía severa asociada al virus pdm A/H1N1, correlacionaba con los niveles de expresión de miR-150 *in vitro*, se realizaron ensayos de infección experimental, de células epiteliales A549 con el virus pdm A/H1N1 o el virus H3N2 estacional durante 10 y 24 horas. La expresión de miR-150 se cuantificó por duplicado usando qRT-PCR. En este ensayo se encontró que la infección con la cepa pdm A/H1N1 induce altos niveles de miR-150, particularmente después de 10 h de infección al compararse con la cepa estacional H3N2 ($p = 0.0004$) o con la expresión en las células no tratadas (Fig. 8C).

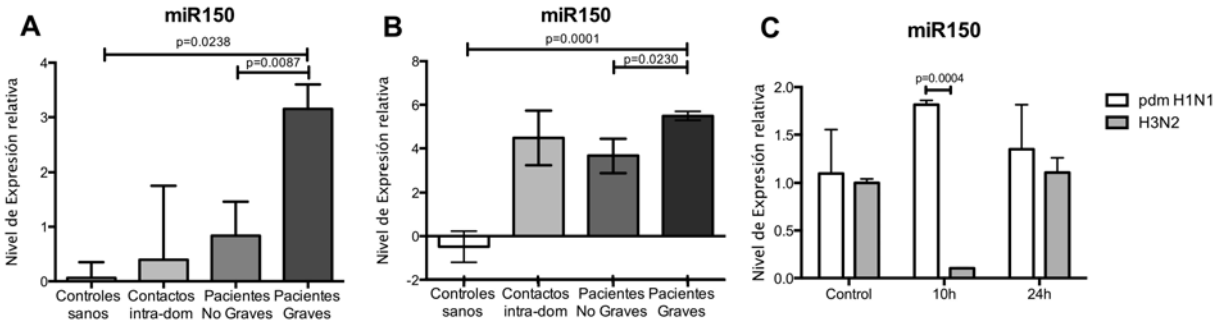

Figura 8. Niveles de expresión de miR-150. A) Lo niveles circulantes de miR-150 analizados por TLDAs se encontraron incrementados en pacientes con la infección severa por el virus A/H1N1 cuando se compararon con los pacientes no graves; B) validación de la expresión de miR-150 por qRT-PRC individual; se confirma la alta expresión de miR-150 en los pacientes graves al compararlos con los sujetos no graves; C) aquí se demuestra que el virus pdm A/H1N1 induce mayores niveles de miR-150 en células epiteliales A549 después de 10 h y 24 h que la cepa estacional H3N2. Los resultados se muestran como unidades de cuantificación relativa (miR-150/U6). Valores de $p < 0.05$ se consideraron como significativos.

Además de miR-150, otros microRNAs incluidos en el microarreglo TaqMan (TLDA: miR-29c, miR-22, miR-145, miR-210, miR-126 and miR-222), mostraron diferencias significativas en sus niveles de expresión en suero de pacientes con la infección por el virus pdm A/H1N1 (Figura 9). La comparación en los niveles circulantes de miR-22 mostró bajos niveles de expresión en los pacientes graves respecto de los pacientes con infección no severa por el virus pdm A/H1N1 ($p < 0.05$). Además también se encontraron diferencias significativas en la expresión de miR-29, miR-210 y miR-145 en los pacientes con infección sintomática de la infección al compararse con la de los contactos intradomiciliarios y/o los sujetos sanos ($p < 0.05$), este perfil de expresión diferencial fue independientemente del curso clínico de la enfermedad. Los niveles de miR-126 y miR-222 estuvieron regulados a la baja en los contactos intra-domiciliarios y los pacientes con neumonía por el virus pdm A/H1N1 (graves y no graves) al compararse con sujetos sanos.

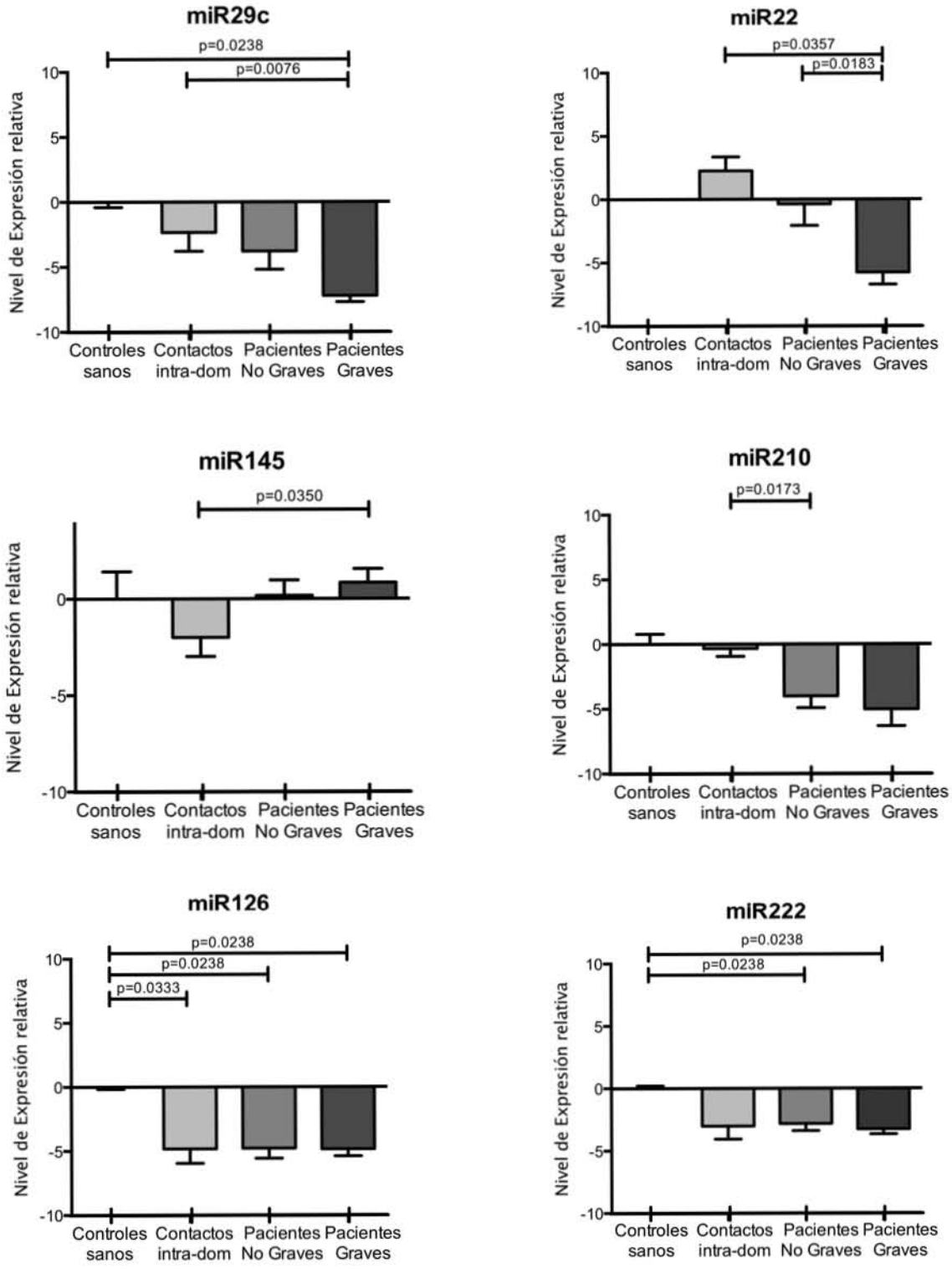

Figura 9. miRNAs diferencialmente expresados en el microarreglo TaqMan (TLDAs). La expresión a la baja de miR-29 y miR-22 se asocia a la forma grave de la infección por el virus pdm A/H1N1. La expresión de miR-145 y miR-210 esta asociada a la forma sintomática de la infección sin distinguir la gravedad y la expresión a la baja de miR-126 y miR-222 se asocia a la infección sin distinguir a ninguna forma clínica de la infección.

LOS PACIENTES CON NEUMONÍA GRAVE ASOCIADA AL VIRUS pdm A/H1N1 MOSTRARON NIVELES INCREMENTADOS DE IL-1RA, IL6, IL-7 Y G-CSF.

Se detecto un incremento de los niveles circulantes de IL-1RA, IL-6, IL-7 y G-CSF en el grupo de pacientes con infección grave por el virus pdm A/H1N1 al compararse con los pacientes con enfermedad no grave asociada al virus pdm A/H1N1 (Figura 10A). Los pacientes con enfermedad severa exhibieron una tendencia a la baja en los niveles circulantes de CCL4 y CCL5 al compararlos con los niveles de los pacientes no graves infectados con el virus A/H1N1; y significativamente disminuidos entre los pacientes graves y los contactos intradomiciliarios ($p \leq 0.0001$).

Todos los individuos que estuvieron expuestos al virus pdm A/H1N1, al comparar sus niveles circulantes de CXCL10 y CXCL8, mostraron niveles incrementados de dichas citocinas con respecto de los sujetos sanos (Figura 10B). Respecto a los niveles circulantes de factores de crecimiento (Figura 10C), se observaron niveles significativamente elevados de G-CSF en los pacientes con neumonía grave asociada al virus pdm A/H1N1, al compararlos con los niveles de los pacientes con neumonía no grave asociada al virus pdm A/H1N1. Los pacientes con enfermedad severa exhibieron una tendencia a la alta en los niveles circulantes de VEGF respecto de los pacientes no graves y un incremente significativo respecto de los sujetos sanos (Figura 10C). Los contactos intradomiciliarios mostraron un incremento en los niveles de PDGF-bb al compararse con los demás grupos estudiados (Figura 10C). Por último, se realizaron correlaciones entre la expresión de miR-150 y los niveles de citocinas/quimiocinas/factores de crecimiento circulantes de los individuos en estudio (Figura 11). La figura 11 muestra las correlaciones significativas ($p < 0.05$) entre la alta expresión de miR-150 y de IL-1b, CXCL8, IFN-γ y G-CSF, particularmente en los pacientes con neumonía severa asociada al virus A/H1N1.

Estos hallazgos en conjunto sugieren que la alta expresión de miR-150 en los pacientes con neumonía grave por el virus de influenza pdm A/H1N1 podría contribuir al fenotipo pro-inflamatorio desregulado en la infección severa por el virus A/H1N1.

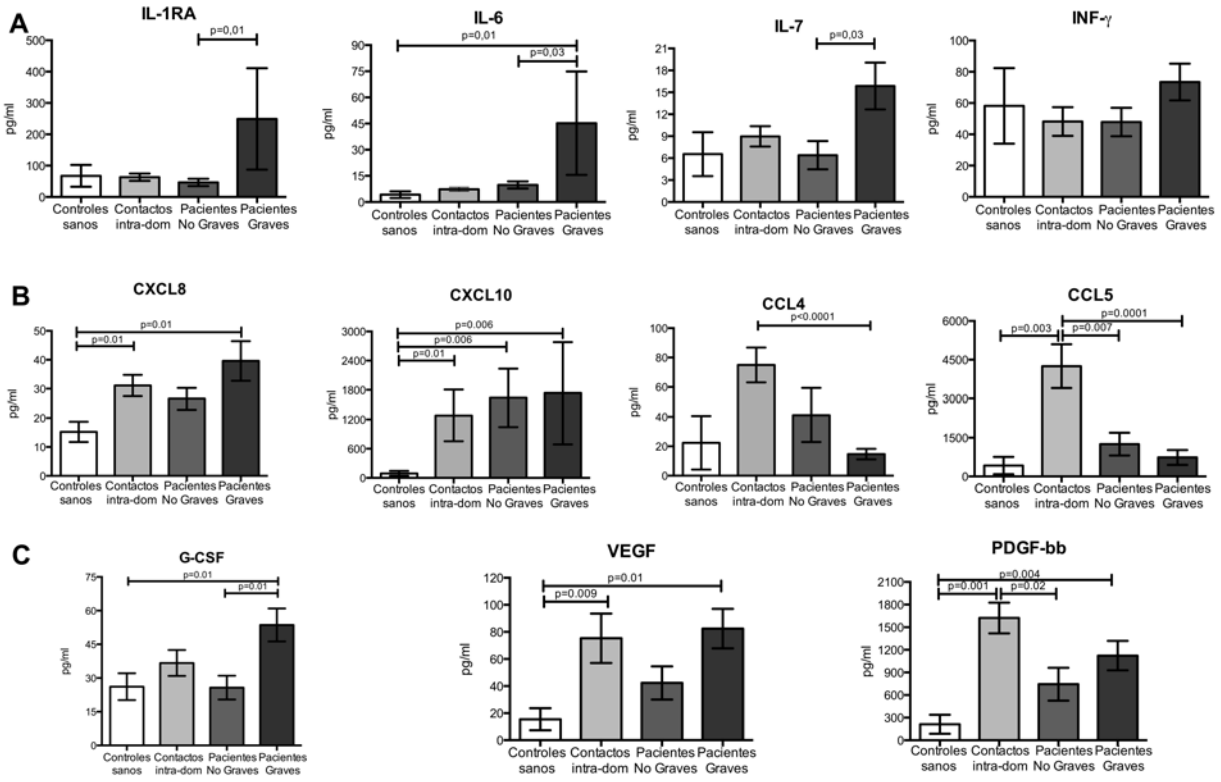

Figura 10. Niveles de citocinas, quimiocinas y factores de crecimiento en suero de pacientes con diferentes formas de la infección por el virus pdm A/H1N1. A) Niveles incrementados de IL-1RA, IL-6 e IL-7 en pacientes graves respecto a los pacientes no graves. Los niveles de IFN-γ fueron similares entre los grupos. B) Los niveles de CXCL8 y CXCL10 se encontraron significativamente elevados en todos los grupos con infección por el virus pdm A/H1N1 respecto de los sujetos sanos particularmente en los individuos graves. C) Los pacientes con enfermedad grave presentaron altos niveles de G-CSF, VEGF y respecto de los sujetos sanos.

ANÁLISIS *IN SILICIO* LA BASE KEGG PARA EL ENRIQUECIMIENTO DE VÍAS DE SEÑALIZACIÓN CELULAR ASOCIADAS A LOS miRNAS DIFERENCIALMENTE EXPRESADOS EN LOS PACIENTES CON LA INFECCIÓN POR EL VIRUS pdm A/H1N1

Para el entendimiento de la participación de la expresión diferencial de miRNAs en la patogénesis de la enfermedad causada por el virus pdm A/H1N1 se analizaron los blancos moleculares de miR-150, miR-29c, miR-22, miR-210, miR-145, miR-126 y miR-222. Posteriormente se realizó un enriquecimiento de vías de señalización celular que determinó a que vía de señalización forma parte cada blanco molecular de los miRNAs diferencialmente expresados en el estudio (Tabla 3). Este análisis mostró que las vías PI3K–Akt–mTOR, cáncer, de respuesta antiviral, apoptosis y vías de adhesión celular están asociadas con el grupo de miRNAs diferencialmente expresados en la infección por el virus pdm A/H1N1.

TABLA 3

miRNA	Expresión	Vía enriquecida	Valor *p*	Genes Blanco
150-5p	↑	PI3K-Akt	0.00925154	MYB, VEGFA
		Cáncer de vejiga	0.00925154	VEGFA
		Vías en cáncer	0.01057432	VEGFA
		Hepatitis B	0.04403999	EGR2
29c-3p	↓	PI3K-Akt	3.20E-05	BCL2,CDK6,COL3A1,COL4A2,COL1A1,COL1A2,LAMC1,Col6a2
		Vías en cáncer	0.004938493	CRKL,BCL2,CDK6,JUN,COL4A2,LAMC1
		Leucemia mieloide crónica	0.02176365	CRKL,CDK6
		Hepatitis B	0.04332757	BCL2,CDK6,JUN
145-5p	↑	Hepatitis B	7.57E-05	IFNB1,TIRAP,MYC,CDKN1A,STAT1
		Cáncer de vejiga	0.000467507	MMP1,MYC,CDKN1A
		Vías en cáncer	0.000522824	IGF1R,TPM3,MMP1,MYC,CDKN1A,STAT1
210	↓	Cáncer de vejiga	0.04300107	E2F3
		Vías en cáncer	0.04772988	APC
		Cáncer de endometrio	0.04964395	APC
222-3p	↓	PI3K-Akt	0.000361926	CDKN1B,DDIT4,PPP2R2A,KIT,FOXO3,PTEN
		Vías en cáncer	0.001791104	FOS,STAT5A,CDKN1B,MMP1,KIT,PTEN
		Hepatitis B	0.003718223	FOS,STAT5A,CDKN1B,PTEN
		Cáncer de endometrio	0.01395174	FOXO3,PTEN
		Leucemia mieloide crónica	0.03403043	STAT5A,CDKN1B
126-5p	↓	Cáncer de endometrio	1.16E-05	KRAS,CTNNB1
		Hepatitis B	0.02422571	KRAS,CDK6,NFAT5
		Leucemia mieloide crónica	0.02422571	KRAS,CDK6

Tabla 3. Análisis de enriquecimiento de miRNAs, sus blancos moleculares y vías de señalización celular implicados en la infección por el virus (pdm) A/H1N1

La red de interacción de los miRNAs diferencialmente expresados en la infección por el virus pdm A/H1N1 muestra la conexión de miR-150, miR-22, miR-210, miR-145 y miR-126 con la vía PI3K–Akt–mTOR, vías de cáncer y la respuesta antiviral. Finalmente, miR-150 y miR-126 interaccionan con la vía de VEGF (Figura 12 y Figura 13). Estos hallazgos demuestran una conexión cercana entre los miRNAs que fueron detectados asociados con la infección por el virus pdm A/H1N1 lo cual caracteriza

molecularmente a las formas clínicas de la enfermedad.

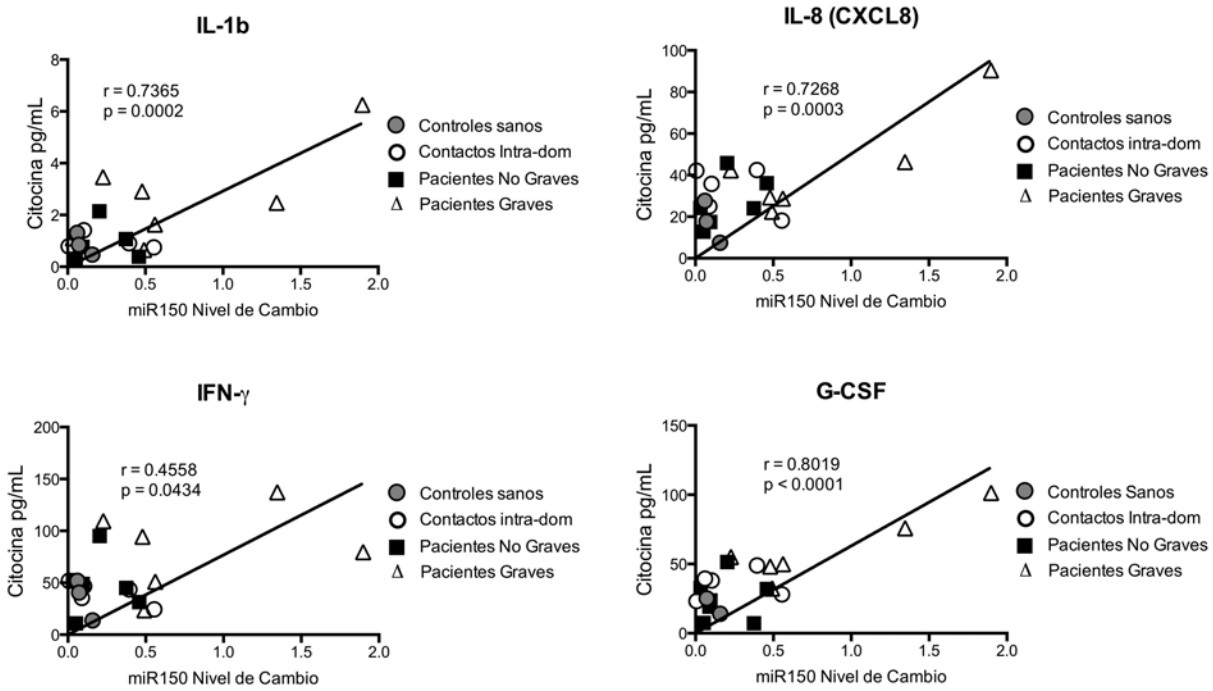

Figura 11. Correlación entre la expresión de miR-150 y los niveles de citocinas, quimiocinas y factores de crecimiento en suero.

DISCUSIÓN

Este estudio ha evaluado el perfil de expresión de miRNAs y de mediadores inflamatorios circulantes en pacientes con formas clínicas diferentes de la infección por el virus pdm A/H1N1. Los altos niveles de miR-150 asociado a la forma severa de la enfermedad por el virus pdm H1N1 fue uno de los hallazgos más relevantes al demostrar el fenómeno de forma independiente al sistema de microarreglos - PCR tiempo real - utilizado en el mapeo inicial y posteriormente encontrar el mismo patrón de expresión en ensayos experimentales de infección de células epiteliales A/549 con la cepa pdm A/H1N1 que induce niveles incrementados de expresión respecto a la cepa estacional H3N2 [9]. Diferentes grupos de investigación han identificado través del uso de microarreglos a miR-150 como uno de sus paneles de miRNAs que estuvieron desregulados en leucocitos y en suero de pacientes con sepsis comparados con sujetos sanos [98-100]. Es importante anotar que estos datos reportan regulado a la baja la expresión de miR-150 en infección bacteriana, enfoque distinto al estudiado en este trabajo acerca de la implicación de miRNAs en una infección viral, sin embargo los autores realzan la importancia de la presencia de este miRNA circulante como marcador pronostico de gravedad. Ma, Y; Persing y

colaboradores encontraron niveles disminuidos de miR-150 en dos cohortes independientes de pacientes: individuos con sepsis y pacientes con síndrome de respuesta inflamatoria sistémica (SIRS, por sus siglas en inglés) respecto de sujetos sanos, correlacionando dichos niveles de miR-150 con niveles elevados del puntaje para la evaluación de fallo orgánico secuencial (SOFA, por sus siglas en inglés), el cual es un indicador clínico importante de gravedad [101] . Además Chorng-Kuang How y colaboradores [100] analizaron el perfil de expresión de miRNAs en sepsis por bacterias gram-negativas encontrando baja la expresión de miR-150 en leucocitos periféricos de pacientes con uro-sepsis. Los mismos autores encontraron bajos niveles en la expresión de miR-150 tras estimular vía receptores tipo Toll (TLRs, por sus siglas en inglés) una línea celular de monocitos THP-1 tratados con LPS e identificaron que la estimulación con poly(I:C), de TLRs de especificidad diferente al LPS (TLR4), tal como TLR3 (involucrado en infecciones virales) incrementa significativamente la expresión de miR-150 [99]. Este estudio está en concordancia con nuestros experimentos de infección de células epiteliales A/549 con el virus estacional H3N2 y el virus pdm H1N1, el cual induce mayores niveles de expresión de miR-150, sugiriendo que la expresión incrementada de miR-150 observada en los pacientes con neumonía severa por el virus de influenza pdm H1N1 es específica de infecciones virales.

Respecto a la participación de miR-150 en el balance de la respuesta inmune, el grupo de Rajewsky y colaboradores demostraron en un modelo de ratón, que miR-150 controla la diferenciación y maduración de células B a través de su blanco molecular c-Myb, el cual es un factor que debe mantener niveles adecuados para el abastecimiento de células B. Dicho factor necesita estar expresado en células precursoras como un factor importante de maduración y diferenciación celular, la expresión de miR-150 en células maduras permite regular a la baja a c-Myb de manera temporal. Es decir una vez que existe un abastecimiento de células B maduras, se mantiene un control en la proporción de las mismas a través de la caída en la expresión de c-Myb pero en caso de las células T CD4, en las que ocurre el mismo patrón de expresión de c-Myb durante su maduración, los autores encontraron que existe una re-expresión de este factor durante la activación de células T CD4 [102].

En el contexto de infecciones, recientemente se ha establecido un vinculo entre miR-150 y el supresor 1 de la señalización de citocinas (SOCS1, por sus siglas en inglés) [103]. Chen y colaboradores, en 2014 evaluaron a través de qPCR la expresión de SOCS1 y miR-150 en células mononucleares de pacientes

con la forma hemorrágica de la infección, es decir la forma grave (DHF, por sus siglas en inglés) por el virus del Dengue (DENV-2, por sus silgas en inglés), así como la infección no hemorrágica (DF, por sus siglas en inglés), la cual es una forma no severa de la infección. Este grupo de investigación encontró que los pacientes con DHF tenían niveles disminuidos de SOCS1 y de IFN-γ y niveles incrementados de miR-150 respecto de los pacientes no graves infectados con DENV-2[103]. En adición este estudio demostró después de enriquecer células CD14+ a partir de sangre periférica de sujetos no infectados por DENV-2 e infectar in vitro con el mismo virus, que existe una caída en la expresión de SOCS1 de manera dosis dependiente tras el tratamiento con miR-150 exógeno (miR-150 mimic), poniendo en evidencia la regulación directa de SOCS1 por miR-150 [103]. SOCS1 forma parte de una familia de proteínas que regulan negativamente la señalización de citocinas a través del control de vías de moléculas transductores de señales y activadores de la transcripción y de la cinasa Janus (JAK/STAT, por sus siglas en ingles), las cuales son parte de la cascada de señalización que se desencadena una vez que el ligando de una citocina se ha unido a su receptor, una JAK se une al dominio citoplasmático de los receptores de citocinas, fosforila la región y permite que la molécula STATs se una también al dominio intracelular citoplasmático donde es fosforilada por JAK para su dimerización, lo cual le permite llegar al núcleo una vez que ha sido activada por JAK y se ha disociado del receptor. Ya en el núcleo STAT induce la expresión de genes blanco uniéndose a una secuencia denominada: secuencia activada gamma (GAS, por sus siglas en inglés) así como a otros motivos específicos en la región promotora [104]. La proteína SOCS1 regula negativamente la producción de las citocinas IFN-α, IFN-γ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-13, IL-15 y TNF-α [104, 105] y existe información en la literatura de que los cambios en la expresión de proteínas SOCS1 y SOCS3 afectan la regulación en la producción de citocinas/quimiocinas durante la infección con el virus de influenza A, tanto en el sistema inmune innato como en el adaptativo [106]. La sobre expresión de miR-150 observada en pacientes graves por la infección con virus pdm A/H1N1 sugiere que este miRNA podría promover un caída en la expresión de SOCS-1 a través de la regulación en la vía JAK/STAT como un mecanismo que induzca la producción elevada de citocinas inflamatorias.

En adición, este estudio se detectaron altos niveles de IL-1RA, IL-6, IL-7, G-CSF, así como un ligero incremento de IFN-γ , CXCL8 y VEGF en suero de pacientes con la infección severa por el virus pdm A/H1N1. El incremento en los niveles de IL-1b, IFN-γ, CXCL8 y G-CSF en los sujetos con neumonía

grave asociada al virus pdm A/H1N1 correlacionaron con la alta expresión de miR-150. El modelo tumoral en ratón de Liu y colaboradores demuestra la que sobre-producción de miR-150 controla la vía de VEGF promoviendo el desarrollo de tumor en un modelo experimental [107]. Estos datos en conjunto, quizá reflejan la participación de miRNAs en la producción de mediadores inflamatorios, así como la falta de balance en su expresión durante la forma grave de la infección por el virus pdm A/H1N1 contribuyendo a la severidad y fatalidad de la enfermedad.

De acuerdo a los datos obtenidos por TLDAs, miR-29c, miR-145 y miR-22 están expresados diferencialmente en pacientes con neumonía severa asociada al virus pdm A/H1N1, mientras que miR-210, miR-126 y miR-122 se encontraron regulados a la baja en individuos que estuvieron expuestos al virus pdm A/H1N1. La literatura reconoce la participación de estos miRNAs en una variedad de procesos celulares; por ejemplo miR-22, que fue encontrado asociado a la forma grave de la infección en este estudio, se ha encontrado alterado en el contexto de cáncer y como marcador pronóstico, promoviendo la transición epitelio-mesénquima en modelos experimentales de cáncer [9, 108, 109]. Respecto a la expresión de miR-29, en este trabajo se encontraron bajos niveles [110] en pacientes con enfermedad severa. Además de su papel en metástasis, miR-29 participa en la regulación de la respuesta inmune y la producción de citocinas pro-inflamatorias de células T. Esta familia de miRNAs inhibe la producción de IFN-γ en células T por la inhibición del factor transcripcional T-box (T-bet) [111] y el modelo experimental en ratones muestra una respuesta Th1 pro-inflamatoria excesiva contra patógenos intracelulares [110]. En concordancia con el patrón de expresión encontrado en este estudio Song y colaboradores encontraron que miR-29a marcaba la infección grave por el virus pdm A/H1N1 a través del uso de microarreglos de expresión y de la validación por qPCR, por lo cual lo identifican como un factor pronóstico de gravedad [112]. En adición a miR-22 y miR-29c, hemos encontrado que miR-126 marcaba la infección de la infección sin distinguir entre la forma clínica de la infección por el virus de pdm A/H1N1. Huang y colaboradores en 2014 encontraron una asociación entre los niveles disminuidos de miR-126 y el síndrome de dificultad respiratoria aguda en un modelo de ratas caracterizado por daño epitelial pulmonar e inflamación extensa de vías respiratorias aguda y crónica [113]. miR-126 es codificado dentro del intrón 7 de la secuencia del gen dominio 7-tipo factor de crecimiento epidermal, (Egfl7, por sus siglas en inglés) con una alta expresión en células endoteliales, está involucrado en el desarrollo vascular. Recientemente se encontró que miR-126 es expresado también en células

dendríticas plasmacitoides (pDCs, por sus siglas en inglés) de manera específica dentro de la estirpe de células del sistema inmune, entre sus funciones, se reconoce que es indispensable para la producción de interferones tipo I [114](IFNs-1, por sus siglas en inglés) por parte de las pDCs, ya que regula la expresión de su blanco molecular tsc-1. TSC-1 es un inhibidor de la vía de mTOR, la cual es importante para la manutención de la vía del VEGF-1 a través de la re-expresión del receptor VEGFR2. En ausencia de miR-126 tsc-1 no es inhibido y éste regula negativamente la vía de supervivencia de miTOR y la re-expresión de VEGR2, particularmente en las pDCs en las que se induce un afecto apoptótico y los bajos niveles de esta subpoblación de células es la causa principal de la ausencia de una respuesta antiviral correcta por la ausencia de las células que son las principales productoras de interferones tipo I. Todos estos hallazgos se identificaron en un modelo experimental en ratones deficientes de miR-126 [114] y abre caminos hacia la búsqueda del mismo fenómeno en un modelo de infección por el virus de influenza A para esclarecer la sub-expresión de miR-126 en la infección humana por el virus pdm A/H1N1.

El análisis de redes de interacción entre los miRNAs diferencialmente expresados y las vías implicadas en el enriquecimiento de blancos moleculares de los mismos, muestra una fuerte interacción con vías de señalización en cáncer, apoptosis, la vía de VEGF y la vía de sobrevivencia celular PI3K-Akt-mTOR. Por lo tanto se puede especular la participación de un grupo de miRNAs diferencialmente expresados en la patogénesis de la infección por el virus de influenza (pdm) A/H1N1 a través de la modulación de diversas vías de señalización celular llevando a una falta de balance de la respuesta inflamatoria en los pacientes que desarrollan una forma severa de la infección. Es importante señalar las limitaciones de este estudio respecto al tamaño de muestra. Debido al enfoque de gravedad y la corta duración del brote epidémico del 2009 no fue posible tener un mayor número de pacientes en beneficio de la calidad en la selección y la clasificación de los mismos. Una segunda limitación es la falta de incluir una cohorte de pacientes con enfermedad respiratoria causada por un virus distinto al pdm A/H1N1 que permitiera evidenciar el efecto directo de esta nueva variante de influenza viral humana en la patogénesis de la infección. Una tercera limitante es la falta de evidencia experimental in vivo e in vitro que permita entender la participación de los miRNAs diferencialmente expresados a nivel sistémico en la respuesta inmune contra el virus de influenza pdm A/H1N1 y la por último la necesidad de esclarecer y validar por qRT-PCRs individuales, la expresión de miR-29c, miR-22 y miR-126 en estudios posteriores.

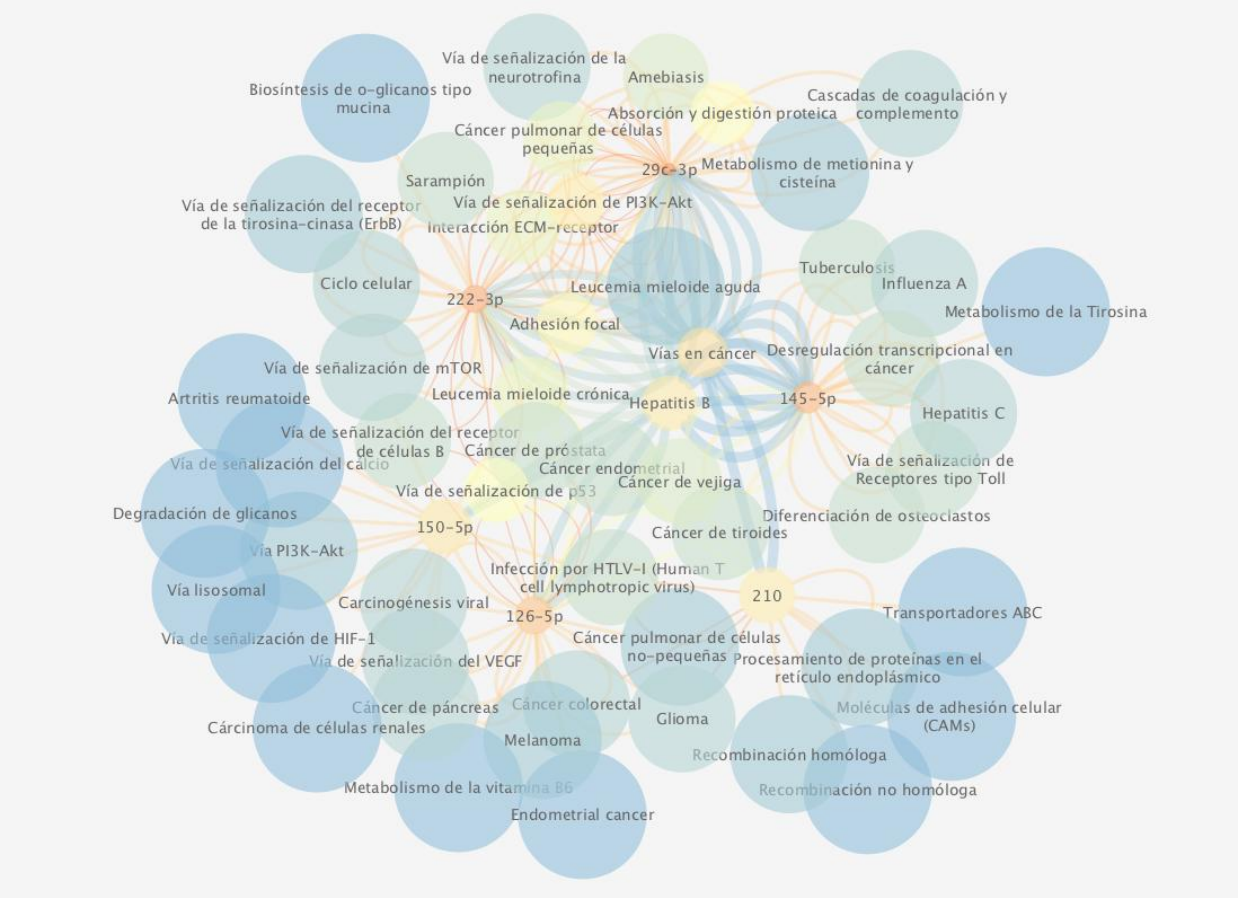

Figura 12. Red de interacción entre los miRNAs diferencialmente expresados y vías de señalización celular. La red de interacción se construyó a través del software Cytoscape v.3.3.0. Los blancos moleculares de la predicción para el grupo de miRNAs diferencialmente expresados en la infección por el virus pdm A/H1N1, son componentes de las vías de señalización que s muestran en el interactoma. Al centro se muestran las interacciones más fuertes de acuerdo al numero de miRNAs que interaccionan con cada vía.

CONCLUSIONES.

1. El presente estudio confirmó la importancia del perfil de expresión de miRNAs circulantes asociados con mediadores inmunológicos como posibles blancos, entre ellos citocinas pro-inflamatorias para el entendimiento de la patogénesis de la infección con el virus A/H1N1.

2. Los resultados obtenidos permiten concluir que la expresión de miR-150, miR-29c, miR-22, miR-210, miR-145, miR-126 y miR222 esta alterada en pacientes con distintas formas clínicas de la infección por el virus pdm A/H1N1.
3. Los niveles elevados de miR-150 están asociados a la forma grave de la enfermedad causada por el virus pdm A/H1N1 existiendo una correlación entre los niveles circulantes de miR-150 y los diferentes mediadores pro-inflamatorios así como factores de crecimiento incluidos: IL-1b, IFN-γ, CXCL8 y G-CSF.
4. Se detectaron niveles significativamente incrementados de IL-1ra, IL6, IL-7 y G-CSF en los pacientes graves por neumonía asociada al virus pdm A/H1N1.

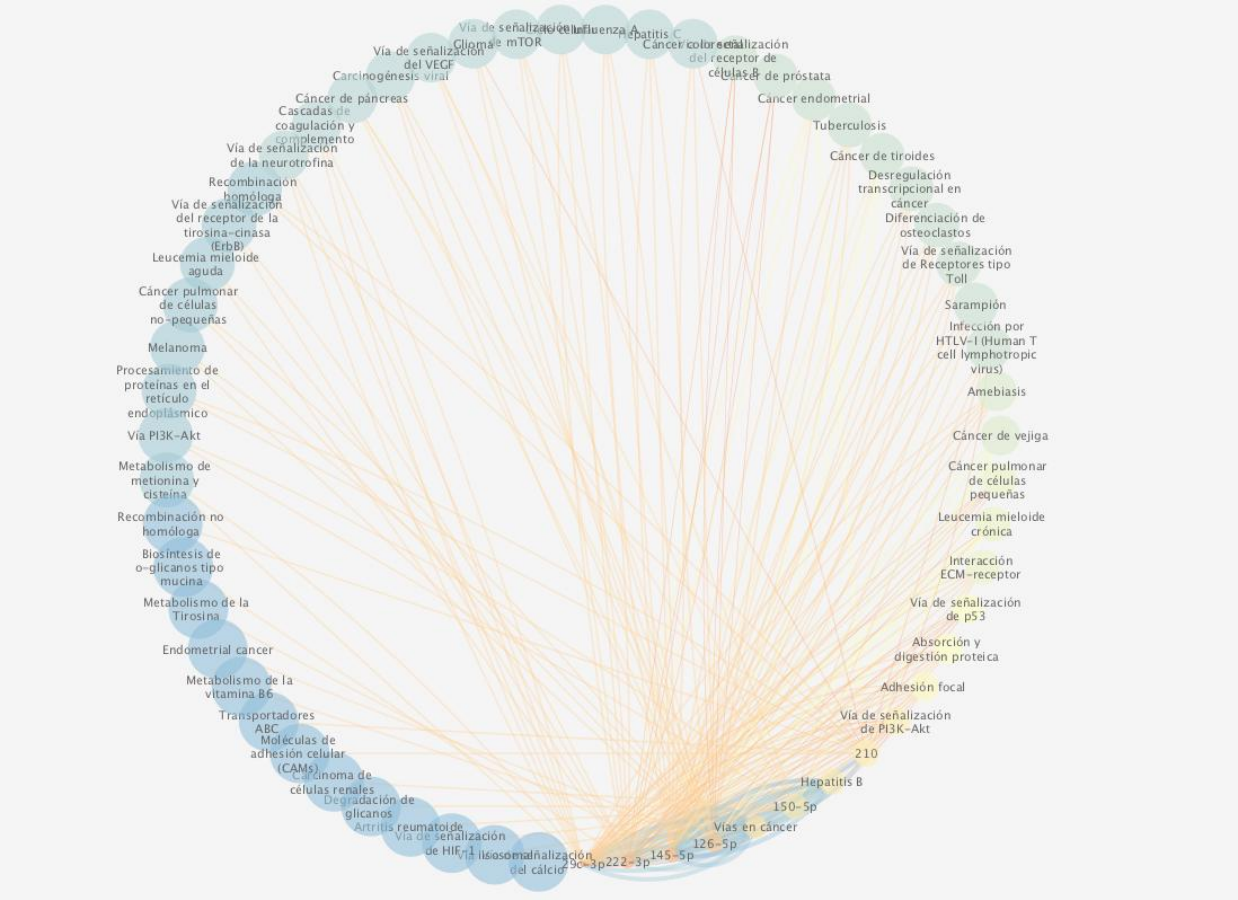

Figura 13. Red de interacción circular entre los miRNAs diferencialmente expresados y vías de señalización celular. La red de interacción se construyó a través del software Cytoscape v.3.3.0. Los blancos moleculares de la predicción para el grupo de miRNAs diferencialmente expresados en la infección por el virus pdm A/H1N1, son componentes de las vías de señalización que se muestran en el interactoma. Representación circular que muestra – de mayor a menor – en un ordenamiento similar al sentido contrario de manecillas del reloj, el grado de interacción de cada vía con el número de miRNAs diferencialmente expresados en la infección por el virus pdm H1N1.

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Circulating levels of miR-150 are associated with poorer outcomes of A/H1N1 infection☆

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ABSTRACT

Background: Overproduction of pro-inflammatory cytokines and chemokines is frequently associated with severe clinical manifestations in patients infected with influenza A/H1N1 virus. Micro-RNAs (miRNAs) are highly conserved small non-coding RNA molecules that post-transcriptionally regulate gene expression and are potential biomarkers and therapeutic targets in different inflammatory conditions.
Methods: We studied the circulating and miRNA profiles in critically ill A/H1N1 patients, A/H1N1 patients with milder disease, asymptomatic housemates and healthy controls. Cytokine, chemokine and growth factors that were potential targets of differentially expressed miRNAs were assessed. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and interactome analysis of these miRNAs were also performed.
Results: Critically ill patients exhibited a significant over-expression of circulating miR-150 ($p < 0.005$) when compared to patients with milder disease. miR-29c, miR-145 and miR-22 were differentially expressed in patients with severe A/H1N1 disease whereas miR-210, miR-126 and miR-222 were downregulated in individuals exposed to the A/H1N1 virus. Significant correlations ($p < 0.05$) between circulating levels of miR-150 with IL-1ra, IL-2, IL-6, CXCL8, IFN-γ, CXCL10 and G-CSF were detected, particularly in critically ill patients.
Conclusion: The up-regulation of miR-150 is associated with poorer outcomes of A/H1N1 infection. The differential expression of miRNAs related with immune processes in severe A/H1N1 disease supports the potential role of these miRNAs as biomarkers of disease progression.

1. Background

Influenza A viruses cause frequent outbreaks of acute respiratory tract infections and represent a significant public health threat (Lapinsky, 2010). The recent pandemic outbreak caused by the swine-origin influenza A/H1N1 virus in 2009 (Smith et al., 2009) affected most countries, and its severe manifestations caused over 18,000 confirmed deaths worldwide (Writing Committee of the WHOCoCAoPI et al., 2010) but estimates of deaths go as high as 270,000 (Dawood et al., 2012). Several studies have revealed that pandemic (pdm) A/H1N1 virus strain infects and efficiently replicates in epithelial cells and macrophages of the lower respiratory tract leading to cell activation and the production of inflammatory mediators that contribute to an acute inflammatory microenvironment in the lung (Itoh et al., 2009).

Even though immune mediators such as cytokines, chemokines and growth factors are critical to controlling influenza A virus infection, their overproduction in an uncontrolled inflammatory response can lead to devastating lung damage (Zuniga et al., 2011; Monsalvo et al., 2011;

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Bermejo-Martin et al., 2010). Humans infected by pdm A/H1N1 strain often-present high serum levels of pro-inflammatory cytokines and chemokines, which are believed to contribute to the disease pathogenesis and the development of acute respiratory distress syndrome (ARDS) (Zuniga et al., 2011; Monsalvo et al., 2011; Bermejo-Martin et al., 2010; Bautista et al., 2013). Experimental and cross-sectional studies have demonstrated that exaggerated production of inflammatory/angiogenic and metabolic mediators is associated with a higher risk to develop ARDS in pdm A/H1N1 infected patients (Bautista et al., 2013; Ubags et al., 2014). However, the mechanisms related with the immunopathogenesis of severe pneumonia by pdm A/H1N1 virus are not fully comprehended. Understanding the factors determining these poor outcomes is therefore of great importance, particularly in the context of the appearance of newly more pathogenic or oseltamivir resistant strains.

In this context, miRNAs regulate the expression of other genes by different mechanisms and influence most of the cellular processes including antiviral inflammatory responses (Perera and Ray, 2007). Circulating miRNA concentrations differ according to physiological and pathological states such as obesity, type 2 diabetes, systemic inflammatory diseases, cancer and infectious diseases (Contu et al., 2010; Zampetaki et al., 2010; Houzet et al., 2008; Pan et al., 2012), suggesting that miRNAs may be useful biomarkers for diagnosis and clinical progression of various diseases.

We studied the expression profile of miRNAs in peripheral blood from critically ill patients with pdm A/H1N1 virus infection as well as in pdm A/H1N1 patients with mild disease, asymptomatic household contacts and healthy donor controls to determine its role as a biomarker related to severe disease and to possibly understand its role on dysregulated inflammatory responses.

2. Methods

2.1. Subjects

Serum samples from pdm A/H1N1 infected patients, healthy donors and household asymptomatic contacts were collected during the 2009 outbreak at the National Institute for Respiratory Diseases (INER) Ismael Cosío Villegas in Mexico City. We analyzed the circulating miRNA profile in 29 individuals (8 with diagnosis of severe pneumonia by A/H1N1 virus, 8 with mild pneumonia by A/H1N1, 8 household asymptomatic contacts and 5 healthy controls). The diagnosis of ARDS was based on standard definitions (Bernard et al., 1994). The asymptomatic household contacts were considered as those individuals that were in close contact with confirmed pdm A/H1N1 patients that did not develop acute respiratory disease. The group of healthy controls had no history of respiratory flu-like illness in the last 6 months. Specific anti-A/H1N1 antibodies were detected in 76.5% of the household contacts (>1:16), whereas significant titers were not detected in the group of healthy controls.

The Institutional Review Board (IRB) of the INER reviewed and approved the protocols for influenza studies. All subjects or their legal responsible, particularly in case of critically ill patients, provided written informed consent for these studies, and they authorized the storage of their DNA samples at INER repositories for this and future studies. In this study, we did not collect samples from minors/children, only young adults older than 17 years were included.

2.2. A/H1N1 virus detection

Nasal swab samples and BAL were obtained from hospitalized patients at the INER, and sent to the INER Microbiology lab for RNA isolation using the viral RNA mini kit (*Qiagen Westburg*, *Leusden*, *The Netherlands*). Detection of swine-influenza viruses in respiratory specimens was assessed by real time RT-PCR according with CDC and World Health Organization guidelines.

2.3. Anti-H1N1 antibody titers

The titers of serum anti-A/H1N1 antibodies were measured by using the hemagglutination inhibition method (HAI). Briefly; serially-diluted aliquots of serum samples (25 μl) in PBS were mixed with 25 μl aliquots of the A/H1N1 virus strain isolated at our Institute (corresponding to four hemagglutination units). The serum-virus dilutions were incubated for 30 min at room temperature. 50 μl of 0.5% chicken erythrocytes were added and after 30 min the HAI activity was evaluated. The serum HAI antibody titer was established as the reciprocal of the last serum dilution with no hemagglutination activity. Those individuals with titers greater than dilution 1:16 were considered as positive for the A/H1N1 infection/exposure.

2.3.1. Circulating miRNA expression profiling in patients with A/H1N1 infection, asymptomatic housemates and healthy controls

Total circulating RNA was isolated from a serum volume of 250 μl using Trizol LS (*Invitrogen*, *Carlsbad*, *CA*). miRNAs were reverse transcribed using the Megaplex™ RT Primers, Human Pool v3.0 (*Applied Biosystems, Foster City, CA*). Amplified products were loaded on TaqMan Low Density Arrays (TLDAs). TaqMan Human MicroRNA Array v3.0 A and B (*Applied Biosystems*, *Foster City*, *CA*) covering 754 miRNA species, were used for miRNA profiling in patients and controls. The TaqMan Low Density Gene Expression Assays for miRNAs are placed in 384-well microfluidic cards and includes three housekeeping genes U6, U44 and U48. Quantitative PCRs on TLDAs were performed on a 7900HT Fast Real Time PCR System (*Applied Biosystems*, *Foster City*, *CA*). U6 reference control was the most stable in expression (around 1.0 fold of change) as we show in four replicates in each group of patients studied, Supplementary Fig. 1. Thus we performed standard normalization using U6 RNA endogenous control, and the data were analyzed using the $\Delta\Delta C_T$ method.

2.3.2. Quantitative RT-PCR for miRNA expression validation

qRT-PCR was used to quantify individual miRNAs that were differentially expressed using TLDA assays. Briefly, cDNA was synthesized from total plasma RNA using specific stem-loop primers. The final volume of PCR reaction mixture was 10 μl and included 2 μl of RT product, 10 μl of SYBR® Premix Ex Taq™ PCR master mix (2× concentration), 1 μl of the following miRNA forward primers: hsa-miR-210: 5′-CTGTGCGTGTGACAGCGGCTGA; hsa-miR-145: 5′-GTCC AGTTTTCCCAGGAATCCCT; hsa-miR-22: 5′-AAGCTGCCAGTTGAAGAA CTGT; hsa-miR29c: 5′-TAGCACCATTTGAAATCGGTTA; hsa-miR-126: 5′-TCGTACCGTGAGTAATAATGCG; hsa-miR-222: 5′-AGCTACATCT GGCTACTGGGT; hsa-miR-150: 5′-TCTCCCAACCCTTGTACCAGTG and U6 Forward: 5′-GCTTCGGCAGCACATATACTAAAAT U6 Reverse: 5′-CGCTTCACGAATTTGCGTGTCAT. qRT-PCR assays were performed in a Quant Studio 12K Flex System real time PCR instrument (*Life Technologies*, *Foster City*, *CA*). The reactions were carried out with a 10 min incubation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate, and the average threshold cycle and SD values were calculated. The relative expression level of the miRNAs was calculated by the $\Delta\Delta C_T$ method using U6 housekeeping expression level.

2.3.3. Measurements of circulating levels of cytokines, chemokines and growth factors

Serum levels of cytokines, chemokines and growth factors (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, CCL2, CCL3, CCL5, CXCL10, G-CSF, GM-CSF, PDGF-bb, FGF and VEGF) were assessed by Luminex using the instrument BioPlex-200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as previously described (Zuniga et al., 2011).

2.4. Seasonal and pdm A/H1N1 influenza virus isolation, identification, and propagation

Influenza pdm A/H1N1 and seasonal H3N2 virus isolates were obtained from patients with severe pneumonia and who signed an informed consent letter, during their hospitalization at the INER. Live influenza pdm A/H1N1 and seasonal H3N2 viruses were isolated in Madin–Darby canine kidney cells (MDCK). They were cloned by limiting dilution, and virus stocks were prepared in MDCK cells. Virus infectivity was assessed by titration of tissue culture infection dose 50% ($TCID_{50}$) in MDCK cells. The titer of the virus stock was adjusted to 1×10^6 $TCID_{50}$/ml.

2.5. In vitro infection of A549 epithelial cells with seasonal H3N2 or pdm A/H1N1 influenza viruses

Epithelial A549 cells were infected with 5×10^5 $TCID_{50}$ of the pdm A/H1N1 or seasonal H3N2 strains. Mock-treated A549 cells received virus-free culture medium. A549 infected cells were cultured during 10 h and 24 h and were harvested for RNA isolation. All assays were performed by triplicate.

2.5.1. Bioinformatics analyses

For miRNA TLDA microarray data analyses were performed with an algorithm designed in Perl language. The percentage of patients that had a defined C_T value for each miRNA was evaluated. For the analysis, we selected the miRNAs with defined C_T values that were present in at least 50% of the studied individuals. Thus miRNAs that showed significant differences in the fold of change among groups were obtained. As we mentioned data were first analyzed as fold of change in each group and data from TLDA microarrays and validations by quantitative RT-PCR were also analyzed after normalization using U6 as housekeeping gene using the $\Delta\Delta C_T$ equation.

Gene and pathway enrichment were performed through the DIANA LAB TOOLS (http://diana.imis.athena-innovation.gr). DIANA TOOLS use the microT-CDS v5 algorithm for target prediction, microT v4 for the experimentally validated miRNA interactions derived from DIANA-TarBase v6.0. The curated pathway database KEGG was used to annotate the biological functions and canonical pathways of the targets. Pathways that were significantly enriched by the predicted target (p-value < 0.05) were selected for network modeling and topological analysis.

To link miRNAs with their target genes within enriched pathways, interactome networks were constructed using the software Cytoscape v3.1.1. For the network topological analysis unidirectional graphs were used. The CentiScaPe 1.0 plug-in was used to calculate network centralities, node degrees for constructed networks.

2.6. Statistical analysis

Demographic characteristics, vital signs, clinical outcomes, treatments, mechanical ventilation and gas exchange parameters, laboratory tests, and lung function scores were determined at the admission to the ICU. Variables were compared using the Student's t test and one-way analysis of variance (ANOVA).

We performed a normality test using Kolmogorov–Smirnov test. The differential expression of microRNAs between groups was assessed by ANOVA and Kruskal–Wallis tests. Mann–Whitney U and Student t-tests were used to analyze differences between two groups. The correlation between cytokine/chemokine/growth factor levels and miR-150 was assessed using Spearman's correlation coefficient. All statistical analyses were performed using the GraphPad v 5.0 software.

3. Results

Clinical and demographic characteristics of the studied groups are summarized in Table 1. Significant differences in the mean of age and body mass index (BMI) were not detected using ANOVA analysis among the groups. All critically ill patients had diagnosis of acute respiratory distress syndrome (ARDS) required mechanical ventilation and were admitted to the Intensive Care Unit (ICU) at the INER in Mexico City during the first A/H1N1 outbreak in 2009. As expected, patients with the diagnosis of ARDS displayed a lower value of Kirby index (PaO_2/FiO_2) than patients with milder A/H1N1 disease (mean: 104.7 vs. 227). The main signs and symptoms at the start of the illness included fever, myalgia, cough, and headaches, while chest pain, dyspnea, and cyanosis occurred after the third day. Critically ill patients received 150 mg/d of Oseltamivir during mechanical ventilation, whereas the remaining patients received it in general during 5 days.

3.1. Pandemic A/H1N1 virus induces a high expression of miR-150 in vivo and in vitro

We found that circulating miR-150 levels were significantly higher in patients with severe A/H1N1 disease when compared with patients with milder A/H1N1 disease ($p = 0.0087$) and to healthy controls ($p = 0.0235$) (Fig. 1A). This significant increase in the circulating levels of miR-150 in critically ill patients was confirmed by qRT-PCR that was used as a validation method, Fig. 1B.

To corroborate if the ex vivo expression levels of circulating miR-150 in critically ill A/H1N1 patients correlate with the miR-150 expression levels in vitro, we performed triplicate series of experimental infection assays of A549 epithelial cells with both pdm A/H1N1 and seasonal H3N2 strains during 10 and 24 h. The expression of miR-150 was measured by qRT-PCR by duplicate. In this assay we found that the infection with the pandemic A/H1N1 strain induces higher levels of miR-150, particularly after 10 h of infection, when compared to the seasonal H3N2 strain ($p = 0.0004$) or mock treated A549 cells, Fig. 1C.

We also found a set of miRNAs (miR-29c, miR-22, miR-145, miR-210, miR-126 and miR-222), included in the TLDA microarrays, which exhibited significant differences in their circulating levels in patients with the A/H1N1 infection, Supplementary Fig. 2. Circulating levels of miR-22 were lower in the group of critically ill A/H1N1 patients when compared to the patients with mild A/H1N1 disease ($p < 0.05$). We also found differential levels of miR-29 miR-210 and miR-145 in patients with A/H1N1 infection independently from the clinical outcome when compared with contacts and controls ($p < 0.05$). Levels of miR-126 and miR-222 were downregulated in household contacts and A/H1N1 patients when compared to healthy controls.

3.2. Patients with severe A/H1N1 disease exhibit differential circulating levels of IL-6, IL-7, CCL5, GCSF and VEGF

A significant increase of the circulating levels of IL-1ra, IL-6 and IL-7 ($p < 0.05$) was detected in the group of patients with severe A/H1N1 infection when compared to the patients with milder A/H1N1 disease, contacts and healthy controls, Fig. 2A. Interestingly, patients with severe disease exhibited markedly lower levels of CCL4 and CCL5 than patients with milder A/H1N1 disease, but particularly when the severe patients were compared to household contacts ($p \leq 0.0001$). All A/H1N1 exposed individuals had significant higher levels of CXCL10 and CXCL8 as compared to healthy controls, Fig. 2B. Regarding growth factors, higher levels of G-CSF and VEGF were observed in patients with severe A/H1N1 disease than in patients with milder disease, contacts and controls. Household asymptomatic contacts exhibited a marked increase in the levels of PDGF-bb when compared to other groups, Fig. 2C.

Additionally, we correlate the miR-150 expression and the cytokine/chemokine/growth factor levels between patients with milder and severe A/H1N1 infection. In Fig. 3 we show the significant correlations

Table 1
Clinical and demographic characteristics of individuals included in the study.

	Healthy Controls (n = 5)	Asymptomatic contacts (n = 8)	A/H1N1 Mild (n = 8)	A/H1N1 Severe (n = 8)	p value
Age mean (±SD)	41.1 (±9.7)	41.6 (±6.6)	40.5 (±13.6)	49.5 (±12.9)	0.47[a]
Male (%)	3 (60)	2 (25)	4 (50.0)	5 (62.5)	–
Mechanical ventilation n (%)	–	–	–	8(100)	–
Days on critical care unit	0	0	0	15(±10)	–
Body mass index (kg/m^2) mean	28.3	29.6	32.0	30.8	0.75[a]
Kirby index mean (±SD)	–	–	227 (±20.8)	104.7 (±45)	0.006[b]
Type 2 diabetes n (%)	0 (0)	1(12.5)	1 (12.5)	1 (12.5)	–

Data are expressed as means ± standard deviation (SD) or number and percentage.
[a] ANOVA (significant differences in age mean and body mass index were not observed using analysis of variance among groups)
[b] Student's t test (significant differences in the Kirby index mean were detected between patients with mild and severe A/H1N1 disease).

($p < 0.05$) between a higher expression of miR-150 and IL-1b, CXCL8, IFN-γ, and G-CSF, particularly increased in patients with severe A/H1N1 pneumonia.

Taking together these findings we suggest that up-regulation of miR-150 observed in critically ill patients but not in patients with milder disease, is contributing to the dysregulated pro-inflammatory phenotype in severe A/H1N1 infection.

3.3. KEGG pathway enrichment analysis of the miRNAs differentially expressed in patients with severe A/H1N1 infection

To understand the potential influence of the differentially expressed miRNAs in the pathogenesis of severe A/H1N1 disease, we analyzed the predicted targets of the differentially expressed miRNAs using the KEGG pathway enrichment analysis. We found eight pathways that were significantly enriched, Table 2. This analysis of the predicted targets shows that PI3K–Akt–mTOR, cancer, antiviral response, apoptosis and cell adhesion molecule pathways are associated with the set of the differentially expressed miRNAs in A/H1N1 infection. Target genes of miRNA-150, that was significantly over-expressed in peripheral blood of patients with severe disease but not in patients with mild disease, were mainly VEGFA, MYB and EGR2 that are related with the phosphatidylinositol 3′-kinase (PI3K)–Akt–mTOR signaling pathway.

The interactome network of the differentially expressed miRNAs in A/H1N1 infection shows a connection between miR-150, miR-210, miR-145, miR-22 and miR-126 networking principally with the (PI3K)–Akt–mTOR pathways, cancer pathways, and antiviral pathways. Finally, miR-150 and miR-126 are linked to the VEGF pathway, Fig. 4 and Supplementary Fig. 3. These findings demonstrate a close connection between the miRNAs that we detected associated with both A/H1N1 infection and characterizing the poor outcome of the disease.

4. Discussion

miRNAs have an important role in the regulation of many processes including inflammation in different clinical conditions (Perera and Ray, 2007). It is well known that uncontrolled immune inflammatory responses may contribute to the pathogenesis of severe lung damage in mice and humans with pandemic A/H1N1 virus infection (Zuniga et al., 2011; Bautista et al., 2013; Ubags et al., 2014; Ramirez-Martinez et al., 2013).

In the current study we analyzed the circulating miRNA profiles and inflammatory mediators circulating levels in patients with different clinical presentations of the A/H1N1 infection and the most striking findings were: 1) high circulating levels of miR-150 are associated with severe A/H1N1 illness; 2) in experimental infection assays pandemic A/H1N1 induces higher levels of miR-150 than seasonal H3N2 strain; 3) miR-29c, miR-145 and miR-22 are differentially expressed in patients with severe A/H1N1 disease whereas miR-210, miR-126 and miRT-222 are downregulated in individuals exposed to the A/H1N1 virus; 4) there is a correlation between circulating levels of miR-150 with different pro-inflammatory mediators and growth factors including: IL-1b, IFN-γ, CXCL8 and G-CSF, particularly in critically ill A/H1N1 patients and 5) PI3K–Akt–mTOR, apoptosis, cancer and antiviral pathways are the most associated with the differentially expressed miRNAs in A/H1N1 infection.

We found a higher expression of miR-150 in the blood from critically ill patients with A/H1N1 infection when compared to A/H1N1 patients

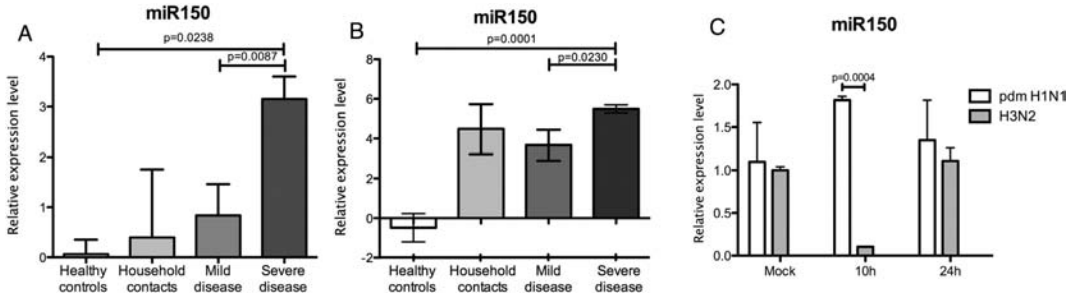

Fig. 1. Circulating levels of miRNAs in patients with A/H1N1 infection, contacts and controls. A) Circulating levels of miR-150 analyzed by TLDA method were higher in patients with severe A/H1N1 infection (critically ill A/H1N1 infected patients) when compared to patients with milder disease; B) in this figure we show the validation of miR-150 expression by qRT-PCR confirming the higher expression in severe A/H1N1 patients; and C) here we demonstrate that the pandemic A/H1N1 virus induces significantly higher levels of miR-150 than the seasonal H3N2 strain in epithelial A549 cells after 10 h and 24 h. Results are shown as means of relative quantitation units (miR-150/U6) +/− range. p values < 0.05, were considered significant.

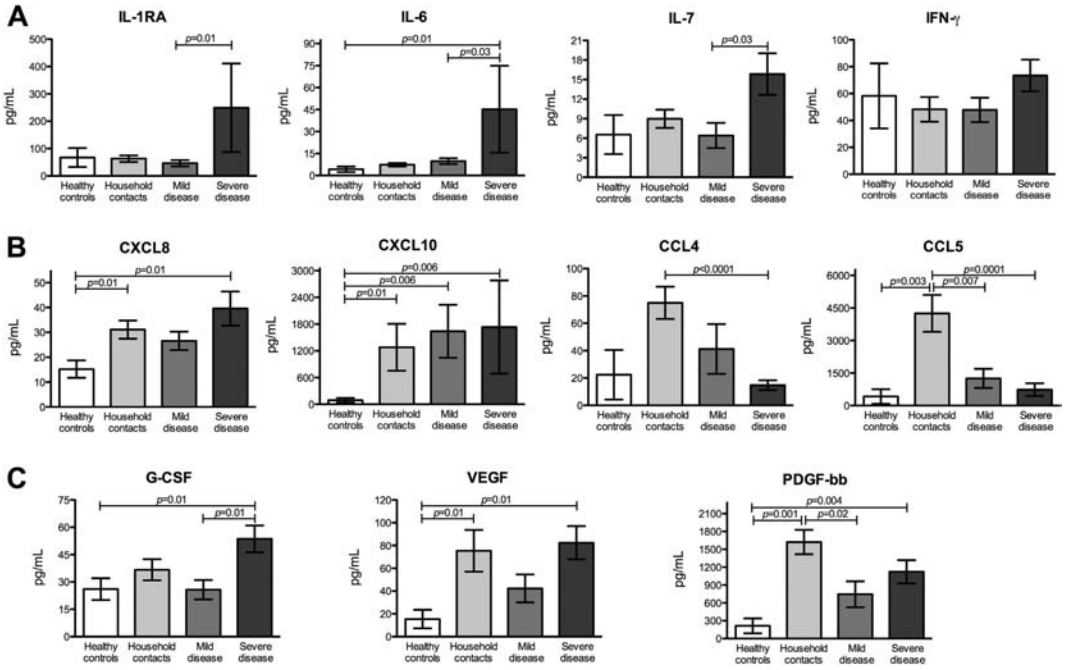

Fig. 2. Cytokine/chemokine/growth factor levels in serum samples from healthy controls, household contacts and pandemic A/H1N1 infected patients with mild and severe illness. A) Higher levels of IL-1RA, IL-6, and IL-7 were found in serum from critically ill patients, but not in non-critical patients and control groups. The levels of IFN-γ were similar among groups. B) The levels of CXCL8 and CXCL10 were markedly higher in all groups exposed to the A/H1N1 virus, particularly in critically ill patients, when compared to controls. The levels of CCL4 and CCL5 were significantly lower in patients with severe illness when compared with asymptomatic contacts. C) Critically ill patients exhibited higher levels of G-CSF and VEGF and lower levels of PDGF-bb than non-critically ill patients and controls. Results are shown as means ± SEM. Differences in the levels of the cytokines/chemokines and growth factors were analyzed by Mann–Whitney U test. p values < 0.05 were considered statistically significant.

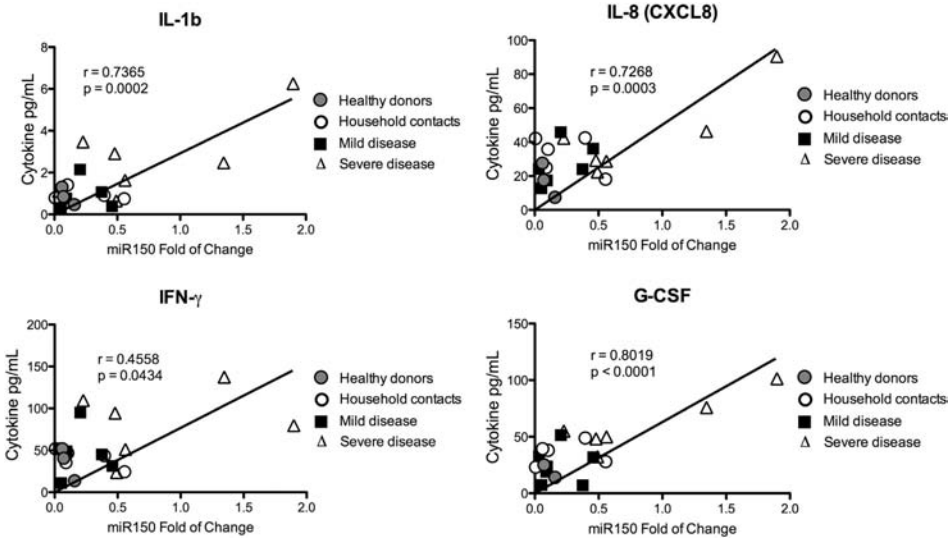

Fig. 3. Significant correlations between circulating levels of miR-150 and different cytokines, chemokines and growth factors in healthy controls, household contacts and pandemic A/H1N1 infected patients with mild and severe illness. In this figure we have shown the correlations between circulating levels of miR-150 and IL-1b, IFN-γ, CXCL8, and G-CSF. These positive correlations were particularly significant ($p < 0.05$) in the group of patients with severe A/H1N1 pneumonia.

Table 2
Pathway enrichment analysis of the circulating miRNAs differentially expressed in patients with severe A/H1N1 infection.

miRNA	Expression	Pathway enrichment	p value	Target genes
150-5p	↑	PI3K–Akt–mTOR	0.009	MYB, VEGFA
		Bladder cancer	0.009	VEGFA
		Pathways in cancer	0.01	VEGFA
		Hepatitis B	0.04	EGR2
29c-3p	↓	PI3K–Akt–mTOR	3.20E−05	BCL2, CDK6, COL3A1, COL4A2, COL1A1, COL1A2, LAMC1, Col6a2
		Pathways in cancer	0.004	CRKL, BCL2, CDK6, JUN, COL4A2, LAMC1
		Chronic myeloid leukemia	0.02	CRKL, CDK6
		Hepatitis B	0.04	BCL2, CDK6, JUN
22-3p	↓	Endocytosis	0.01	ZFYVE20, RAB5B
		Non-small cell lung cancer	0.02	E2F2, CDK6
		Viral carcinogenesis	0.03	CDK6, HDAC4, PRKACA
		TGF-beta signaling pathway	0.04	SP1, BMP7
145-5p	↑	Hepatitis B	7.57E−05	IFNB1, TIRAP, MYC, CDKN1A, STAT1
		Bladder cancer	0.0004	MMP1, MYC, CDKN1A
		Pathways in cancer	0.0005	IGF1R, TPM3, MMP1, MYC, CDKN1A, STAT1
210	↓	Bladder cancer	0.04	E2F3
		Pathways in cancer	0.04	APC
		Endometrial cancer	0.04	APC
222-3p	↓	PI3K–Akt–mTOR	0.0003	CDKN1B, DDIT4, PPP2R2A, KIT, FOXO3, PTEN
		Pathways in cancer	0.001	FOS, STAT5A, CDKN1B, MMP1, KIT, PTEN
		Hepatitis B	0.003	FOS, STAT5A, CDKN1B, PTEN
		Endometrial cancer	0.01	FOXO3, PTEN
		Chronic myeloid leukemia	0.03	STAT5A, CDKN1B
126-5p	↓	Endometrial cancer	1.16E−05	KRAS, CTNNB1
		Hepatitis B	0.02	KRAS, CDK6, NFAT5
		Chronic myeloid leukemia	0.02	KRAS, CDK6

with milder disease. In addition, we demonstrate that pandemic A/H1N1 strain but not seasonal H3N2 strain induces significantly higher levels of miR-150 in A549 epithelial cells after 10 h of infection. These findings suggest that factors inherent to pandemic A/H1N1 influenza strain may in part influence the expression of miR-150 and possibly incite the development of severe inflammatory disease.

Recent studies have provided important insights about circulating and cellular miRNA-150 deregulation in different disorders, including leukemia (Fayyad-Kazan et al., 2013), HIV (Munshi et al., 2014) and dengue virus infection (Chen et al., 2014). In this respect, altered circulating levels of miR-150 (and lack of c-Myb posttranscriptional regulation) have been associated with poor outcomes in patients with sepsis (Roderburg et al., 2013). miR-150 is expressed in several immune cells and is critical in the cellular transition mechanism from pro-B to pre-B and, in the NK and iNKT cell development and functional activity (Xiao et al., 2007; Bezman et al., 2011). An important link between miR-150, c-Myb (one of its principal targets) and a reduced expression of the suppressors of cytokine signaling 1 (SOCS1) in PBMCs derived from patients with dengue hemorrhagic fever has been recently described (Chen et al., 2014). SOCS are a family of 7 proteins that downregulate cytokine signaling by negatively regulating JAK/STAT-mediated signal transduction (Naka et al., 2005; Nakagawa et al., 2002). SOCS-1 negatively regulates the production of several cytokines including IFN-α, IFN-γ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-13, IL-15 and TNF-α (Naka et al., 2005; Nakagawa et al., 2002). There is evidence that supports that changes in the expression of SOCS proteins play a critical role in the regulation of cytokine/chemokine production in both innate and adaptive immune and in the regulation of immunity against the influenza A virus (Pothlichet et al., 2008).

In this study, high levels of IL-1RA, IL-6, IL-7 and G-CSF and a slight increase in the levels of IFN-γ, CXCL8 and VEGF were detected in the blood from patients with severe A/H1N1 disease. The differential levels of these immune-mediators and growth factors between patients with severe and mild A/H1N1 disease correlated with the expression of miR-150, that was significantly associated with severe A/H1N1 infection. It has been described that miR-150 targets up-stream proteins involved in the regulation of VEGF, thus the over-expression of this miRNA promotes the production of high levels of VEGF in experimental models of tumor development (Liu et al., 2013). Our findings support the hypothesis that specific miRNA profiles may influence the production of pro-inflammatory mediators; these immune mediators possibly contribute to the poor regulated inflammatory phenotype characteristic of patients with severe A/H1N1 infection.

In previous published studies, we have described that the infection with the pandemic A/H1N1 viral strain may increase the production of inflammatory mediators by inhibiting SOCS-1 and promoting high production of IL-6, CXCL8, MCP-1 and TNF-α by human macrophages (Ramirez-Martinez et al., 2013). Activated macrophages infiltrate to the lungs throughout early stages of influenza A virus infection (Perrone et al., 2008). Although macrophages are essential in the innate protection against pathogens in the lung (Peters et al., 2004), their polarization to an M1 pro-inflammatory phenotype may promote tissue injury due to the uncontrolled production of inflammatory mediators (La Gruta et al., 2007). Our findings are relevant because the overexpression of miR-150 may promote a diminished or delayed induction of SOCS-1, through a deficient regulation of the JAK/STAT pathway, as a possible mechanism that induces a greater production of pro-inflammatory cytokines by A/H1N1-infected infiltrated macrophages, and as a consequence may be deleterious.

Our results also showed a downregulation of miR-22 in severe A/H1N1 disease. miR-22 is associated with a variety of cellular processes including apoptosis, cell cycle, and motility and its expression is altered in cancer and correlates with tumorigenicity and cancer prognosis (Zhang et al., 2010), eliciting epithelial–mesenchymal transition in experimental models of cancer metastasis (Song et al., 2013a). Supporting our findings of the involvement of miR-22 in the lack of regulation of inflammatory responses in influenza, previous studies have reported that miR-22 controls the signaling cascade of the PTEN/AKT/FoxO1 pathway (Bar and Dikstein, 2010).

Other findings include differential expression of miR-29, miR-145, miR-126, miR-210 and miR-222 in serum from all the patients with A/H1N1 infection and contacts in relation to controls. Besides the role of miR-29 family in cancer metastasis, these miRNAs have been involved in immune regulation and pro-inflammatory cytokine production by T cells. miR-29 family inhibits the production of IFN-γ by T cells by targeting the T-box transcription factor (T-bet) and eomesodermin (EOMES) genes (Steiner et al., 2011). Furthermore, miR-29 deficient mice exhibit an exuberant Th1 pro-inflammatory response against

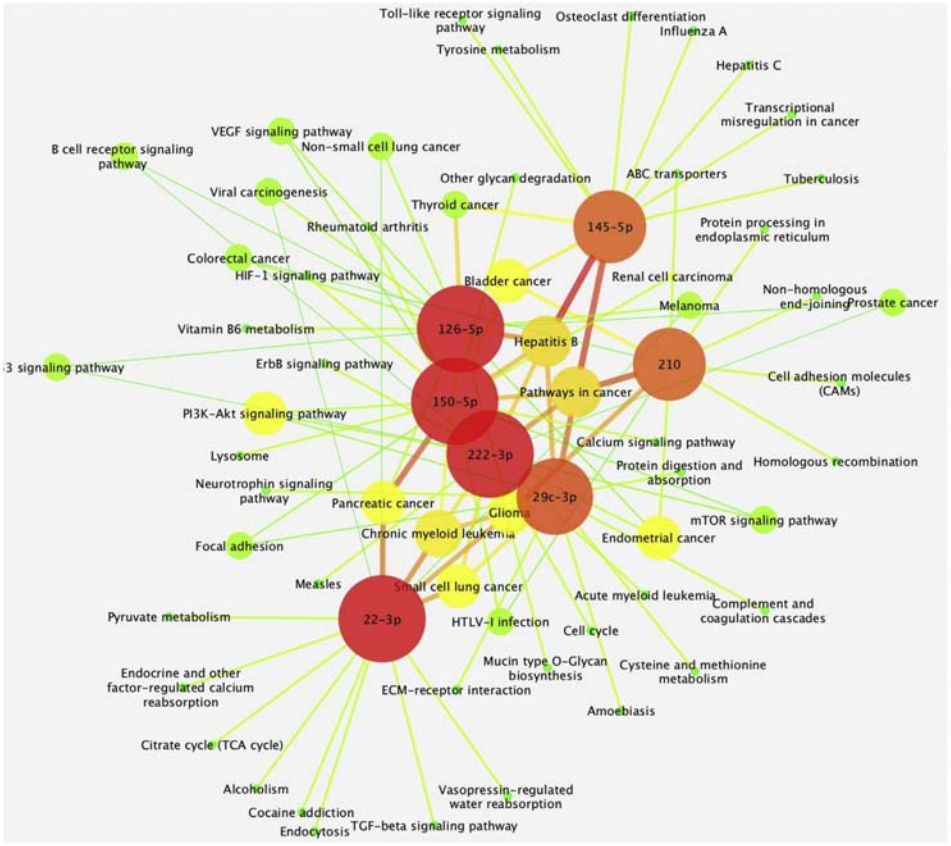

Fig. 4. Interaction network of differentially expressed miRNAs in A/H1N1 infection and the predicted pathways. Interactome networks were constructed using the software Cytoscape v3.1.1. The interactome analysis of the predicted targets shows that PI3K–Akt–mTOR, cancer, antiviral response, apoptosis and cell adhesion molecule pathways are associated with the set of the differentially expressed miRNAs in A/H1N1 infection.

intracellular pathogens (Ma et al., 2011). Recently miR-29a was considered a suitable prognostic factor of poor outcomes in A/H1N1 infection (Song et al., 2013b). In addition low expression of miR-126 has been associated with ARDS (Huang et al., 2014) and cystic fibrosis (Oglesby et al., 2010), both disorders characterized by pulmonary epithelial injury and extensive acute and chronic airway inflammation. miR126 is encoded within intron 7 of the EGF-like domain 7 (Egfl7) gene sequence with a high expression in endothelial cells and is involved in vascular development because of its role in the VEGF signaling pathway (Fish et al., 2008).

Interactome network analysis revealed a strong connection of miR-150, miR-210, miR-145, miR-126 and miR-29c that are interacting mainly with the (PI3K)–Akt–mTOR pathways (Supplementary Fig. 2). miR-150 and miR-126 are interacting through the VEGFA pathway. This is an interesting finding because recent studies have described the critical role of miR-126 deficiency and the hypo-production of type I interferons in viral infections (Agudo et al., 2014). The interaction between miR-145 and miR-210 with cancer pathway was also observed. Finally, miR-29c–miR-210 interacts through the cell adhesion molecule pathway. Based on these findings, we can speculate that miR-126 downregulation and miR-150 over-expression may lead to exacerbated inflammatory responses in patients infected with A/H1N1 virus. And miR-29c may be also associated with a deficient response by type I interferons; these possible mechanisms may be contributing to the pathogenesis of lung damage and poor outcomes of the disease.

We therefore hypothesized that miRNAs involved in immune feedback or regulatory mechanisms that normally control host inflammatory responses to pathogens may be absent or impaired in severe pdm A/H1N1 infection, due to the virus itself. The identification of differential expression of miR-150, involved in the regulation of inflammation and apoptosis, among patients with severe disease and patients with milder disease will help to understand the implications of this miRNA in the clinical outcome of the disease and to estimate the risk to develop severe disease in patients with alterations in the expression of these miRNAs.

This study has some limitations, including the relatively small number of patients that were studied. Sample size was restricted by the study's focus on critically ill patients during a short duration outbreak in 2009. Moreover, we choose not to include those patients with influenza like illness likely associated with A/H1N1 infection without viral corroboration. However, we choose to include patients that were well clinically classified and carefully followed during their hospital stay. In

this regard, we were able to reduce the effect of confusing factors at the moment of the interpretation of the results, limiting the influence of clinical presentation variation. A second limitation is our inability to include a replication cohort of patients with ARDS associated with other respiratory viruses different from A/H1N1 strain and third, the lack of experimental assays to understand the possible role of miRNAs in the pathogenesis of severe A/H1N1 infection.

5. Conclusions

In summary, our study underlines the importance of circulating miRNA profile in understanding severe A/H1N1 disease pathogenesis. We conclude that the expression of circulating miRNAs is altered in patients with different clinical outcomes of A/H1N1 infection and that high levels of circulating miR-150 are associated with poor outcomes of the disease by the A/H1N1 virus. The evidence of a dysregulated miRNA expression during the A/H1N1 infection provide insights that may help to identify novel biomarkers and molecular mechanisms involved in the pathogenesis of severe viral pneumonia.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2015.07.001.

Abbreviations

miRNAs	microRNAs
pdm	pandemic
KEGG	Kyoto Encyclopedia of Genes and Genomes
ARDS	acute respiratory distress syndrome
IL-	interleukin-
CXCL	C-X-C motif chemokine
CCL	chemokine (C–C motif) ligand
IFN-γ	interferon gamma
TNF	tumor necrosis factor alpha
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
PDGF-bb	platelet-derived growth factor subunit B
FGF	fibroblast growth factors
VEGF	VASCULAR endothelial growth factor
INER	National Institute of Respiratory Diseases
HAI	Haemagglutination inhibition method
TLDAs	TaqMan Low Density Arrays
HIV	Human Immunodeficiency Virus
SOCS	suppressors of cytokine signaling
T-bet	T-box transcription factor

Competing interests

The authors have no conflict of interest to declare.

Authors' contributions

JM carried out the experimental assays of miRNA expression analyze the data and drafted the manuscript, GR, LJ, AC, SPP, AH, LO, AM, LP, BOQ, JG, and ARV participate in the collection of samples, classification and in the analysis of data and drafted the methodology section of the manuscript, FAM and CC participate in the data analysis and virus isolation and cultures, and GMRP, AZ, EM, and JZ conceived and designed the study, analyze data and drafted the manuscript. All authors read and approved the final manuscript.

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Serum Surfactant Protein D (SP-D) is a Prognostic Marker of Poor Outcome in Patients with A/H1N1 Virus Infection

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Abstract
Introduction Surfactant protein D (SP-D) plays an important role in the innate responses against pathogens and its production is altered in lung disorders.
Methods We studied the circulating levels of SP-D in 37 patients with acute respiratory distress syndrome due to the A/H1N1 virus infection and in 40 healthy controls. Cox logistic regression models were constructed to explore the association of SP-D levels and risk of death.
Results Mortality rate after a 28-day was 32.42 %. Significant higher levels of SP-D were detected in A/H1N1 patients with fatal outcome ($p < 0.05$). After adjusting for confounding variables, levels of SP-D $\geq$250 ng/mL were associated with increased the risk of death (HR = 8.27, 95 % CI 1.1–64.1, $p = 0.043$).
Conclusions Our results revealed that higher circulating levels of SP-D are associated with higher mortality risk in critically ill A/H1N1 patients. SP-D might be a predictive factor of poor outcomes in viral pneumonia.

Keywords Surfactant protein D (SP-D) · A/H1N1 virus · Influenza · ARDS · Mortality · Biomarker

Introduction

Host factors including cardiac, respiratory, and metabolic comorbidities are associated with poorer outcomes of A/H1NI infection and development of acute respiratory distress syndrome (ARDS) [1–3]. Recent studies support the hypothesis that a dysregulated immune activation is a key factor that determines the development of severe pneumonia [4–6].

Surfactant protein D (SP-D) is synthesized by type II pneumocytes, belongs to the collectin family and its main function is to recognize pathogen associated molecular patterns allowing microbial elimination. SP-D participates in neutralization and clearance of influenza viruses given its molecular affinity to viral hemagglutinin [7, 8]. SP-D levels correlates with pro-inflammatory immune responses, mainly when alveolar macrophages interact with the trimeric form

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trough their CD91 receptor, leading the activation of p38 MAPK signaling pathway to elicit Th1 responses [8]. SP-D production is influenced by diverse lung disorders [9, 10], and its role as a biomarker of lung inflammation has been described [11, 12]. Based on the central role of SP-D in the pulmonary host defense and in the regulation of inflammatory responses and its dysregulation in lung diseases, we hypothesize that circulating levels of SP-D are modified as a result of lung tissue damage in critically ill A/H1N1-infected patients, and that SP-D is a useful biomarker to predict poor outcomes in ARDS patients with A/H1N1 infection.

We aimed to determine if circulating levels of SP-D correlates with the risk of mortality after a 28-day follow-up in critically ill patients with A/H1N1 infection.

Materials and Methods

Patients

A group of 37 patients with ARDS associated to pandemic A/H1N1 virus infection was included. Patients were hospitalized at the intensive care unit (ICU) of the National Institute for Respiratory Diseases (INER) in Mexico City during the A/H1N1 virus outbreak in April 2009. Our Institute is a reference center for emerging respiratory diseases in Mexico. In the outbreak of influenza in April 2009, the Medical and Research staff was early involved in the description of clinical characteristics of patients with severe respiratory illness associated to the novel A/H1N1 virus. The infrastructure of the ICU consists in 15 beds for critical patients and 230–250 patients with different respiratory diseases including COPD, HIV, TB, pneumonia, and influenza-like disease are admitted annually. The overall mortality per year in critically ill patients at the ICU is around 25–32 %.

Diagnosis of A/H1N1 infection was assessed by the presence of influenza-like symptoms, bilateral pulmonary infiltrates, and a positive test RT-PCR for the A/H1N1 virus. The diagnosis of ARDS was based on standard definitions [13].

A group of 40 asymptomatic household contacts, which were in close contact with A/H1N1 patients but did not developed acute respiratory disease, was recruited. Importantly, 76.5 % of the contacts exhibited significant titers of anti-A/H1N1 antibodies (1:16), supporting the fact that they were in contact with the A/H1N1 virus.

The Institutional Review Board of the INER reviewed and approved the study. All subjects or their legally authorized relatives provided written informed consent.

A/H1N1 Virus Detection

Nasopharyngeal samples were obtained and viral RNA was isolated using the viral RNA-mini kit (Qiagen Westburg, Leusden, The Netherlands). Detection of A/H1N1 virus was assessed by real time RT-PCR.

SP-D Levels Determination

Circulating levels of SP-D were measured by ELISA (BioVendor Research Products ELISA kit, Asherville, NC, USA). Briefly, 100 μL of serum or standards were incubated during 1 h with 100 μL of anti-SP-D antibody. After three washes, 100 μL of HRP solution was added and incubated by 1 h. One-hundred microliters of TMB substrate was added to each well and reaction was stopped after 15 min by adding 100 μL of stop solution. Absorbance at 450 nm was measured with a microplate ELISA reader (Bio-Rad Laboratories, Inc., CA, USA).

Anti-A/H1N1 Antibody Titers

The titers of serum anti-A/H1N1 antibodies were measured using the hemagglutination inhibition (HAI) assay. Briefly, serially diluted serum samples (25 mL) were mixed with 25 mL of the A/H1N1 virus strain. The serum/virus dilutions were incubated for 30 min at room temperature. Fifty milliliters of 0.5 % chicken erythrocytes were added and after 30 min, HAI activity was evaluated. Serum antibody titers were established as the reciprocal of the last serum dilution with no hemagglutination activity and titers greater than 1:16 were considered positive.

Statistical Analysis

Demographic characteristics, vital signs, clinical outcomes, treatments, mechanical ventilation and gas exchange parameters, laboratory tests, and APACHE II scores were determined at the admission to the ICU. Continuous variables were compared using the Student's t test for variables with normal distribution and the Mann–Whitney U test otherwise. Categorical variables were analyzed using χ^2 test. Cox's multivariate proportional hazard models were constructed to explore the factors associated with risk of death. The independent variables tested in the models were demographic and anthropometric data, lung function, laboratory findings, and comorbid conditions.

We chose the cut-off of 250 ng/mL to determine the two groups for survival analysis. This cut-off was arbitrarily selected after a extensive review of the literature in which we found that in general serum levels of SP-D in healthy volunteers is typically lower than 100 ng/mL. In addition, in certain pathological conditions such as idiopathic pulmonary fibrosis, COPD, and interstitial pneumonia serum mean levels of SP-D are usually higher than 300 ng/mL [14]. The correlation between SP-D levels and BMI was assessed using Spearman's correlation coefficient.

The significance level was set at two-tailed $p < 0.05$, and at $p < 0.01$ in case of multiple comparisons. All analyses were performed using Stata v.10.0 (StataCorp, College Station, TX, USA).

Results

The clinical and demographic characteristics of the patients are presented in Table 1.

The median age of the studied A/H1N1 09 patients and asymptomatic controls was similar. Twenty-three of the patients were male (62 %) and 57 % (21/37) were current or former smokers. No significant differences were detected in the body mass index (BMI). Eighty-nine percent of the patient's required invasive mechanical ventilation due to respiratory failure defined as PaO_2 value <60 mmHg (normal value at the altitude of Mexico City: 70 ± 3 mmHg) despite $FiO_2 > 60$ % or an increased breathing effort with accessory respiratory muscle usage. The mortality rate after a 28-day follow-up was 32.42 % (12 of 37 patients).

The main signs and symptoms at the start of the illness included fever, myalgias, cough, and headaches, while chest pain, dyspnea, and cyanosis occurred usually after the third day. All patients were treated with oseltamivir (150 mg/day) during the period of required mechanical ventilation. In addition, all patients received ceftriaxone and clarithromycin from the time of admission, and treatment was sustained, while the patients were on mechanical ventilation, but was upgraded in case of fever, if leukocytosis developed again, if new infiltrates on chest X-ray appeared, or if antibiotic resistance was documented in samples from bronchial aspirates. Blood, urine, and bronchial secretion cultures performed at the time of hospitalization in the ICU revealed neither bacterial, fungal, nor mycobacterial secondary infections. Systemic corticosteroids were administered to all patients at a dose of 1 mg/kg/day of methylprednisolone during the mechanical ventilation period. No other clinical or biochemical variable, including the APACHE II score showed significant difference among these subgroups.

Fatal Outcome in Critically Ill A/H1N1 Patients is Associated to Increased Levels of Circulating SP-D

As illustrated in Table 1 and Fig. 1, circulating SP-D values were significantly higher in patients who died (630 ng/mL) when compared to survivors (172 ng/mL, $p < 0.02$) and to healthy controls (49.5 ng/mL, $p < 0.0001$). After adjusting for confounding variables including age, gender, and PaO_2/FiO_2, we found that a serum SP-D concentration ≥250 ng/mL significantly increased the risk of death in the study population (HR = 8.27, 95 % CI 1.1–64.1, $p = 0.043$). In addition, Kaplan–Meier's survival estimates were calculated for the referred value. Patients with SP-D concentrations <250 ng/mL showed a 28-day survival estimate of 0.91 (95 % CI 0.51–0.98) meanwhile this probability was 0.38 (95 % CI 0.16–0.61) for the ≥250 ng/mL group, ($p < 0.021$), Fig. 2. Significant correlations between SP-D levels with BMI were not detected.

Discussion

Among its many effects, SP-D has been shown to be a strong chemoattractant for both monocytes and neutrophils with important effects in the pathogenesis of lung disorders [8–10]. In this study, we analyzed the circulating levels of

Table 1 Demographic characteristics, clinical features, and serum SP-D values of the study population at study entry

Variable	All patients, $n = 37$	Non-survivors, $n = 12$	Survivors, $n = 25$	p[a] Value
Age, years ($IQR_{25–75}$)	40 (29–52)	43.5 (28.5–48.5)	39 (29–52)	0.90
Male, *n* (%)	23 (62)	9 (75)	14 (56)	0.35
Body mass index ($IQR_{25–75}$)	29.3 (27.1–33.3)	31.1 (27.2–35.2)	28.9 (26.9–32.3)	0.45
Smokers, *n* (%)	21 (57)	8 (67)	13 (52)	0.68
Symptom onset to admission ($IQR_{25–75}$)	7 (2–21)	5.5 (3–13)	7 (2–21)	0.58
Dyspnea onset to discharge or death, days ($IQR_{25–75}$)	16 (1–38)	11 (6–22)	20 (1–38)	0.08
Body temperature, °C ($IQR_{25–75}$)	37.1 (36.5–38)	36.8 (36.2–38.2)	37.1 (36.8–37.8)	0.73
Pulse rate, bpm ($IQR_{25–75}$)	100 (95–112)	104 (92–113)	100 (95–100)	0.9
Pulse-oximetry, % ($IQR_{25–75}$)	76 (69–82)	71 (68–81)	78 (70–85)	0.37
PaO_2/FiO_2 ($IQR_{25–75}$)	190.4 (161.9–248.1)	186.6 (161.9–214.7)	202.8 (174.7–253.8)	0.51
SP-D, ng/mL ($IQR_{25–75}$)	434.5 (105–776)	630 (439–931)	172 (56–476)	0.02
APACHE II, score ($IQR_{25–75}$)	9 (7–12)	9 (7–12)	9.5 (7–12.5)	0.65
Diabetes mellitus, *n* (%)	5 (13.5)	0 (0)	5 (20)	0.16

[a] Comparisons between non-survivors and survivors were performed with Student's *t* test for continuous variables with normal distribution and the Mann–Whitney *U* test otherwise. Categorical variables were analyzed using χ^2 test. Data are shown as medians ($IQR_{25–75}$) and percentages

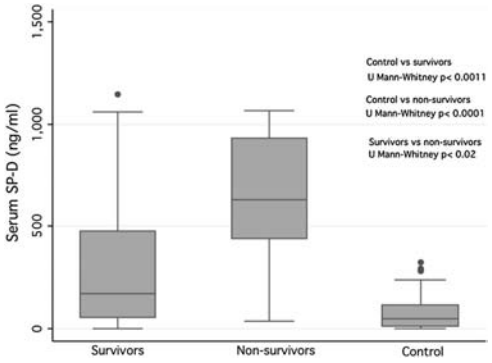

Fig. 1 Circulating levels of SP-D in patients with ARDS-A/H1N1 09 infection with fatal outcome, survivors, and controls. A marked increase in the circulating levels of SP-D was observed patients with A/H1N1 09 infection with fatal outcome (non-survivors) when compared to A/H1N1 patients that become recovered (survivors) and asymptomatic controls. Results are shown as medians and interquartile range ($IQR_{25–75}$). Comparisons among groups were analyzed using the Mann–Whitney *U* test and *p* values <0.05 were considered statistically significant

SP-D in a group of patients with severe disease associated to the A/H1N1 virus with and without fatal outcome. The most striking findings were that the patients with fatal outcome displayed a marked increase in the circulating levels of SP-D. After adjustment by age, gender, comorbidities, and APACHE II score, we found that a serum SP-D concentration >250 ng/mL could predict an increased risk of death at 28 days in patients with pneumonia associated to the A/H1N1 virus infection.

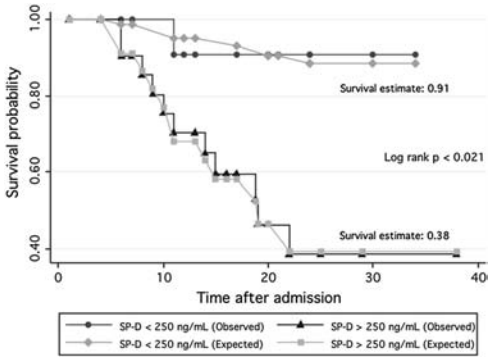

Fig. 2 Kaplan–Meier's survival estimates of all patients with ARDS and A/H1N1 09 infection according to the SP-D circulating levels. Patients with SP-D concentrations <250 ng/mL showed a 28-day survival estimate of 0.91 (95 % CI 0.51–0.98), whereas survival estimate in patients with levels ≥250 ng/mL was 0.38 (95 % CI 0.16–0.61) ($p < 0.021$)

SP-D is mainly secreted into the alveoli, thus a rise in serum concentrations may reflect protein translocation caused by the abnormal increase in alveolar-capillary membrane permeability, possibly due to loss of its structural integrity [12, 15]. Serum SP-D has been proposed as a biomarker for acute and chronic respiratory diseases including COPD and IPF in diverse clinical studies [16–18]. These studies support the hypothesis of the potential deleterious effect of the overproduction of SP-D which was directly associated with worse clinical outcomes in lung diseases. Nishida and coworkers [19] reported a non-significant correlation of SP-D and KL-6/MUC1 mucin levels with the clinical progression or poorer outcomes in pediatric A/H1N1 patients. These differences might be explained because pediatric patients had a milder disease and did not require mechanical ventilation, whereas more than 80 % of our patients required mechanical ventilation at the ICU. Interestingly, other studies have described that low levels of SP-D expression in lungs are associated with fatal influenza by A/H1N1 strain [20]. Our findings indicate that serum SP-D levels might be useful to estimate the risk of poorer outcomes in critical patients with severe lung disease associated to A/H1N1.

It is well documented that SP-D is a glycoprotein involved in the regulation of innate immunity and antiviral responses in the lung [9–11]. This protein often translocates to the systemic circulation in clinical conditions in which lung permeability is increased. Different studies have demonstrated that SP-A and SP-D are important biomarkers of IPF where high levels of these surfactant proteins are predictors of mortality [21]. In addition, evidence in large cohorts also supports its importance as a prognostic marker of clinical exacerbations and in COPD progression [18]. Recent reports suggest that the analysis of circulating levels of SP-D is useful as a diagnostic tool in severe sepsis patients with ARDS [22] and to evaluate the progression of lung injury in critically ill patients with mechanical ventilation in whom circulating levels of this protein positively correlates with the lung injury score as a parameter to measure the pathophysiological features of ARDS [23] In this scenario, clinical and experimental studies have demonstrated that the increased plasma SP-D levels could reflect acute alveolar damage and type II cell hyperplasia [24, 25]. Thus, SP-D level appears to be a reliable indicator of alterations in alveolar epithelial permeability because is more hydrophilic than other surfactant proteins such as SP-A that possibly results in a higher capacity to enter to the circulation.

SP-D plays a critical role in the regulation of macrophages, neutrophils, and fibrocytes infiltrates promoting the production of pro-inflammatory and pro-fibrotic cytokines including TGF-β and PDGF-AA [26]. In viral lung infections, SP-D may participate in the recruitment of

macrophages contributing to a dysregulated inflammatory response in A/H1N1 critically ill patients. Non-regulated inflammatory responses may exert a deleterious effect in the lung tissue resulting in the development of severe A/H1N1 disease. We and other investigators have reported higher levels of the pro-inflammatory mediators including IL-6, CXCL8, CCL2, and CCL5 in serum and bronchoalveolar lavage from A/H1N1-infected patients with severe pneumonia than those in either the household contacts or healthy controls [4, 27, 28].

Corticosteroid treatment may help to reduce circulating and lung levels of pro-inflammatory mediators. However, recent reports demonstrate that early use of corticosteroid therapy does not reduce mortality of critically ill patients with influenza A/H1N1 virus infection [29, 30]. In contrast, the corticosteroid treatment significantly decreases lung injury and mortality in patients with severe A/H1N1 infection [31] and septic shock [32]. In our study, a dose of 1 mg/kg/day of methylprednisolone was administered to ICU admitted patients during the mechanical ventilation during the outbreak in April 2009. As we mentioned in the results section, a mortality rate of 32.4 % was observed in our patients after 28 days, whereas 54 % of mortality was observed in A/H1N1 patients that received similar doses of steroids after 90 days of follow-up [30]. It is possible that the use of corticosteroid therapy may result in increased susceptibility of secondary bacterial infections due to the pronounced reduction of protective immune mediators [33].

This study has some limitations, including the relatively small number of patients that were studied. Sample size was restricted by the study's focus on critically ill patients during a short duration outbreak in 2009. Moreover, we choose not to include those patients with influenza-like illness likely associated with A/H1N1 infection but without viral corroboration. A second limitation is our inability to include a replication cohort of patients with ARDS associated to other respiratory viruses different from A/H1N1 strain and third, the lack of experimental assays to understand the possible role of SP-D in the pathogenesis of severe A/H1N1 infection.

In summary, our results indicate that higher circulating levels of SP-D are associated with higher mortality risk in patients with pneumonia due to the A/H1N1 virus. This protein is a potential prognostic biomarker of poorer outcomes of viral pneumonia. Further work is necessary to identify the mechanisms that determine the possible pathogenic effect of high levels of SP-D in infected lung with A/H1N1.

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Conflict of interest The authors declare that they have no conflict of interest.

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Obesity and pro-inflammatory mediators are associated with acute kidney injury in patients with A/H1N1 influenza and acute respiratory distress syndrome

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ABSTRACT

Background: The obesity has been shown to increase the severity of A/H1N1 infection and the development of acute respiratory distress syndrome (ARDS) and organ involvement.
Methods: Circulating levels of C-peptide, insulin, glucagon, leptin, acute phase reactants (procalcitonin, C-reactive protein, tissue plasminogen activator, and serum amyloids A and P), were measured in samples from 32 critically ill patients with A/H1N1 virus infection, 17 of whom had ARDS complicated by acute kidney injury (AKI) and 15 of whom had ARDS but did not develop AKI.
Results: Patients with ARDS and AKI (ARDS/AKI) had higher BMI and higher levels of C-peptide, insulin, leptin, procalcitonin and serum amyloid A compared to those ARDS patient who did not develop AKI. Adjusting for confounding variables using logistic regression analysis, higher levels of C-peptide (>0.75 ng/mL) (OR = 64.8, 95% CI = 2.1–1980, p = 0.0006) and BMI > 30Kg/m^2 (OR = 42.0, 95% CI = 1.2–1478, p = 0.04) were significantly associated with the development of AKI in ARDS patients.
Conclusion: High levels of C-peptide and BMI > 30 kg/m^2 were associated with the development of AKI in ARDS patients due to A/H1N1 infection. These metabolic/obesity indicators, together with the profiles of pro-inflammatory acute phase proteins, may be important links between obesity and poor outcomes in A/H1N1 09 infection.

1. Introduction

The A/H1N1 2009 virus (Smith et al., 2009) continues circulating every year in Mexico and many other countries and is associated with the development of acute respiratory distress syndrome (ARDS) in individuals with risk factors including obesity, diabetes, and chronic respiratory diseases (Bautista et al., 2010; Perez-Padilla et al., 2009). A growing body of evidence supports the hypothesis that the poor outcomes in patients with A/H1N1 09 infection is associated with a combination of host susceptibility and pathogen virulence factors that influence the immune activation and the viral infection (La Gruta et al., 2007; Lapinsky, 2010; Ramirez-Martinez et al., 2013). In addition, a high incidence of acute kidney injury (AKI) has been reported in patients with severe pneumonia from A/H1N1 2009 virus infection, with an associated high mortality rate (Nin et al., 2011; Pettila et al., 2011).

Viral pneumonia and ARDS are characterized by an extensive inflammatory response in the lung (Itoh et al., 2009; La Gruta et al., 2007). We, and others have previously examined the inflammatory mediator profiles of patients with severe forms of A/H1N1 09 disease (Bautista et al., 2013; Bermejo-Martin et al., 2010; Itoh et al., 2009; Monsalvo et al., 2011; Ramirez-Martinez et al., 2013; Zuniga et al., 2011). We have reported increased levels of several cytokines and chemokines such as IL-6, IL-10, CXCL8, and CCL5 in serum and bronchoalveolar lavage samples from these patients (Zuniga et al., 2011) and recently we showed that angiogenic and metabolic mediators such as

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VEGF, MCP-1 (Bautista et al., 2013) and leptin (Ubags et al., 2014) may participate in the pathogenesis of ARDS.

Obesity and metabolic syndrome are frequently associated with poorer outcomes from pneumonia and ARDS, yet the mechanisms of disease progression and organ involvement are not fully understood. Given this association, mediators related to obesity and metabolic disorders could play a role in and predict the development of severe disease in patients with A/H1N1 09 virus infection. Thus, in the present study, we analyzed the serum levels of various inflammatory and metabolic mediators related to obesity and metabolic syndrome in blood from patients with ARDS associated with the A/H1N1 09 virus infection with and without AKI.

2. Materials and methods

2.1. Patients

In this study we included a cohort of 32 critically ill patients admitted to the intensive care unit (ICU) at the National Institute of Respiratory Diseases (INER) with diagnosis of ARDS secondary to infection with A/H1N1 virus (Bernard et al., 1994). Patients were divided into two groups, patients with diagnosis of ARDS who developed AKI (ARDS/AKI, N = 17), and patients with ARDS but without evidence of AKI (ARDS, N = 15). The diagnosis of renal failure was established according to the Acute Kidney Injury Network criteria as previously described (Bautista et al., 2013).

Approval for this study was obtained from the Institutional Review Board from INER and informed consent was provided according to the Declaration of Helsinki. Written informed consent was obtained from all studied patients and/or from their relatives or authorized legal representative.

2.2. A/H1N1 virus detection

Nasal swab samples were obtained from ICU patients. RNA isolation was performed using the viral RNA mini kit (Qiagen Westburg, Leusden, The Netherlands) and the detection of the A/H1N1 strain sequences was performed by real time RT-PCR using primers and probes approved by the US Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO).

2.3. Serum levels of obesity related mediators and acute phase reactants

Levels of metabolic mediators (C-peptide, insulin, glucagon and leptin) and acute phase proteins (procalcitonin, tissue plasminogen activator, serum amyloid A, C reactive protein and serum amyloid P) were measured in serum samples obtained from ARDS/AKI and ARDS patients during the first three days after ICU admission. Measurements were performed using the Bio-Plex Luminex 200 instrument (Bio-Rad Laboratories, Hercules CA, USA).

2.4. Muscle biopsies

In order to rule out muscle destruction and rhabdomyolysis, muscle specimens were obtained from the vastus lateralis from patients with AKI. Biopsies were stained using hematoxylin and eosin (HE) and Masson's trichrome stain, and were examined by a qualified pathologist.

2.5. Statistical analysis

Clinical and demographic characteristics of the studied patients are summarized in Table 1. Univariate analysis was done using the Mann–Whitney–Wilcoxon or X^2 test when was appropriate. Cox logistic regression models were used to analyze the variables associated with the risk to develop AKI in ARDS patients. The independent variables were: demographic and anthropometric data, lung function, laboratory findings, and comorbid conditions. We performed a multivariate analysis adjusting with variables that proved to be significant in the univariate analysis or variables that could be relevant for susceptibility to AKI including age and gender.

Table 1
Demographic and clinical characteristics of patients with ARDS/AKI and ARDS associated with A/H1N1 infection.

Variable	A/H1N1 ARDS/AKI (N = 17)	A/H1N1 ARDS (N = 15)	p value*
Age mean (±SD)	41.4(±11.8)	39.6(±11.5)	0.7
Gender M/F (%)	13/17	9/15	0.3
BMI ≥ 30 kg/m^2 (%)	13/17(76.5)	7/15(46.7)	0.08
Mechanical ventilation mean (days)	14.2 (±5.8)	16.1(±9.1)	0.5
APACHE II score	21.4 (±5.3)	18.1(±2.8)	0.05
Hospitalization (days)	22(±8.3)	28.7(±16.1)	0.1
Time from onset of symptoms to ICU admittance (days)	10 (±9.3)	9 (±8.0)	0.6
Co-morbidities (%)	13/17(76.5)	5/15(80)	0.8

ARDS: Acute respiratory distress syndrome; AKI: acute kidney injury. Data are means ± standard deviation, or number and percentage.
* Comparisons of the continuous variables among groups were performed using the Mann–Whitney–Wilcoxon test, whereas the X^2 test was used to compare frequencies.

The correlation between BMI and the metabolic (C-peptide, insulin, leptin) and acute phase reactants (procalcitonin, SAA), was analyzed using Pearson's correlation coefficient. p values < 0.05 and p < 0.01 (in case of multiple comparisons) were considered statistically significant. Statistical analyses were performed using Stata v. 10.0 (StataCorp, College Station, TX, USA) and GraphPad Prism software v 5.04 (GraphPad Software, La Jolla, CA).

3. Results

All ARDS patients with and without AKI required mechanical ventilation and were positive for A/H1N1 virus RT-PCR test. Patients were admitted to the ICU at the INER in Mexico City during the first A/H1N1 outbreak between April and September of 2009. We observed a mortality rate of 21.5% among ARDS and 35.2% in ARDS/AKI patients. No significant differences in mean age were observed among groups. A marginal difference was observed in the APACHE II score between groups (p = 0.05). The frequency of BMI ≥ 30 kg/m^2 was higher in the ARDS/AKI group when compared with the ARDS only group (76.5% vs 46.7%, respectively, p = 0.08). Ninety four percent of ARDS/AKI group was overweight or obese (BMI ≥ 25 kg/m^2) whereas 60% of ARDS group was overweight or obese (p < 0.05). Marked differences in oseltamivir treatment delay, mechanical ventilation days, hospitalization days and the prevalence of comorbidities were not detected. The time of onset of symptoms to the ICU admittance was also similar among groups, Table 1.

Laboratory findings revealed significant differences in levels of creatinine (ARDS/AKI: 1.4 mg/dL vs ARDS: 0.8 mg/dL, p = 0.03), albumin (ARDS/AKI: 3.4 g/dL vs ARDS: 2.9 g/dL, p = 0.04) and diuresis (ARDS/AKI: 1.2 L/day vs ARDS: 3.5 L/day, p = 0.003) between ARDS/AKI and ARDS groups. Levels of BUN, LDH, CPK, Na, and glucose were similar among groups, data not shown.

Histological analysis of the muscle biopsies revealed discrete lymphocyte infiltrates, myositis and atrophy rather than rhabdomyolysis. Bronchial secretion cultures obtained at the ICU admission neither reveal bacterial, fungal, nor mycobacterial secondary infections.

3.1. BMI, diabetes markers and acute phase reactant levels in patients with A/H1N1-related ARDS with and without AKI

Patients with AKI exhibited considerable higher levels of markers of metabolism and acute phase response. The ARDS/AKI group exhibited higher levels of C-peptide (ARDS/AKI: 1.26 ng/mL vs ARDS: 0.53 ng/mL, p < 0.0001); insulin (ARDS/AKI: 390 pg/mL vs ARDS: 221 pg/mL,

$p = 0.001$) and leptin (ARDS/AKI: 2951 pg/mL vs ARDS: 1461 pg/mL, $p = 0.05$) when compared with the ARDS group patients. We also detected higher serum levels of procalcitonin (1254 pg/mL vs. 954.1 pg/mL, $p = 0.0003$) and serum amyloid A (12.04 ng/mL vs 6.23, $p < 0.0001$) in the ARDS/AKI group compared to ARDS group, Table 2 and Fig. 1.

BMI was correlated with the circulating levels of the metabolic (C-peptide, insulin, leptin) and acute phase reactants (procalcitonin, SAA) using Pearson's correlation coefficient. There was significant correlation between BMI and levels of C-peptide ($r = 0.469$, $p = 0.008$), insulin ($r = 0.424$, $p = 0.03$), leptin ($r = 0.553$, $p = 0.001$) and procalcitonin ($r = 0.423$, $p = 0.01$), Fig. 2.

After multivariate analysis adjusted for confounding factors including age, gender, LDH, glucose, CPK and albumin we found a significant association between high levels of C-peptide (>0.75 ng/mL) with the risk of developing AKI in ARDS patients (adjusted OR = 64.8, 95% CI = 2.1–1980, $p = 0.01$). Obesity (BMI > 30 kg/m^2) was also associated with a higher risk of developing AKI (adjusted OR = 42.0, 95% CI = 1.2–1478), Table 3.

4. Discussion

Influenza A virus infections are the most common viral etiology of ARDS. Several viral and host factors influence the clinical outcome of severe pneumonia and the development of ARDS. AKI is a frequent complication of ARDS due to A/H1N1 influenza virus infection, and is associated with a high incidence of subsequent mortality (Nin et al., 2011; Pettila et al., 2011).

In this study, we analyzed circulating levels of metabolic syndrome/diabetes markers and acute phase reactant levels in a cohort of patients with A/H1N1-related ARDS with and without complicating AKI.

We found that, in this cohort, ARDS patients who developed AKI exhibited higher circulating levels of C-peptide, insulin, procalcitonin, serum amyloid A, and leptin than patients without AKI. After adjustment for confounding variables, we found that elevated circulating concentrations of C-peptide and the BMI > 30 kg/m^2 were significantly associated with higher risk to the development of AKI in ARDS patients.

Inflammation and metabolism are two closely related processes. Several studies have suggested that metabolic mediators such as leptin, C-peptide, and insulin are not only operative in the pathogenesis of obesity and the metabolic syndrome, but also exert pro- or anti-inflammatory activities in other contexts (Hotamisligil, 2006; Lumeng and Saltiel, 2011).

Diverse biological effects of C-peptide have been described, including a potentially anti-inflammatory effect in experimental models of hemorrhagic shock (Chima et al., 2011a, 2011b). In addition to this, some studies have demonstrated that C-peptide may be important in the prevention of diabetic nephropathy and improvement of renal function in patients with type 1 diabetes (Johansson et al., 2000). Contrary to its anti-inflammatory role, however, C-peptide may also exert deleterious effects through its ability to drive inflammatory cell infiltration into early arteriosclerotic lesions (Marx, 2008; Vasic et al., 2012). High levels of C-peptide in AKI patients have also been suggested to reflect compensatory reno-protective mechanisms (Chima et al., 2011b). However, the potential contributions of C-peptide to the lung and kidney damage seen in patients with severe A/H1N1 pneumonia are as yet unknown.

In our study, we also find high levels of the acute phase reactants, procalcitonin and serum amyloid A, in patients with ARDS complicated by AKI. Importantly, exaggerated immune response in the setting of acute A/H1N1 09 influenza virus infection has been consistently associated with poorer outcomes (Bermejo-Martin et al., 2010; Lee et al., 2011; Zuniga et al., 2011). The production of pro-inflammatory molecules and acute phase proteins in response to more pathogenic viral strains induce early antiviral responses by recruiting neutrophils, macrophages, activated T cells and dendritic cells to the lung, but a failure to regulate of these inflammatory responses may exert a deleterious effect, promoting tissue damage (Itoh et al., 2009; Perrone et al., 2008). Previous studies have suggested that VEGF, MCP-1, IL-6, IL-8 and C-reactive protein may be useful as early biomarkers of kidney damage in different pathological conditions in both humans and mice (Bautista et al., 2013; Kwon et al., 2010; Liu et al., 2009; Nechemia-Arbely et al., 2008; Schrijvers et al., 2004; Tang et al., 2014).

In the current study, we also find a significant association between obesity BMI ≥ 30 kg/m^2 and the development of AKI in H1N1-related ARDS patients. Obesity triggers a chronic inflammatory state that involves increased levels of systemic pro-inflammatory cytokines as well as acute phase reactants, and has been previously associated with increased risk for ARDS-associated AKI (Soto et al., 2012). Furthermore, in experimental mouse models of obesity, an augmented recruitment of activated leukocytes to inflamed tissues has been described (Cao, 2014), and such an exaggerated inflammatory response has been implicated in the pathogenesis of H1N1 in the lung (Zhang et al., 2013).

In humans, elevated levels of IL-6 and MCP-1 are associated with obesity and may either positively or negatively regulate systemic glucose and lipid metabolism (Galic et al., 2010; Trayhurn and Wood, 2004). In addition high levels of TNF-α also have been correlated with insulin resistance (Hotamisligil et al., 1993, 1994). Many signaling pathways appear to be involved in the interface of inflammation and metabolism, implicating the pattern recognition receptors (PRRs) such as TLR4 and NOD, the intracellular downstream mediators of the TLR activation such as IKK and NF-κB, ceramides and sphingolipids, and the JUN kinase pathways (Arkan et al., 2005; Cai et al., 2005; Hannun and Obeid, 2008).

In addition, we have recently demonstrated that leptin, a mediator previously associated with appetite control and obesity, is important in the immunopathogenesis of acute lung injury associated with both bacterial and viral infections (Ubags et al., 2014). Our findings here suggest that obesity may contribute at least in part to the inflammatory phenotype and severity in patients with ARDS by A/H1N1 09 virus.

Lastly, we found that procalcitonin, a novel biomarker associated with the severity of inflammation in infectious diseases, was markedly increased in the serum of patients with severe pneumonia who developed AKI compared to non-AKI patients. Similar results have been observed in recent studies of critically ill patients with severe A/H1N1pneumonia (Cuquemelle et al., 2011) and, in general, higher levels of procalcitonin correlate with severity of pneumonia and risk of associated mortality (Bloos et al., 2011).

Table 2
Diabetes associated markers, acute phase proteins and pro-inflammatory cytokines levels in patients with H1N1-related ARDS with and without AKI.

Variable	A/H1N1 ARDS/AKI (N = 17)	A/H1N1 ARDS (N = 15)	p value*
Obesity and diabetes markers			
C-peptide (ng/mL)	1.26 (0.09–6.7)	0.53(0.08–2.1)	<0.0001
Insulin (pg(mL)	390 (5.0–3307)	221 (5.6–1497)	0.001
Glucagon (pg/mL)	97.12 (16.9–301.6)	106.20 (16.3–240.4)	0.6
Leptin (pg/mL)	2951 (83.7–29737)	1461 (66.1–32098)	0.05
Acute phase reactants			
Procalcitonin (pg/mL)	1254 (322–2024)	954.1 (158.7–322.3)	0.0003
Tissue plasminogen activator (pg/mL)	4432 (391–34594)	6913 (438–39898)	0.6
Serum amyloid A (ng/mL)	12.04 (2.5–49.1)	6.23 (2.5–52.7)	<0.0001
C reactive protein (ng/mL)	31.52 (5.8–238.8)	10.34 (3.5–47.0)	0.2
Serum amyloid P (ng/mL)	23.20 (10.30–60.3)	22.07 (24–98.6)	0.4

ARDS: Acute respiratory distress syndrome; AKI: acute kidney injury. Data are shown as Medians (Range).

* Comparisons of the continuous variables among groups were performed using the Mann–Whitney–Wilcoxon test.

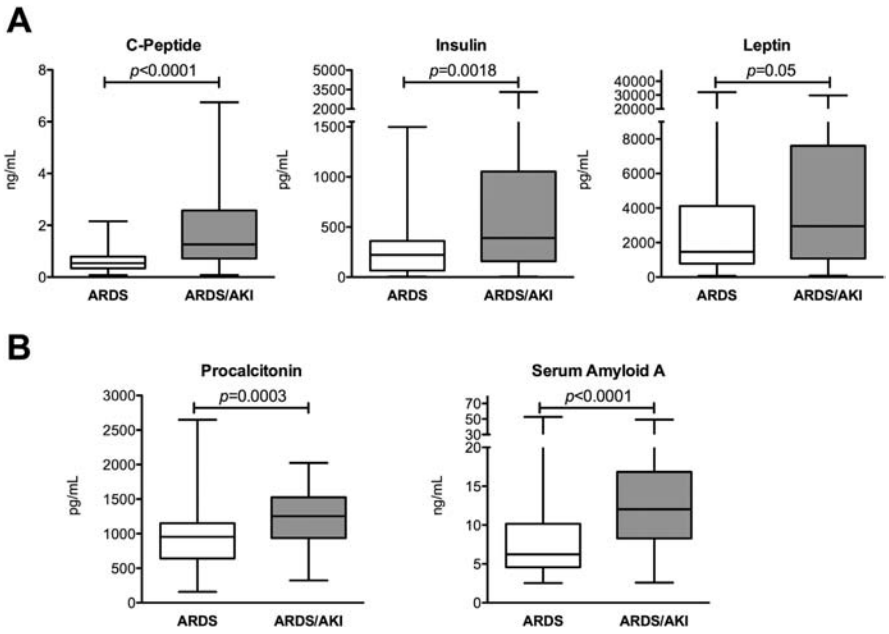

Fig. 1. Diabetes markers and acute phase reactants levels in patients with A/H1N1-related ARDS with and without AKI. ARDS: Acute respiratory distress syndrome; AKI: acute kidney injury. A marked increase in the levels of obesity/diabetes related mediators (A): C-peptide, insulin and leptin; and Acute phase proteins (B): procalcitonin, serum amyloid A were observed in the group of ARDS/AKI patients. Data are shown as medians (range). *p values were calculated using the Mann–Whitney–Wilcoxon test. p values < 0.05 were considered significant.

This study has some limitations, including its relatively small sample size, restricted by the study's focus on patients with severe pneumonia that were hospitalized at the ICU during a short-duration outbreak. In addition, we chose to include only patients with severe pneumonia that were confirmed to be infected with A/H1N1, instead of the many that were suspected. A second limitation is that this is a descriptive cross-sectional study and therefore we are unable to define the direction of the changes in the levels of metabolic/inflammatory mediators,

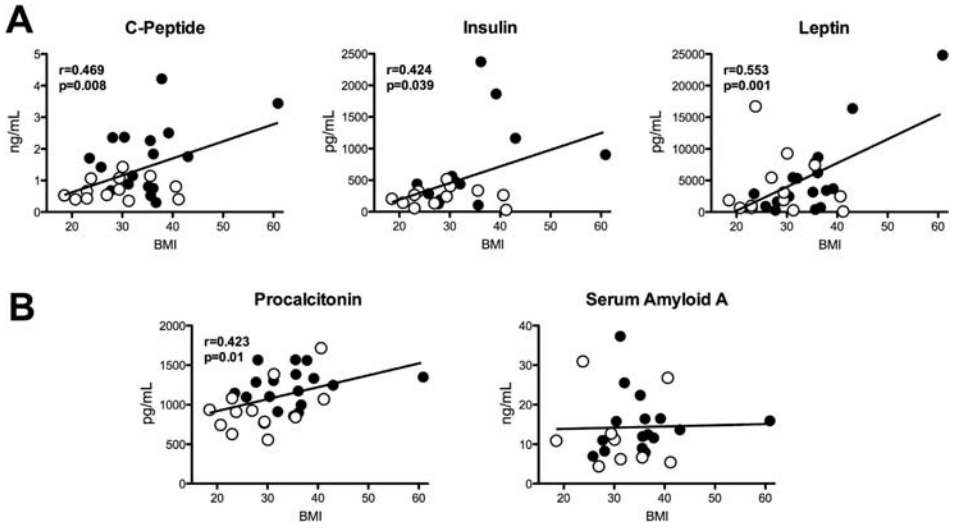

Fig. 2. Correlation between BMI and circulating levels of metabolic and acute phase proteins in A/H1N1 patients with ARDS/AKI and ARDS. There was a significant correlation between BMI and C-peptide, insulin, leptin, and procalcitonin (PCT) in ARDS/AKI (●) and ARDS (○) patients. Correlations were calculated using Pearson's correlation coefficient. p values < 0.05 were considered significant.

Table 3
Factors associated with AKI in ARDS patients after logistic regression analysis.

Variable	Adjusted OR (95%CI)	p*
Obesity (BMI ≥ 30Kg/m2)	42.0 (1.2–1478)	0.04
C-peptide (>0.75 ng/mL)	64.8(2.1–1980)	0.0006

ARDS: Acute respiratory distress syndrome; AKI: acute kidney injury. The analyses were adjusted by confusing variables including glucose, DHL, CPK, albumin, pH, CPK3, and PCT.
* The variables of age and gender did not modify the associations.

thus we cannot confirm if our findings are the cause or consequence of renal involvement in severe A/H1N1 09 influenza virus infection.

5. Conclusions

In summary, high levels of C-peptide and BMI > 30 kg/m^2 were found to be associated with the development of AKI in ARDS secondary to A/H1N1 influenza. Given the previously established link between obesity and a chronic pro-inflammatory state, and the association between high levels of acute phase proteins and inflammatory mediators and poor outcomes from A/H1N1, our findings may reflect a synergy between the obese state and H1N1-induced inflammation, leading to greater tissue injury. Whether the increased levels of these metabolic/pro-inflammatory mediators contribute directly to the kidney injury, is currently unknown. The described profiles of metabolic, acute phase proteins and cytokines may be helpful as clinical biomarkers of risk of severe disease and kidney involvement associated with the A/H1N1 infection and could be important links between obesity and poor outcomes in A/H1N1 09 infection.

Conflict of interest statement

The authors declare that they have no competing interests.

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Seasonal and pandemic influenza H1N1 viruses induce differential expression of SOCS-1 and RIG-I genes and cytokine/chemokine production in macrophages

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ABSTRACT

Background: Infection with pandemic (pdm) A/H1N1 virus induces high levels of pro-inflammatory mediators in blood and lungs of experimental animals and humans.
Methods: To compare the involvement of seasonal A/PR/8/34 and pdm A/H1N1 virus strains in the regulation of inflammatory responses, we analyzed the changes in the whole-genome expression induced by these strains in macrophages and A549 epithelial cells. We also focused on the functional implications (cytokine production) of the differential induction of suppressors of cytokine signaling (SOCS)-1, SOCS-3, retinoid-inducible gene (RIG)-I and interferon receptor 1 (IFNAR1) genes by these viral strains in early stages of the infection.
Results: We identified 130 genes differentially expressed by pdm A/H1N1 and A/PR/8/34 infections in macrophages. mRNA levels of SOCS-1 and RIG-I were up-regulated in macrophages infected with the A/PR/8/34 but not with pdm A/H1N1 virus. mRNA levels of SOCS-3 and IFNAR1 induced by A/PR/8/34 and pdm A/H1N1 strains in macrophages, as well as in A549 cells were similar. We found higher levels of IL-6, TNF-α, IL-10, CCL3, CCL5, CCL4 and CXCL8 ($p < 0.05$) in supernatants from cultures of macrophages infected with the pdm A/H1N1 virus compared to those infected with the A/PR/8/34 strain, coincident with the lack of SOCS-1 and RIG-I expression. In contrast, levels of INF-α were higher in cultures of macrophages 48 h after infection with the A/PR/8/34 strain than with the pdm A/H1N1 virus.
Conclusions: These findings suggest that factors inherent to the pdm A/H1N1 viral strain may increase the production of inflammatory mediators by inhibiting SOCS-1 and modifying the expression of antiviral immunity-related genes, including RIG-I, in human macrophages.

1. Introduction

The 2009 outbreak of swine-origin influenza A/H1N1 [1,2] continues to affect many countries, having caused over 18,000 deaths worldwide [3]. A growing body of evidence supports the hypothesis that the development of severe pneumonia in patients with pandemic (pdm) A/H1N1 infection is associated with increased immune activation and immune complex deposition [4,5]. Therefore, it is of great importance to understand the factors that determine the development of severe disease. In this regard, high levels of pro-inflammatory cytokines and chemokines have been detected in peripheral blood and lung tissue from patients with severe pneumonia associated to the pdm A/H1N1 infection [4,6]. Even though cytokines, chemokines, and growth factors are required to control influenza virus infection, their overproduction in an

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uncontrolled inflammatory response can lead to lung tissue damage [4,6,7]. The suppressors of cytokine signaling (SOCS) are a family of proteins that down-regulate cytokine signaling [8–11] by negatively regulating JAK/STAT-mediated signal transduction [9–11]. Experimental infection of A549 epithelial cells with seasonal influenza A virus up-regulates the expression of SOCS-1 and SOCS-3. These proteins regulate the immune response against influenza A viruses through a retinoid-inducible gene (RIG)-I/mitochondrial antiviral signaling protein (MAVS)/interferon (alpha and beta) receptor 1 (IFNAR1)-dependent pathway [12].

On the other hand, the combination of gene segments from North American and Eurasian swine lineages of the pdm A/H1N1 virus [13] create unique molecular structures [14,15] that could regulate host immune responses differently than seasonal influenza strains, and contribute to the particular clinical presentation of pandemic influenza infections.

We therefore hypothesized that immune feedback or regulatory mechanisms that normally control host inflammatory responses to pathogens may be absent or impaired in severe pdm A/H1N1 infection, due to the virus itself.

To establish if particular patterns of gene expression and alterations of immune regulation are attributable to specific viral factors, we analyzed the whole genome expression patterns induced by the pdm A/H1N1 and A/PR/8/34 strains through microarray analysis of infected human macrophages and A549 cells. In addition, we performed *in vitro* assays of macrophages and A549 cells in order to evaluate the differences between the pdm A/H1N1 and A/PR/8/34 in their capacity to induce SOCS-1, SOCS-3, and the antiviral response molecule RIG-I, as well as the production of pro-inflammatory cytokines, chemokines and growth factors.

2. Materials and methods

2.1. Ethics statement

The Institutional Review Board of the National Institute of Respiratory Diseases (INER) reviewed and approved this protocol (protocol number B27-10), under which all subjects were recruited. All subjects provided written informed consent, and authorized the storage of their samples at INER repositories for this and future studies.

2.2. Seasonal and pandemic A/H1N1 influenza virus isolation, identification, and propagation

Influenza pdm A/H1N1 virus isolates were obtained from patients with severe pneumonia, who signed an informed consent letter, during the 2009 outbreak in Mexico City, at the National Institute for Respiratory Diseases. Detection of pdm A/H1N1 viral RNA from the respiratory specimens was assessed by real time RT-PCR according with CDC and WHO guidelines. Live influenza pdm A/H1N1 and seasonal A/PR/8/34 viruses were isolated in Madin–Darby canine kidney cells (MDCK). Virus infectivity was assessed by determination of tissue culture infection dose 50% ($TCID_{50}$) in MDCK cells. The titers of virus stocks were adjusted to 1×10^6 $TCID_{50}$/mL. The H1N1 strain (A/PR/8/34) was obtained from the American Type Culture Collection (ATCC) and titrated to the same concentration as pdm A/H1N1.

2.3. PBMC isolation, monocyte isolation and macrophage differentiation

Buffy coats from five healthy blood donors, who signed an informed consent letter, were obtained from the Blood Bank of the INER. Total peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Lymphoprep (*Axis-Shield*, *Oslo*, *Norway*). $CD14^+$ monocytes were purified using magnetic beads (*Miltenyi*, *Auburn*, *CA*, *USA*). Purity of isolated monocytes was assessed by flow cytometry using anti-human monoclonal antibodies: CD14-FITC and CD3-PE (*BioLegend*, *San Diego*, *CA*, *USA*), obtaining a 99% purity. Isolated monocytes were seeded at a concentration of $5x10^5$ cells per well onto 24-well low-adherence culture plates (*Corning Life Sciences*, *Corning*, *NY*) in 10% FBS, 1% L-glutamine (*Gibco BRL*, *Life Technologies*, *Gaithersburg*, *MD*) supplemented RPMI-1640 culture medium (*Sigma Chemical Co.*, *St. Louis*, *MO*, *USA*) with penicillin (0.6 mg/mL), and streptomycin (60 mg/mL) (*Gibco BRL*, *Life Technologies*), and were incubated at 37 °C and 5% CO_2 during 14 days. At day 14, 98% of macrophage differentiation was obtained, as assessed by flow cytometric analysis of CD11b, HLA-DR and CD14 expression (*BD Biosciences*, *San José*, *CA*, *USA*).

2.4. In vitro infection of macrophages and epithelial cells with seasonal A/PR/8/34 or pdm A/H1N1 influenza viruses

Macrophages were infected with 5×10^5 $TCID_{50}$ of the pdm A/H1N1 or seasonal A/PR/8/34 strains. Mock-treated cells received virus-free culture medium. Culture supernatants were collected 30 min, 1 h, 2 h, 5 h, 10 h, 15 h, 24 h, and 48 h later for cytokine, chemokine, and growth factor measurements. Macrophages were harvested for RNA isolation. All assays were performed by triplicate. A549 epithelial cells were infected with pandemic or A/PR/8/34 virus under the same conditions used for human macrophages. The infection of macrophages was confirmed using monoclonal antibody anti-influenza A virus hemagglutinin (HA) (*Light Diagnostics*, *Millipore*, *Billerica*, *MA*, *USA*), after 6 and 48 h of infection (Supplementary Fig. 1A and B). In addition, we analyzed the viral titers using the haemagglutination inhibition (HAI) assay. Briefly, two fold dilutions of supernatants from infected macrophages or A549 cells were prepared and mixed with chicken red blood cells and incubated at 37 °C during 90 min. A significant rise of the viral titers after 5 h of infection of macrophages and A549 cells was detected. However, higher titers of pdm A/H1N1 in cultures of macrophages were detected earlier (Supplementary Fig. 1C).

2.5. Microarray gene expression analysis

Total RNA was obtained from macrophages and A549 epithelial cell cultures 10 h after infection with either the A/PR/8/34 or pdm A/H1N1 strains and from uninfected cells (Mock). Equimolar concentrations of total RNA from five independent *in vitro* experiments were pooled for microarray gene expression analysis. Each RNA pool was processed in duplicate. cDNA synthesis, amplification, and gene expression profiling were done according to the manufacturers instructions (Affymetrix WT Sense Target labeling assay manual). Labeled DNA was added to hybridization cocktail and the sample was injected into the array, (GeneChip Human Gene 1.0 ST Array). Wash and stain processes were performed in the GeneChip Fluidics Station 450. The probe arrays were scanned using The GeneChip® Scanner 3000 7G (*Affmetrix*, *Santa Clara CA*, *USA*).

2.6. SOCS-1, SOCS-3, RIG-I and IFNAR1 mRNA expression

Total RNA was isolated from macrophages and A549 epithelial cells using the RNA easy isolation Kit (*Qiagen*, *Valencia CA*, *USA*). SOCS-1, SOCS-3, RIG-I and, IFNAR1 mRNA expression levels were measured by real time RT-PCR using validated TaqMan assays from Applied Biosystems (SOCS-1: hs00864158_g1, SOCS-3: hs01000485_g1, RIG-I: hs00204833 and IFNAR1: hs01066116). β-

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actin expression was used as an endogenous control (*Life Technologies/Applied Biosystems*, *Foster City*, CA). qRT-PCR was performed for each target gene and β-actin as house-keeping gene. Triplicate cycle threshold C_T values were analyzed using the comparative C_T ($\Delta\Delta CT$) and then presented as relative quantification (RQ) units.

2.7. Western blot assays for SOCS-1 and SOCS-3 protein detection

Cells were lysed in RIPA buffer M-PER (*Pierce*, *Cheshire*, *UK*) containing a protease inhibitor cocktail (*P8340; Sigma Aldrich*, *St. Louis MO*, *USA*). Protein concentrations were determined by the Bradford method. Equal amounts of proteins (30 μg) were resolved on SDS-PAGE in 12.5% acrylamide gels and transferred to nitrocellulose membranes (*Bio-Rad Laboratories*, *Inc.*, *Hercules*, *CA*, *USA*). After blocking with non-fat dried milk, the membranes were incubated with primary anti-SOCS-1 (1:500 dilution; *Santa Cruz Biotechnology*, *Santa Cruz*, *CA*, *USA*), anti-SOCS-3 (1:500 dilution; *Santa Cruz Biotechnology*) and anti-β-tubulin antibodies (1:200 dilution; *Santa Cruz Biotechnology*) followed by horseradish peroxidase-labeled antibody (*Santa Cruz Biotechnology*). Immunodetection was performed by chemiluminescence using the Molecular Imager ChemiDoc XR + System and Quantity One software version 4.6.9 (*Bio-Rad Laboratories*, *Inc.*, *Hercules, CA*, *USA*).

2.8. Cytokine and chemokine quantitation in culture supernatants by Luminex and ELISA

Macrophages from five healthy donors were cultured with either pdm A/H1N1 or seasonal A/PR/8/34 strain as described above. Culture supernatants, collected at 30 min, 1 h, 2 h, 5 h, 10 h, 15 h, 24 h and 48 h, were assayed in triplicate. The levels of IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, CXCL10, CCL2, CCL3, CCL4, PDGF-BB, CCL5, TNF-α and VEGF were determined in a Bio-Plex Luminex 200 instrument (*Bio-Rad Laboratories*, *Inc.*, *Hercules, CA*, *USA*) as previously described [4].

Levels of IFN-α and IFN-β in supernatants from infected macrophages were measured by ELISA (*VeriKine ELISA kit; Piscataway Township*, *NJ, USA*). Briefly, for IFN-α, 50 μL of sample or standards were incubated with equal volumes of sample diluent whereas for IFN-β 100 μL of undiluted sample or standards (standard curve range 12.5–5000 pg/mL for IFN-α and 25–2000 pg/mL for IFN-β, respectively) were used and incubated during 1 h. After three washes, samples and standards were incubated with 100 μL of diluted antibody during 1 h. After three new washes, 100 μL of diluted HRP solution was added to each well and incubated by 1 h. A volume of 100 μL of TMB substrate were added to each well and the reaction was stopped after 15 min by adding 100 μL of stop solution. Absorbance at 450 nm was measured with a microplate ELISA reader Bio-Rad model iMark (*Bio-Rad Laboratories*, *Inc.*, *Hercules, CA*, *USA*).

2.9. Statistical analysis

Microarray background correction and normalization were done using the Oligo package in R Bioconductor. Differentially expressed genes comparing the infections with the pandemic and seasonal viruses were determined using the Limma package, considering a B value threshold of $\geqslant 3$ and a fold change (logFC) value of at least 1.4. Unsupervised hierarchical clustering analysis with the differentially expressed genes was used to identify clusters of genes with differential expression between the two infection conditions. The microarray data were deposited in the Geo-Omnibus database with the MIAME guidelines under the number GSE36012. To identify the biological processes in which the differentially expressed genes are involved, an enrichment analysis was done using the DAVID (http://david.abcc.ncifcrf.gov/) and Reactome databases (http://www.reactome.org/ReactomeGWT/entrypoint.html). For pathway visualization we used the Pathvision software obtaining the cellular pathways from KEGG and WikiPathways.

Differences in mRNA expression and in the levels of cytokines/chemokines/growth factors between cells infected with pandemic A/H1N1 versus seasonal A/PR/8/34 strains were evaluated by the Wilcoxon signed rank test or the Friedman's test using the Graph Pad Prism software version 5.04 (GraphPad Software, La Jolla, CA). p values < 0.05 were considered significant.

3. Results

3.1. Differential expression of antiviral immune response-related genes is induced by pandemic A/H1N1 and A/PR/8/34 strains

In order to compare the transcriptional profiles induced by different influenza A virus strains, we compared genome-wide expression induced by infection (10 h) with the pdm A/H1N1 and A/PR/8/34 viruses in macrophages and A549 cells using the exon array 1.0 ST. We also analyzed the gene expression profile of macrophages without virus infection (Mock at 10 h of infection) and the results were used as basal expression to determine the differences induced in the gene expression provoked by the infection of macrophages with the seasonal and pandemic virus. Considering a B value threshold of $\geqslant 3$ and a fold change value of at least 1.4, the comparison between macrophages infected with the seasonal A/PR/8/34 and the pdm A/H1N1 virus revealed 130 genes with statistically significant differential expression (seven over-expressed genes and 123 down-regulated genes in the pdm A/H1N1 compared with the A/PR/8/34 infection), (see online Supplementary list). Unsupervised clustering analysis using the 130 differentially expressed genes showed that this set of genes is capable of distinguishing macrophages infected with pdm A/H1N1 from those infected with A/PR/8/34, Fig. 1. Enrichment analysis of the differentially expressed genes identified several pathways including antiviral response, cytokine signaling regulation, innate response and proliferation that are significantly enriched in our dataset, Supplementary Fig. 2. Several genes encoding immune response proteins involved in antiviral and inflammatory immune responses against pathogens, including SOCS-1, RIG-I (DDX58), CXCL11, CXCL10, CXCL9, CCL8, CD69, TLR3, JAK2, IFIT3, IFITM3, TRIM5, DHX58, DDX60 and IRF7 amongst others, were significantly down-regulated (considering a B value threshold of $\geqslant 3$ and a fold change value $\leqslant -1.4$) in macrophages infected with pdm A/H1N1 virus. In contrast, CCL1 and other genes including RASGRP1, ETS2, EBI3, ASAP2, ITGB8, and THBS1, were significantly up-regulated in macrophages infected with the pdm A/H1N1 strain (see online Supplementary list).

Furthermore, in correlation with the high levels of IL-6, TNF-α, and CXCL8 proteins observed in 10 h cultures of pdm A/H1N1 infected macrophages, these genes were up-regulated in the microarray analysis, although they did not reach statistical significance.

In agreement to previous studies, the gene expression characteristics of A549 cells induced by the pdm A/H1N1 strain were similar to those induced by a H1N1 seasonal strain [16]. In our analysis, we did not detect significant differential expression of genes in A549 cells infected with the pdm A/H1N1 compared to A/PR/8/34 after 10 h of infection.

3.2. Induction of SOCS-1 and RIG-I mRNA by pdm A/H1N1 and A/PR/8/34 viral strains

In order to validate the microarray expression profiles of genes involved in the regulation of the production of cytokines and che-

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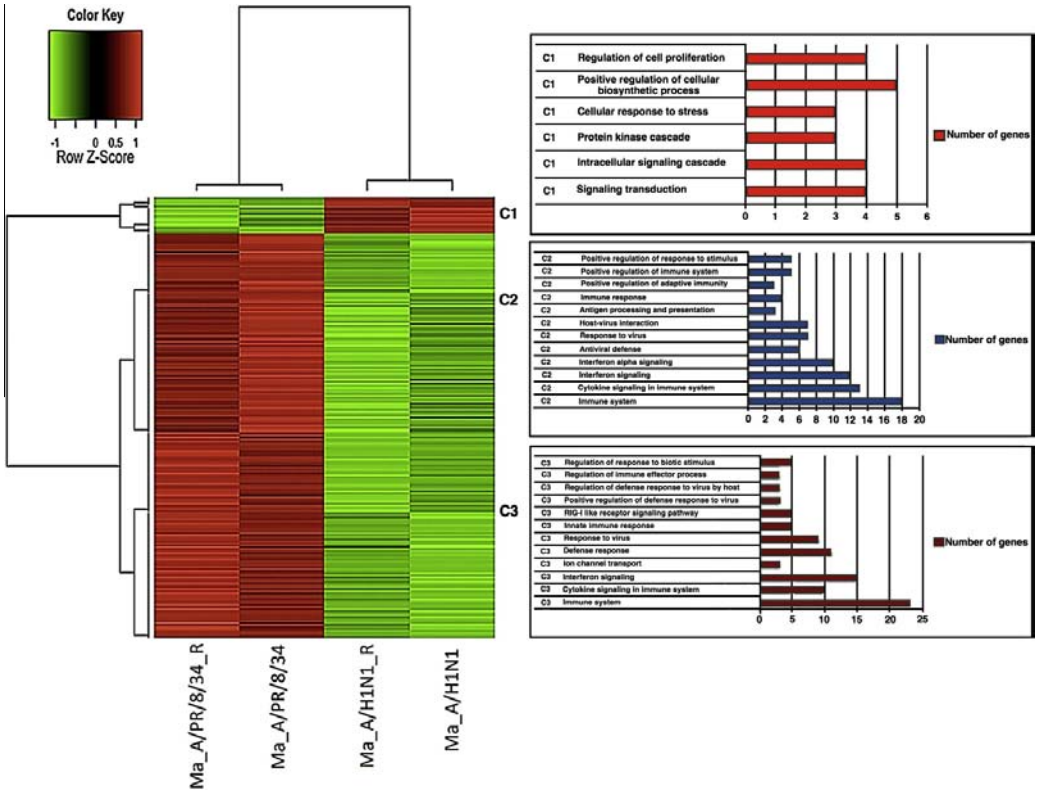

Fig. 1. Heatmap showing differences in gene expression by macrophages infected with pdm A/H1N1 compared to seasonal A/PR/8/34 influenza viruses. Samples and replicates are listed in columns; red highlighting indicates high expression and green highlighting indicates low expression. Dendograms indicating the similarity of gene expression between the macrophages infected with different viral strains are shown (left). The numbers of genes in each cluster involved with specific pathways are shown in the right panel.

mokines and antiviral response, we analyzed the kinetics of the mRNA expression levels of SOCS-1, SOCS-3, RIG-I and IFNAR1 by qRT-PCR in uninfected human macrophages and A549 epithelial cells and those in the early stages of infection. We found significantly greater SOCS-1 mRNA expression in macrophages at 10 h, 15 h, 24 h and 48 h of infection with the A/PR/8/34 virus in comparison with those infected with pdm A/H1N1 virus. At these times, the A/PR/8/34 virus induced a 25-fold or greater increase in SOCS-1 expression compared to mock-treated macrophages, whereas pdm A/H1N1 virus induced less than a 5-fold increase ($p < 0.05$, Fig. 2A).

Expression of RIG-I mRNA was also significantly up-regulated in macrophages infected with A/PR/8/34 virus at 5, 10, 15 and 24 h ($p < 0.05$), compared to those infected with the pdm A/H1N1 virus, which expressed RIG-I mRNA levels only slightly greater than those observed in mock-treated macrophages. Importantly, the levels of RIG-I mRNA after 48 h of infection were also significantly higher in macrophages infected with the seasonal A/PR/8/34 strain. mRNA levels of SOCS-3 and IFNAR1 induced by APR/8/34 and the pdm A/H1N1 strains in macrophages were similar. However, levels of SOCS-3 mRNA were substantially higher in macrophages infected with either virus compared with mock-treated macrophages, consistent with the known induction of SOCS-3 by seasonal influenza A strains [12], while IFNAR1 mRNA was only slightly increased after infection with either viral strain, Fig. 2A. mRNA expression levels of SOCS-1 and RIG-I in A/PR/8/34- or pdm A/H1N1-infected A549 cells were not statistically different. However, these genes were slightly up-regulated in the A549 cells infected with the A/PR/8/34 virus compared with the pdm A/H1N1 virus, Fig. 2B.

The production of SOCS-1 protein was assessed by Western blot assays in whole-cell lysates of macrophages and A549 cells. In line with the qRT-PCR results, this protein was detected in lysates of macrophages infected with A/PR/8/34 after 5 h, 10 h, 24 h and 48 h, yet in macrophages infected with the pdm A/H1N1 virus, SOCS-1 was detected only in the 24 h and 48 h post-infection samples. The same pattern was observed in lysates of A549 infected cells. SOCS-3 was detected in all lysates from macrophages and A549 cells infected with both viral strains, Fig. 2C. As expected, the levels of the protein RIG-I were decreased in the lysates of macrophages infected with the pdm A/H1N1 virus (data not shown).

3.3. The infection of macrophages with the pdm A/H1N1 or A/PR/8/34 strains induces different levels of inflammatory mediators

The levels of cytokines, chemokines and growth factors in supernatants of macrophages infected with A/PR/8/34 or pdm A/H1N1 strains are shown in Fig. 3. Higher levels ($p < 0.05$) of the

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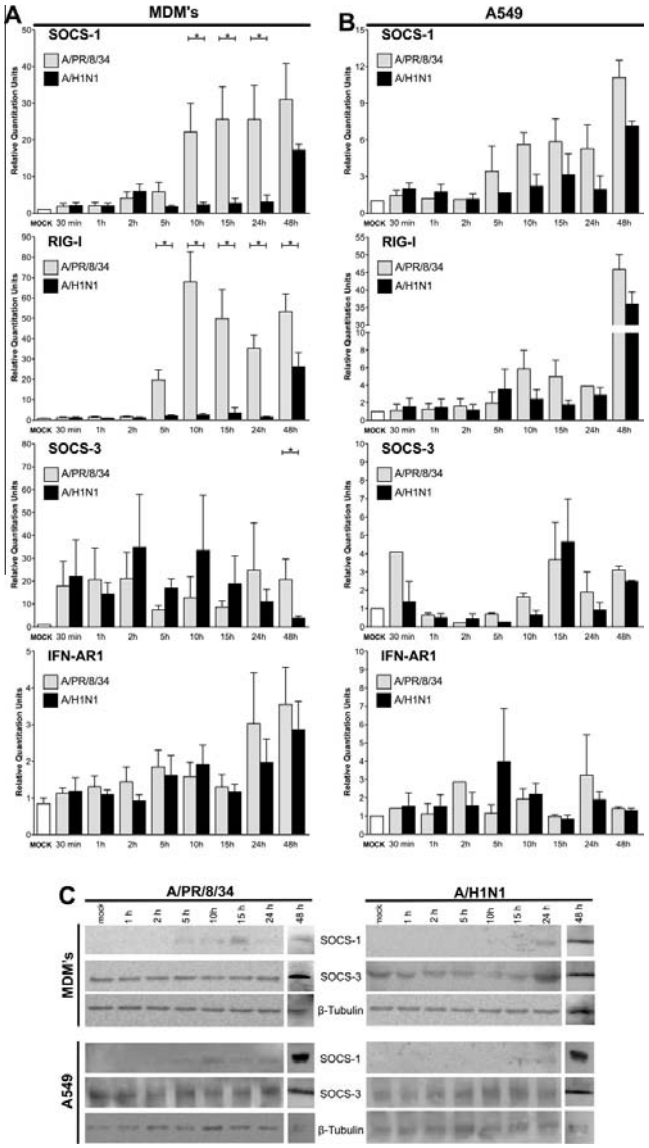

Fig. 2. mRNA levels of SOCS-1, SOCS-3, RIG-I and IFNAR1 induced by the infection with seasonal (A/PR/8/34) or pdm A/H1N1 influenza strains. Levels of SOCS-1, SOCS-3, RIG-I and IFNAR1 mRNA induced by the seasonal or pdm A/H1N1 influenza strains in macrophages (A) and A549 cells (B) were determined by qRT-PCR. Significantly higher levels of SOCS-1 and RIG-I mRNA were detected in macrophages at 5 h, 10 h, 15 h, 24 h and 48 h of infection with the seasonal virus compared to those infected with pdm A/H1N1 virus. SOCS-1 and SOCS3 protein production by macrophages and A549 cells after infection with A/PR/8/34 and A/H1N1, as determined by Western blotting (C). Differences in the gene expression were analyzed by Wilcoxon signed-rank test. p values < 0.05 (*) were considered statistically significant.

cytokines IL-6, TNF-α and IL-10 were detected in supernatants from macrophage cultures infected with pdm A/H1N1 virus compared with those infected with the A/PR/8/34 virus. Both strains induced similar kinetics and levels of IFN-γ production. Regarding chemokines, levels of CCL3, CCL4, CCL5 and CXCL8 were considerably higher ($p < 0.05$) in cultures treated with the pdm A/H1N1 virus than those treated with A/PR8/34. In contrast, high levels of CXCL10 were induced by A/PR8/34, while this cytokine was almost undetectable in pdm A/H1N1-infected cultures. Significantly higher levels of G-CSF, particularly at 10 h post-infection, were induced by the pdm A/H1N1 strain. Levels of IFN-α and IFN-β were also measured in cultures from infected macrophages. Here, higher production of IFN-α was detected at 48 h in the cultures infected by the A/PR/8/34 strain. IFN-β was undetectable, Fig. 3. In contrast

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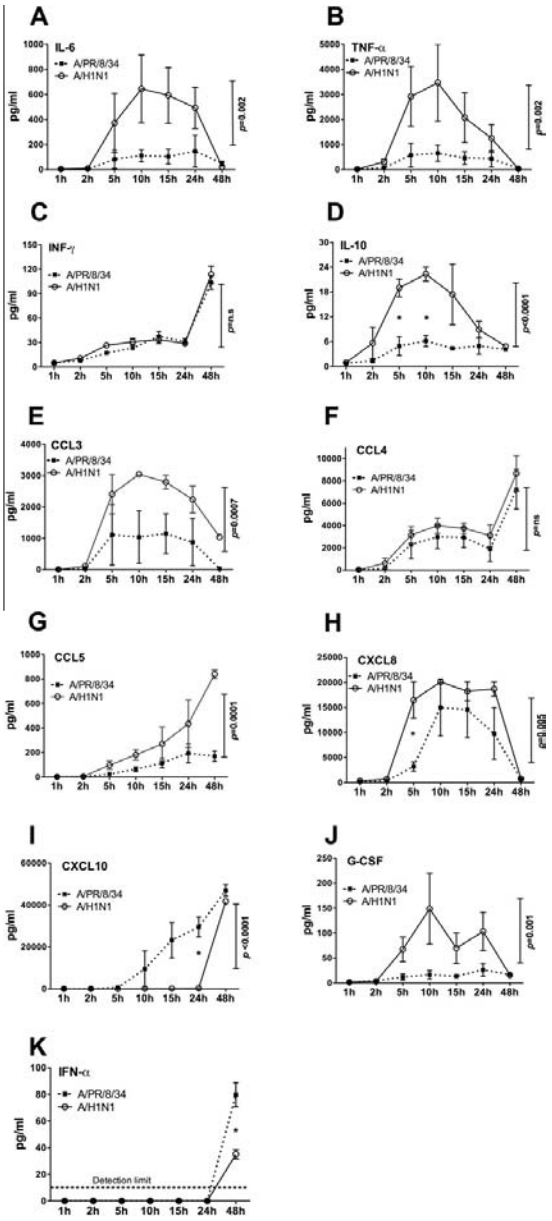

Fig. 3. Cytokine/chemokine/growth factor levels in culture supernatants of macrophages infected with seasonal or A/H1N1 influenza strains. Results are shown as means ± SEM of 3 independent experiments per strain. Significantly higher levels of IL-6, TNF-α and IL-10 in supernatants from macrophage cultures infected with pdm A/H1N1 strain were observed compared to those infected with the seasonal (A/PR/8/34) strain. Both strains induced similar production of IFN-γ. Levels of CCL3, CCL4, CCL5 and CXCL8 were considerably higher in cultures treated with the pdm A/H1N1 virus. Differences in the levels of the cytokines/chemokines and growth factors induced by the different viral strains on macrophages from the same donors at each time point (1 h, 2 h, 5 h, 10 h, 15 h, 24 h and 48 h) were analyzed by Wilcoxon signed-rank test (*). The differences including all time points in seasonal and pdm A/H1N1 strains treatment were evaluated by using the Friedman's test. *p* values < 0.05 were considered statistically significant.

to the pattern seen with infected macrophages, no significant differences in the levels of TNF-α, IL-10, CCL3, and CCL5 in supernatants of A549 cells infected with pdm A/H1N1 or seasonal virus were observed. However, a slight increase in the levels of IL-6,

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IFN-γ, CCL4, CXCL8, CXCL10 and G-CSF was observed, particularly in 10 h supernatants from A549 cultures, infected with the A/PR/8/34 strain (Supplementary Fig. 3).

4. Discussion

In previous studies, elevated levels of pro-inflammatory cytokines and chemokines in blood and lung from patients with severe pneumonia associated with pdm A/H1N1 virus have been identified, suggesting that hypercytokinemia may contribute to the severity of the disease [4,6].

Here, we analyzed the ability of pdm A/H1N1 and A/PR8/34 viruses to induce specific gene expression patterns in infected macrophages and A549 epithelial cells. These analyses allowed us to identify 130 genes differentially induced (B value threshold of $\geqslant 3$ and a fold change value $\geqslant 1.4$) by these strains, particularly in macrophages. These genes are mainly involved in the antiviral response, cytokine signaling regulation, innate immune responses against pathogens, and in cell proliferation pathways (see Supplementary list and the enriched pathways in Supplementary Fig. 2). Findings from experimental *in vitro* infection assays provide evidence that in macrophage cultures, the pdm A/H1N1 strain induces a significant attenuation of SOCS-1 and RIG-1 mRNA expression compared to the A/PR/8/34 strain. Moreover, we detected that the lack of expression of SOCS-1 induced by the pdm A/H1N1 strain in early phases of infection (particularly between 10 and 15 h) is associated with higher production of immune mediators, including IL-6, TNF-α, IL-10, CCL3, CCL4, CCL5, and CXCL8.

Changes in the expression of SOCS proteins play a critical role in the regulation of cytokine/chemokine production in both innate and adaptive immune responses [8–11], and in the regulation of immunity against the influenza A virus [12].

Previous studies have demonstrated that seasonal influenza A viruses are able to induce the expression of both SOCS-1 and SOCS-3 mRNA in human respiratory epithelial cells [12]. However, our findings suggest that the pdm A/H1N1 virus efficiently induces SOCS-3 but not SOCS-1 in early stages of infection. Furthermore, we find that the pdm A/H1N1 virus induces lower SOCS-1 mRNA levels than the A/PR/8/34 virus after 24 h of infection, as well as a delay in the production of the SOCS-1 protein.

SOCS-1 is the best studied of the SOCS family members. Its expression is induced by many cytokines, in particular by IFN-γ [17], and is critical in the activation of macrophages through TLR signaling [18,19]. Functional studies have revealed that SOCS-1 interacts with JAK kinases, inhibiting their tyrosine kinase activity [20]. SOCS-1 negatively regulates the production of multiple cytokines, primarily IFN-α, IFN-γ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-13, IL-15 and TNF-α [10].

Monocyte/macrophage lineage cells are abundantly recruited to the lungs during the initial stages of influenza virus infection [21]. Our findings are relevant because the delayed induction of SOCS-1, through a deficient regulation of the JAK/STAT pathway, may allow an over-production of inflammatory mediators by infected infiltrating macrophages, and may thus be deleterious.

While macrophages are critical to the defense against lung pathogens as revealed by infection assays in macrophage-deficient experimental animals [22], the over-activation and augmented recruitment of circulating monocytes/macrophages to the virus-infected lung may in fact promote progressive tissue damage due to the production of inflammatory mediators [23]. In this context, we believe that studies of circulating monocyte/macrophages isolated after their activation, possibly secondary to the initial *in vivo* infection of the respiratory epithelium, might help to understand the role of infiltrating macrophages in the development of exuberant inflammatory responses and pathogenesis of severe pneumonia.

Importantly, the exclusive induction of SOCS-3, in absence of SOCS-1, in initial phases of the pdm A/H1N1 virus infection, may be consistent with classical macrophage activation or M1 polarization [24], which promotes tissue inflammation and destruction [25]. In accordance with this possible profile of M1 polarization, we found that macrophages stimulated with pdm A/H1N1 characteristically produced G-CSF, TNF-α, and IL-6 [26,27].

Among the *in vitro* elicited molecules, IL-6 and CXCL8 stand out, since they have been detected in blood of pdm A/H1N1 influenza-infected subjects with severe pneumonia in concentrations significantly higher than in subjects exposed to the virus but not developing severe disease [4]. Our findings suggest that IL-6 and CXCL8 induction appears to be stimulated, at least partially, by viral factors, and these cytokines, therefore, could serve as biomarkers of severe manifestations of pdm A/H1N1 infection. The over-production of pro-inflammatory mediators induced by the pdm A/H1N1 strain in circulating macrophages suggest that this strain could elicit the same phenotype in alveolar macrophages, inducing a local and systemic inflammatory state. Our results also suggest that over-activated lung macrophages might be critical in the development of pathogenic responses, contributing in a greater extent to immune dysregulation and lung damage than respiratory epithelial cells.

Concerning regulatory cytokines, high levels of IL-10 have been consistently detected in experimental models [28] and in patients with severe pneumonia caused by pdm A/H1N1 infection [6]. In our study, high levels of IL-10 were induced by the pdm A/H1N1, but not by the A/PR/8/34 strain. However the relevance of IL-10 in the severe forms of pneumonia associated to the pdm A/H1N1 remains unclear.

Interestingly, significant amounts of CXCL10 were selectively induced by the A/PR/8/34 seasonal strain. Microarray analysis also revealed that mRNAs of CXCR3 ligands (CXCL10, CXCL11 and CXCL9), as well as CCL8, were significantly up-regulated by A/PR/8/34 virus, and, in contrast, they were down-regulated during infection by the pdm A/H1N1 strain. Although IFN-γ is a major inducer of CXCL10, we did not detect significant differences in the induction of IFN-γ between the two viral strains, consistent with previous reports [4,28]. The expression of CXCL10 may be enhanced directly by pathogens and other pro-inflammatory cytokines other than IFN-γ [29]. Further functional studies are required to determine the mechanisms underlying the failure of pdm A/H1N1-infected macrophages to express CXCL10, as reported in our findings.

Innate immunity to viral infections is induced by pattern recognition receptors such as toll-like receptors (TLR's) and the retinoic acid-inducible gene I (RIG)-like helicase (RLH) receptors [30]. Our results show that, in contrast to the A/PR/8/34 strain, pdm A/H1N1 virus failed to induce RIG-I mRNA. In concordance with this result, the microarray analysis of gene expression demonstrates that several antiviral proteins normally induced by the RIG-I activity, such as OAS, MX1, RIG-I itself, IFIT2, IFIT3, TLR3, TRIM25, DHX58, DDX60 and ISG15, among others, were considerably down-modulated in pdm A/H1N1 infected macrophages.

RIG-I is critical in the detection of and response to viral infection in host cells. Upon influenza virus infection, the recognition of viral ssRNA by RIG-I induces the TRIM25-dependent ubiquitination of RIG-I and promotes its interaction with MAVS/IPS-1. This triggers the activation of IRF3, an NFkB transcription factor that in turn induces IFN production and other innate response genes [31]. The importance of RIG-I has been demonstrated in experimental models of infection with respiratory viruses in RIG-I-deficient cells [32]. Among other immune escape strategies so far identified, the influenza virus A NS1 protein inhibits the TRIM25-dependent ubiquitination of RIG-I, and in turn the production of type I IFN [33]. Therefore, NS1 is considered a major virulence fac-

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tor, and there has been great interest in studying the possible correlation of virulence with strain-specific differences in the NS1 protein [34,35]. Interestingly, gene profile analysis of human cells infected with genetically engineered influenza A virus has demonstrated that strain specific differences in the NS1 protein correlate with different expression patterns of type I IFN-related genes [36]. In this context, it has been shown that A/PR/8/34 NS1 protein induces strong expression of SOCS-1 and RIG-I compared with NS1 protein of the 1918 pandemic virus [37]. The NS1 gene of the pdm A/H1N1 virus is of swine origin [13–15], and was not previously found in human seasonal viruses. Keeping this in mind, we cannot rule out that strain-specific differences in the NS1 protein of seasonal A/PR/8/34 versus pdm A/H1N1 could in part explain the different responses we found in macrophages infected with these viruses.

Our study has some limitations, particularly regarding the selection of the seasonal H1N1 viral strain (A/PR/8/34) used in the comparative assays with pdm A/H1N1. The A/PR/8/34 strain has been laboratory adapted by several passages in mice and chicken embryos, but has nevertheless been widely used for direct comparisons of pathogenic characteristics with different influenza A strains. In this study, we did not use a contemporary non-pdm influenza A H1N1 strain to perform the functional assays and compare its effects with the pdm A/H1N1 virus. However, preliminary data generated in our laboratory using a circulating H3N2 strain, contemporary to the pdm A/H1N1 strain, suggest that similar to the pdm A/H1N1 virus, the H3N2 strain failed to induce SOCS-1. Interestingly, infection with the seasonal H3N2 virus induces a high expression of RIG-1 in macrophages, of the same magnitude as the A/PR/8/34 (Cruz-Lagunas A, in preparation), contrasting the lack of induction of RIG-I by the pdm A/H1N1 strain.

In conclusion, our results suggest that factors inherent to pdm A/H1N1 influenza strain may in part incite the development of severe inflammatory disease through inhibiting SOCS-1 and RIG-1 expression, resulting in the increased production of inflammatory mediators (cytokine storm) and the inhibition of the antiviral response. These effects could, along with host-related factors, explain the associated poor clinical outcomes observed in some patients. Further work to identify the responsible virulence factors of pdm A/H1N1 is warranted.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2013.01.018.

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