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Pregnant women infected with pandemic influenza A(H1N1)pdm09 virus showed differential immune response correlated with disease severity

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ABSTRACT

Background: During pregnancy, immunological and hormonal alterations place women at increased risk for influenza-related severe illnesses including hospitalization and death. Although A(H1N1) pdm09 infection resulted in increased disease severity in pregnant women, the precise mechanisms responsible for this risk have yet to be established.
Objectives: The present study was aimed to investigate the role of host chemokines and cytokine profiles in A(H1N1) pdm09 infection regarding disease severity in pregnant women.
Study design: This retrospective survey examined 41 pregnant women with confirmed A(H1N1) pdm09 infection. Of them, 12 died (D), 29 survived (S), and 17 remained uninfected and served as controls (C). Antiviral response was evaluated for IFN-β expression and gene expression profiles of cytokines (TNF-α, IL-6, IL-12, TGF-β) and chemokines (IL-8, RANTES, MCP-1, IP-10), and the viral Matrix (M1) gene was quantified and normalized using the housekeeping gene product β-actin mRNA.
Results: Higher IL-8 and TNF-α mRNA expression were found in D and S compared with C, while IL-6 showed higher expression in D. Interestingly, these results were associated with a decrease in the anti-inflammatory response of TGF-β mRNA and IFN-β. These alterations would lead to an imbalance in the immune response of those patients.
Conclusions: Pregnancy-related reductions in IFN-β and TGF-β expression levels and elevated levels of pro-inflammatory cytokines could explain the increased severity of infection and death of pregnant women. These findings may help improve the understanding of the high susceptibility and disease severity to influenza virus infection during pregnancy.

1. Background

Increased morbidity and mortality rates over the course of pregnancy have been documented all through pandemic influenza and seasonal influenza where virus infection rates are particularly high [1]. Pregnancy has been associated with decreased inflammatory responses and stable/increased anti-inflammatory responses to immune challenges in women as well as in animal models [2–7]. Abnormalities in immune adaptation to pregnancy may affect pregnancy outcomes such as risk of preeclampsia, poor fetal growth, and preterm birth [8–11]. Many clinical studies have been performed to empirically define the increased severity of influenza infections during pregnancy. Epidemiological evidence and animal studies have shown that influenza infections are more severe in the second and third trimesters of pregnancy, resulting in greater morbidity and mortality, although the reason for this is still unclear [1,12,13].

Immunological alterations during pregnancy may help explain the increased severity of and susceptibility to infectious diseases.

Abbreviations: A(H1N1)pdm09, pandemic influenza H1N1, 2009; IL, interleukin; IFN-β, interferon beta; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-β.

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As pregnancy progresses, hormone levels change dramatically and are considerably higher than those in nonpregnant females [14]. The interplay between sex hormones and the immune system is complex and multifactorial, affecting thus many organ systems.

The increased morbidity due to influenza infection is associated with higher levels of circulating estrogen. Estrogens have long been known to possess potent immunomodulatory effects in various models of disease [15–17].

The innate immune system, the first line of defense against invading viruses, involves two types of cytokine responses: a pro-inflammatory response and an antiviral response.

Inflammatory cytokines and chemokines play a key role in the pathogenesis of virus infections [18]. Influenza viruses primarily infect the epithelial cells of the upper respiratory tract, evoking release of an array of host inflammatory and antiviral cytokines and chemokines and the recruitment of antiviral immune cells to the infection site.

The increased mortality rate detected in pregnant female mice infected with influenza A(H1N1)pdm09 virus is associated with increased infiltration of neutrophils and macrophages in the lungs of these animals. In addition, pregnant mice showed higher levels of chemokines and pro-inflammatory cytokines, lower respiratory epithelial regeneration and poorer fetal development than non-pregnant mice [19,20].

The increased mortality rate observed in pregnant female mice correlates with greater induction of pro-inflammatory cytokines and chemokines, including TNF-α, MPC-1 in the lungs following infection [20,21].

On the other hand, respiratory epithelial cells have been shown to produce antiviral cytokines such as interferon (IFN-β) [22]. *In vitro* data showed that pregnant women have an attenuated innate interferon response in peripheral blood mononuclear cells (PBMCs) stimulated with A(H1N1)pdm09 compared with PBMCs from nonpregnant women [23].

Despite improvements in healthcare, the bad prognosis of influenza virus infection during pregnancy remains a health concern, as most recently demonstrated by the 2009 influenza virus A subtype H1N1 (H1N1/09) outbreak.

In this study, we hypothesized that the alterations in the pro-inflammatory response and antiviral response may contribute to the severity of the infection in pregnant women infected with A(H1N1)pdm09.

2. Objectives

In an attempt to elucidate the innate immune response to A(H1N1) pdm09 infection and to gain further insight into cytokine-mediated pathogenesis, we retrospectively evaluated the expression levels of a panel of cytokines, chemokines, and viral replication in different groups of pregnant women according to the severity of the infection.

3. Study design

3.1. Subjects and samples

Samples were nasopharyngeal swabs collected from July to September 2009 and sent to the National Influenza Reference Laboratory which houses the WHO National Influenza Center (NIC) for A(H1N1)pdm09 diagnosis.

This study included 41 pregnant women with confirmed A(H1N1)pdm09 infection during the 2009 pandemic. Of them, 29 survived (*S*), and 12 died as a result of infection (*D*). Cardio-respiratory failure was the leading cause of death. Samples of 17 pregnant women non infected with influenza virus A or B were included as control (*C*); this group was enrolled as non-influenza acute respiratory infections (ARI). Samples were obtained in the second and third trimester of pregnancy, because susceptibility and severity of influenza virus infection increases with gestational age [13,24].

Samples were collected with an average of 4.5 days after onset of symptoms for all groups.

This study was approved by the Independent Ethics Committee of the School of Medicine, University of Buenos Aires, Argentina, informed of internationally endorsed standards for the application of the Helsinki Declaration.

Table 1
Primers, probes sequences, and quantitative PCR conditions.

	Sequence		Annealing temperature	Cycle number	Size (bp)
β-Actin	Forward	5′-ATGGGTCAGAAGGATTCCTATGTG-3′	60	40	435
	Reverse	5′-CTTCATGAGGTAGTCAGTCAGGTC-3′			
RANTES	Forward	5′-GTCGTCTTTGTCACCCGAAAG-3′	60	40	65
	Reverse	5′-TCCCGAACCCATTTCTTCTCT-3′			
MCP-1	Forward	5′-CAAACTGAAGCTCGCACTCTCGCC-3′	60	40	354
	Reverse	5′-ATTCTTGGGTTGTGGAGTGAGTGTTCA-3′			
IL-8	Forward	5′-ACTGAGAGTGATTGAGAGTGGAC-3′	60	40	112
	Reverse	5′-AACCCTCTGCACCCAGTTTTC-3′			
IL-6	Forward	5′-TCCACAAGCGCCTTCGGTCCAG-3′	60	40	191
	Reverse	5′-CTCAGGGCTGAGATGCCGTCG -3′			
TNF-α	Forward	5′-CCCAGGCAGTCAGATCATCTTC -3′	60	40	85
	Reverse	5′- AGCTGCCCCTCAGCTTGA -3′			
IL-12	Forward	5′- TGTCACCGAGAAGCTGATGT-3′	60	40	278
	Reverse	5′-GAGGTTTCTGGCCAAACTGA-3′			
IL-10	Forward	5′- TTACCTGGAGGAGGTGATGC -3′	60	40	285
	Reverse	5′- GCCACCCTGATGTCTCAGTT -3′			
TGF-β	Forward	5′-GGACACCAACTATTGCTTCAG-3′	60	40	159
	Reverse	5′-TCCAGGCTCCAAATGTAGG-3′			
IFN-β	Forward	5′-CTTACAGGTTACCTCCGAAACTGAA-3′	60	40	80
	Reverse	5′-GGTTGAAGAATGCTTGAAGCAA-3′			
IP-10	Forward	5′-ATTATTCCTGCAAGCCAATTTTG-3′	60	40	65
	Reverse	5-TCACCCTTCTTTTTCATTGTAGCA-3			
M	Forward	5′- ATTATTCCTGCAAGCCAATTTTG -3′			
	Reverse	5′-AGGGCATTYTGGACAAAKCGTCTA-3′	50	45	244
	PROBE	5′-TGCAGTCCTCGCTCACTGGGCACG-3′			

M, Matrix gen; bp, base pairs.

Table 2
Characteristics and medical conditions in pregnant women with severe A(H1N1)pdm09 infection and controls (*C*).

Patient characteristics	Pregnant women		
	Non-infected controls (*C*)*n* = 17	Survived (*S*) *n* = 29	Died (*D*) *n* = 12
Age mean (SD)	23 ± 4.0	25 ± 5.8	21 ± 4.4
Gestational age, weeks, mean ± SD	24 ± 7.8	29 ± 7.8	20 ± 9.4
Underlying medical conditions			
Asthma	0	10	2
Pneumonia	0	5	4
Diabetes	2	0	0
Morbid obesity	0	1	0
Oxygen requirement	1	3	5

Data are given as ± median.

3.2. RNA extraction and reverse transcription

Total RNA for viral load extracted from whole sample. Viral RNA from nasopharyngeal swabs (whole sample) was obtained using automatic extractors purchased from QIAamp® Viral RNA Mini kit QIAGEN™ and QIAcube (QIAGEN), according to manufacturer's instructions.

Viral presence was then assessed by qRTPCR using primers and probes described in the CDC A(H1N1) pdm09 influenza guidance [WHO-CDC protocol of real-time RT-PCR for swine influenza A(H1N1)pdm09, April 2009], provided by the Centers for Disease Control (Atlanta, GA, USA). After that, cDNA from total RNA was synthesized in two steps. First, Oligo-(dT) primers were added to 1 μg of total RN, heated to 70° C for 10 min, and subsequently cooled on ice. Second, SuperScript® First-Strand Synthesis System for reverse transcriptase M-MLV Reverse Transcriptase (PROMEGA) was added in the presence of 50 mM Tris–HCl, pH 8.3, 75 mM KCl, $MgCl_2$, dNTPs, dithiothreitol (DTT) and kept at 42° C for 80 min.

3.3. Quantitative real time polymerase chain reaction

Influenza real-time RT-PCR was performed in all swabs using primers and probes described in the CDC A(H1N1) pdm09 influenza guidance [WHO–CDC protocol of real-time RT-PCR for swine influenza A(H1N1)pdm09, April 2009].

RNA from the reference isolate named A/Chicken/HK/D11–748-1/2011 was used as positive control for viral load quantification, with 4000 copies/ul of the matrix gene (M1). The gene expression profile of chemokines (IL-8, RANTES, MCP-1, IP-10) and cytokines (IFN-β, TNF-α, IL-6, IL-12, TGF-β) were normalized according to the level for the corresponding housekeeping gene β-actin in the same sample. We used a SYBR green-based real-time PCR reaction in a volume of 25 mL using the ABI Prism 7500 sequence detection system (SDS) from Applied Biosystems.

For quantification of mRNA expression a relative quantification is used, because variations in efficiency of the reverse transcription step are not controlled. The amount of target, normalized to an endogenous housekeeping gene and relative to the calibrator is given by $2 - \Delta\Delta Ct$, where $\Delta\Delta CT = \Delta CT$ (sample)-ΔCT (calibrator). All analyses utilized log-transformed cytokine and chemokines data to normalize the data distributions.

Primers for all chemokines and cytokines were designed using the computer program Primer-Blast. The melting temperature (Tm) of all primers was 58–60° C.

To prevent co-amplification of genomic DNA all the targets the primers are located in two different exons.

All amplicons obtained of chemokines and cytokines were visualized on agarose gel to confirm the molecular weight.

Controls used for mRNA expression of chemokines and cytokines were: A549 cell line (a human alveolar type II epithelial cell line from an adenocarcinoma) defined as a model of human AECII (type II alveolar epithelial cells) as positive control, and Madin–Darby canine kidney (MDCK) as negative control.

Primer sequences, annealing temperatures, cycle numbers, and PCR product sizes are shown in Table 1.

3.4. Statistical analysis

All data were analyzed using the GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The Mann–Whitney *U* test or Kruskal–Wallis variance analysis was used to compare data among groups. Values were expressed as median values. Statistical significance was considered when the *p* value was equal or less than 0.05.

4. Results

4.1. Demographic, clinical, and laboratory features

All pregnant women were <30 weeks of gestation. The most common symptoms at onset were fever >38 °C, myalgias, cough and dyspnea.

Nine patients required oxygen (1 control, 3 survivors, and 5 dead).

Other nine patients presented with influenza-associated pneumonia (5 survivors and 4 dead). Twelve patients had concomitant preexisting conditions such as asthma, diabetes, and morbid obesity (Table 2). All of the deceased pregnant women were at the ICU, requiring ventilator support; the X-ray report indicated bilateral infiltrates and in some cases bacterial pneumonia.

None of the infected pregnant women were treated with antiviral therapy at sampling time.

4.2. IL-8, IL-6 and TNF-α mRNA induction by influenza virus in pregnant women

In order to investigate whether a possible differential expression of chemokines and cytokines could be associated with severity of influenza A(H1N1)pdm09 virus infection in pregnant women, we evaluated the expression of mRNA using qRT-PCR.

The IL-8 mRNA expression was increased in both groups of infected pregnant women compared with controls (Fig. 1a) while no statistical differences were observed in the MCP-1, RANTES and IP-10 expression among the three groups (Fig 1b–d).

In the analysis of acute-phase proteins, we found that the IL-6 mRNA expression showed a marked increase in *D* compared with *S* and *C*, (Fig 2a).

Regarding the TNF-α mRNA expression, we found a higher level in both groups of infected women compared with C (Fig 2b). On the

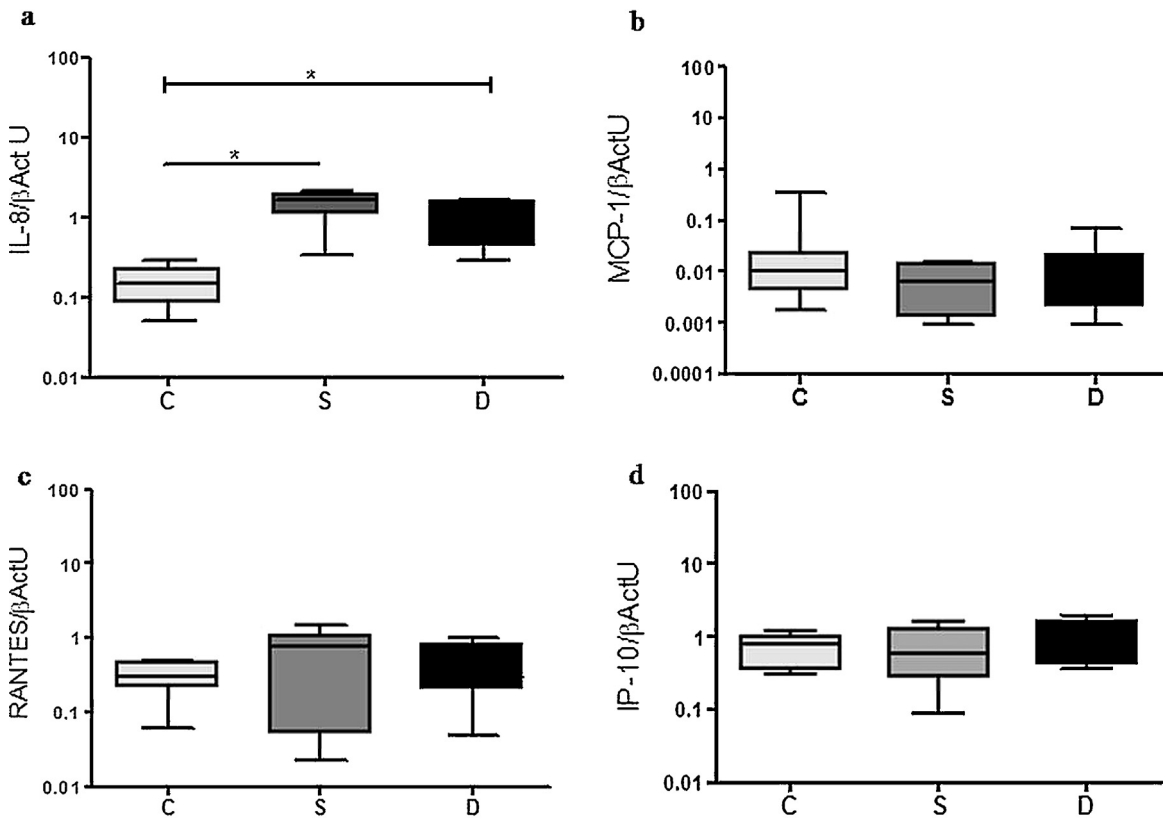

Fig. 1. mRNA expression of IL-8 (a) MCP-1 (b), RANTES (c), and IP-10 (d) was measured in 17 non-infected pregnant women (*C*), 29 survived (*S*), and 12 dead pregnant women (*D*) by Real Time PCR. Horizontal bars indicate median values. *Indicates significant differences ($p \leq 0.05$) using two-tailed Mann–Whitney *U* test with a Bonferroni correction. mRNA expression is represented as fold-change from media (2-ΔΔct) and graphed as median value (range).

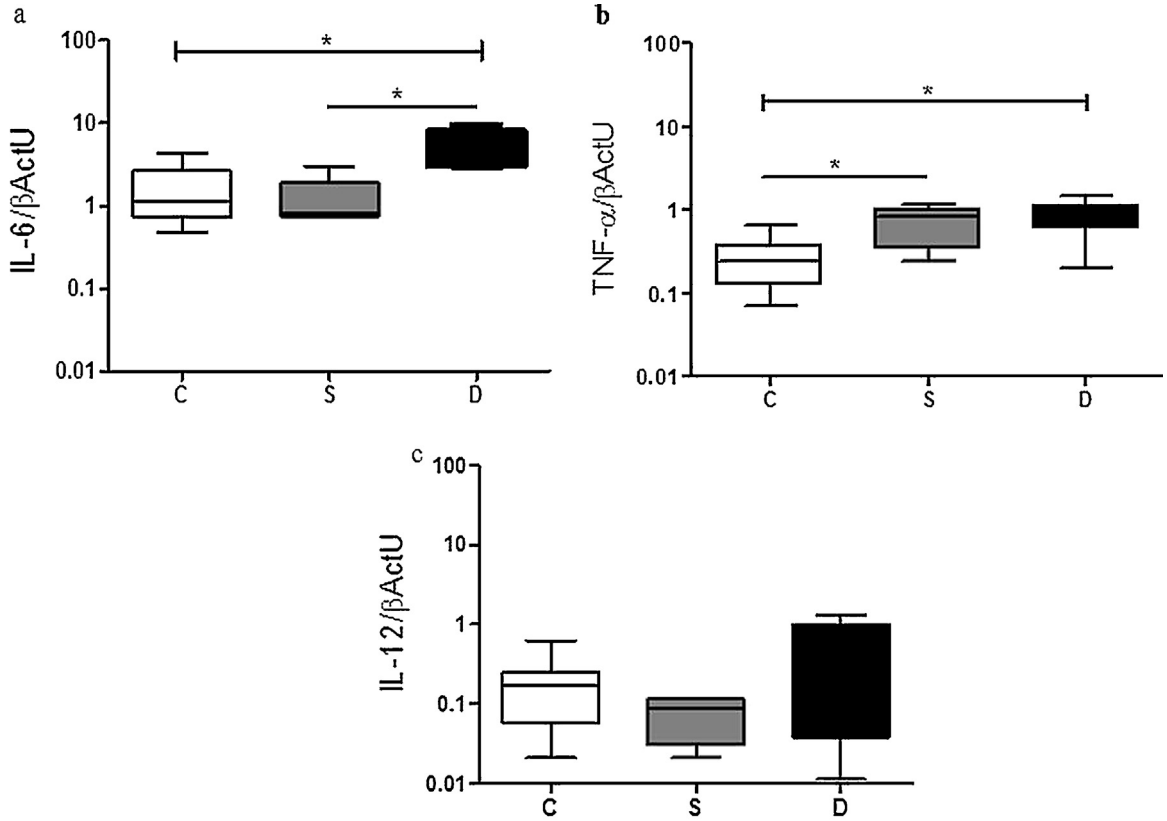

Fig. 2. The mRNA expression of IL-6 (a), TNF-α (b), and IL-12 (c) was evaluated in 17 non-infected pregnant women (*C*), 29 survived (*S*), and 12 dead pregnant women (*D*). Horizontal bars indicate median values. *Indicates significant differences ($p \leq 0.05$) using two-tailed Mann–Whitney *U* test with a Bonferroni correction. mRNA expression is represented as fold-change from media (2-ΔΔct) and graphed as median value (range).

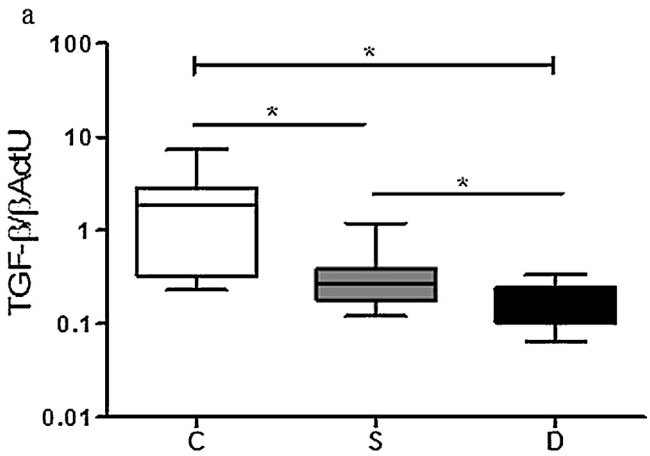

Fig. 3. The mRNA expression of TGF-β mRNA was measured from 13 non-infected pregnant women (*C*), 18 survived (*S*), and 8 dead pregnant women (*D*). Horizontal bars indicate median values. *Indicates significant differences ($p \leq 0.05$) using two-tailed Mann–Whitney U test with a Bonferroni correction. mRNA expression is represented as fold-change from media (2-ΔΔct) and graphed as median value (range).

other hand, no significant differences were found in the expression of IL-12, (Fig 2c) among all the study groups.

4.3. Low TGF-β mRNA expression in D

Next, we investigated whether the expression of anti-inflammatory cytokines like TGF-β was involved during the influenza virus infection. We observe significant difference in the expression of TGF-β between *S* and *C*. Interestingly, the A(H1N1)pdm09 virus infection was accompanied by a significant decrease in TGF-β mRNA expression in *D* compared with *S* and *C* (Fig 3).

4.4. Impaired IFN-β induction was strongly associated with disease severity

One of the critical early signaling events during an influenza virus infection is the release of type-I interferon (IFN).

IFN-β mRNA expression was markedly lower in *D* compared with *S* and *C* (Fig 4a).

In order to compare viral replication among the infected pregnant women, the matrix (*M*) gene copy number was measured by quantitative PCR as a measurement of viral replication. However, we did not find any significant differences in the copy numbers expression of the *M* gene in *D* compared with *S* (Fig 4b).

5. Discussion

Early data from the influenza A(H1N1)pdm09 virus infection indicates that pregnant women were four times more likely to be hospitalized than the general population, highlighting the urgent need for ongoing, national surveillance in this vulnerable population [13]. In response, the centers for disease control and prevention (CDC) implemented the pregnancy flu line (PFL) to monitor the impact of influenza on maternal and fetal/infant health and provided consultation to clinicians and health departments [25].

During the 2009H1N1 pandemic in Argentina, it was estimated that the mortality rate per 100,000 person-years (py) ranged from 1.5 among persons aged 5–44 years to 5.6 among persons aged ≥65 years [26]. An analysis of 332 case fatalities infected with A(H1N1)pdm09 virus showed that twenty (6%) were among pregnant or postpartum women of whom only 47% had been diagnosed with comorbid disorders [27].

While other research groups reported an increase in morbidity due to influenza virus infection during pregnancy, anti-inflammatory mechanisms in the context of infection have not been extensively characterized as they have been in the context of disease [5,6] and of fetal rejection [2,4,28].

Studies have demonstrated that the over-production of specific inflammatory cytokines, such as the tumor necrosis factor (TNF)-α, interleukin (IL), IL-6 and IL-10, as well as the polymorphonuclear neutrophil CC chemokine - IL-8, is the hallmark of viral infection [29].

First we observed an increase in the levels of IL-8 in infected pregnant women compared to controls. This induction is consistent with the finding of neutrophil infiltration in the initial phase of influenza virus infection. However, inflammatory responses were not significantly different in those pregnant women infected compared with controls for MCP-1, IP-10 and RANTES. These results contrast with previous analysis showing increased mortality rates in pregnant female mice, which correlated with higher induction of chemokines in lung following infection. However, these studies only compared the pathogenesis in the mice model [20,21,30].

Previous results [31] have shown high concentrations of IL-6 protein in patients who required critical care support compared to patients who did not. In line with those results, we observed an increase in IL-6 mRNA in infected dead pregnant women. These results suggest a prominent role of the IL-6 associated inflammation in the host response to A(H1N1)pdm09 during pregnancy.

The increased mortality rates found in pregnant female mice correlates with greater induction of proinflammatory cytokines and chemokines, including TNF-α, in the lungs following infection [19,20]. Over-expression of TNF-α has been associated with morbidities in influenza infection [32] and spontaneous pregnancy loss [33].

Among the different cytokines analyzed in our study, the mRNA levels of TNF-α were considerably higher in infected pregnant women than in the control group, but this response was generally similar among infected dead pregnant women and those who survived. These data suggest that TNF-α expression did not correlate with the severity of the infection during pregnancy.

IL-12 is a strong inducer of gamma interferon (IFN-γ) [34], and it has also been shown to be important in the development of type 1 T helper cells [35].

IL-12 appears to be important primarily in early activation of the immune response during primary influenza virus infection. However, IL-12 is no longer required in the pulmonary environment for mechanisms instrumental in mediating virus clearance, namely, activation of effector T cells and secretion of neutralizing antibodies. As a result, infected BALB/c mice are able to recover from influenza virus infection in the absence of IL-12 [36].

The presence and function of IL-12 during influenza infection in pregnancy have not been yet analyzed and relatively few published studies have addressed the antiviral activity of IL-12. In the present study, we examined IL-12 expression in infected pregnant women and we did not detect any differences in the expression of mRNA levels among groups.

The role of TGF-β is to modulate trophoblastic proliferation and differentiation in human fetal growth and preeclampsia [37]. However, the effect of influenza virus infection during gestation on TGF-β levels has not been thoroughly examined.

One report showed that the values of TGF-β in the plasma of infected pregnant women were much higher than those in non-pregnant women, thus suggesting that TGF-β 1 levels rise during pregnancy [38]. In pregnant women, the balance between pro- and anti-inflammatory factors seems to play a key role [39]. We observed a decrease in mRNA expression for TGF-β in infected preg-

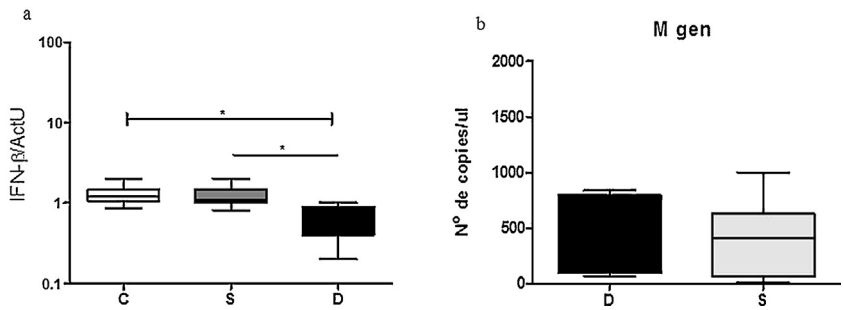

Fig. 4. The mRNA expression of IFN-β (a) was measured from 9 non-infected pregnant women (*C*), 21 survived (*S*), and 12 dead pregnant women (*D*). Horizontal bars indicate median values. *Indicates significant differences ($p \leq 0.05$) using two-tailed Mann–Whitney *U* test with a Bonferroni correction. The influenza matrix (M1) gene copy number (b) was measured by quantitative PCR as a measure of viral replication in each sample Unpaired two tailed Student's *t*-test, $n = 10$ (*D*), $n = 18$ (*S*). mRNA expression is represented as fold-change from media (2-ΔΔct) and graphed as median value (range).

nant women compared with controls, with a lower expression in the deceased patients.

Despite the importance of the innate immune system in host defense, little is known about alterations in IFNs during pregnancy, especially in response to influenza virus infection. Type-I IFNs play a critical role in early immune responses to influenza, and the impairment of this pathway during pregnancy implicates a significant alteration in the response to the virus. Interestingly, we observed that IFN-β expression was lower in those pregnant women who died. These findings suggest that IFN-β might contribute to the pathogenesis and severity of respiratory complications in pregnant women infected with A(H1N1)pdm09. In this regard, it is intriguing that the pregnant mice with elevated estrogen levels result in an attenuated anti-viral immune response. Estrogen treatment leads to early reductions in cytokine production, in particular type-I interferon [40].

Our results are consistent with another study which observed that PBMCs from pregnant women had an attenuated innate antiviral immune response, showing a significant reduction in IFN-α and IFN-λ production following *in vitro* stimulation with A(H1N1)pdm09, indicating that the altered antiviral activity is implicated in the increased morbidity during pregnancy following the influenza pandemics [23].

There are some reports which state that pregnant females have greater virus replication in their lungs than non-pregnant females, while others suggest that virus replication is not affected by pregnancy [19,21]. According to that, we did not find any significant differences in the copy numbers expression of the M gene from dead pregnant women as compared to those who survived (Fig 4b). Our results then support previous evidence showing that the lethality of influenza infections occurs as a result of immune-based tissue injury rather than exacerbated viral burden [41–43]. Furthermore, our findings demonstrate that pregnancy alters the innate host response, which changes the course of A(H1N1)pdm09 infection regardless of the enhanced virus replication, suggesting that the host response may contribute to disease severity through immune pathology-based mechanisms.

In this study, there was no important differences in the underlying clinical conditions between the groups of infected pregnant women surviving the infection, compared to those who died. Therefore, our results could shed light on the understanding the pathogenesis of the infection.

In summary, our findings demonstrate that the damaging effect of the pandemic influenza virus in pregnant women was associated with an increased expression of IL-8, IL-6 and TGF-α. We further identified a strong decrease in INF-β and TGF-β expression in the dead pregnant women. Understanding the interactions would make important contributions to the elucidation of the pathogenesis of influenza virus infection during pregnancy.

It would also be interesting to measure the levels of cytokines/chemokines production by ELISA and to relate these studies with our results.

Additionally, these findings reinforce the public health message that pregnant women should be a high-priority group targeted for vaccination.

Conflict of interests

The authors declare no competing interests or conflict of interest.

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Ethical approval

This study was approved by the Independent Ethics Committee of the Faculty of Medicine, University of Buenos Aires, Argentine.

Authors' contributions

NP, LMP and EB conceived and designed the experiments. NP wrote the paper. All authors read and approved the final manuscript. Contributed to make experiments/reagents/materials/analysis tools: MA, AP, AC, MR, EB, AC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2015.01.009.

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