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HOST IMMUNE RESPONSE TO SARS-CoV-2 INFECTION BY *TLR3*, *TLR4* AND *TLR7* GENE EXPRESSION THROUGH SEMI-QUANTITATIVE RETRO-TRANSCRIPTION PCR

RESPUESTA INMUNE DEL HUÉSPED A LA INFECCIÓN POR SARS-CoV-2 MEDIANTE LA EXPRESIÓN DE LOS GENES *TLR3*, *TLR4* Y *TLR7* A TRAVÉS DE RT-PCR SEMICUANTITATIVA

Martinez Marignac V.L.[1,2], Lirussi D.[3], Oertlin G.[1,2], Favant J.L.[1,2], Fleischman E.[2], Salinas M.[4], Marchetti G.[5], Gassali Z.[5], Richard S.M.[6]

[1] IBIOGEM, Laboratorio Interdisciplinario de Biología y Genética Molecular, Centro de Investigación Científica y de Transferencia Tecnológica a la Producción-CICYTTP, CONICET-UADER, Entre Ríos, Argentina.

[2] Laboratorio Dr. Néstor Bianchi, Hospital San José, Diamante, Entre Ríos, Argentina.

[3] Instituto Nacional de Medicina Tropical ANLIS-Malbrán, Puerto Iguazú, Misiones, Argentina.

[4] Laboratorio Provincial de Epidemiología, Ministerio de Salud, Entre Ríos, Argentina.

[5] Oficina Director – Hospital San José Diamante, Entre Ríos, Argentina.

[6] Instituto Multidisciplinario de Biología Celular (IMBICE), La Plata, Buenos Aires, Argentina.

Corresponding author:
Verónica L. Martinez
vmartinezmarignac@cicyttp.org.ar

ORCID 0000-0002-0486-7280

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ABSTRACT

Toll-like receptors (*TLRs*) involved in viral detection or overexpressed during infection, play also a main role in the subsequent development of fatal clinical manifestations in COVID-19 patients. We characterized by semi-quantitative retro-transcription PCR the expression of *TLR* 3, 4, and 7 in nasopharyngeal total RNA samples from 150 and 152 individuals, positive and negative for COVID-19, respectively. All patients were grouped in accordance with the presence of respiratory symptoms, and results on rapid molecular diagnostic test (NeoKit S.A.) during 2021. Four groups were analyzed: a) COVID-19 RNA detected with severe symptomatology; b) COVID-19 RNA detected with low/mild symptomatology; c) COVID-19 RNA not detected with severe symptomatology; and d) COVID-19 RNA not detected with low/mild symptomatology. Other variables studied were age and sex. Our results show (while correcting the sample size bias of previous reports), a non-significant difference in the expression of *TLR4*/7 between COVID-19 positive and negative patients. Noteworthy, the expression of *TLR3* was augmented in patients that resulted negative for COVID-19. When we compared the expression among the four groups, a significant positive correlation between severe symptomatology and *TLR4* expression in patients positive and negative for COVID-19 was found. Furthermore, *TLR4* gene expression was significantly amplified in those COVID-19 patients with severe symptomatology when compared with non-severe COVID-19 cases. Our data suggest that our innate *TLRs* immune system may respond differently to respiratory infections with similar symptomatology. We confirmed that mainly *TLR3* and *TLR4* could be involved in the response to respiratory pathogenesis and particularly *TLR4* in COVID-19 infection with severe symptoms. In concordance with previous studies, we found that in inflammatory respiratory diseases it is important to focus on *TLR3*, and *TLR4* gene expression to understand severe symptoms development. Research on these pathways may help to find modulators or antagonists to these genes, for future treatments.

Key words: gene expression, isothermal amplification, SARS-CoV-2 diagnoses, semi-quantitative retro-transcription PCR, toll-like receptor genes.

RESUMEN

Los receptores tipo Toll (*TLRs*) implicados en la detección de virus o sobreexpresados durante la infección, desempeñan un papel principal en el desarrollo de manifestaciones clínicas fatales en pacientes con COVID-19. Caracterizamos, mediante retro transcripción y PCR semi-cuantitativa, la expresión de los genes *TLR* 3, 4 y 7 en muestras de ARN nasofaríngeo de 150 individuos positivos y 152 negativos para COVID-19. Todos los pacientes fueron agrupados de acuerdo con la presencia de síntomas respiratorios y los resultados de diagnóstico molecular rápido (NeoKit S.A.) durante 2021. Se analizaron cuatro grupos: a) COVID-19 detectado con sintomatología grave; b) COVID-19 detectado con sintomatología leve/mínima; c) COVID-19 no detectado con sintomatología grave; y d) COVID-19 no detectado con sintomatología leve/mínima. Otras variables estudiadas fueron la edad y el sexo. Los resultados muestran, corrigiendo el sesgo de tamaño de muestra de informes previos, una diferencia no significativa en la expresión de *TLR4/7* entre pacientes positivos y negativos para COVID-19. Se destaca la expresión de *TLR3* incrementada en pacientes que resultan negativos para COVID-19. Al comparar la expresión entre los cuatro grupos, se encontró una correlación positiva significativa entre la sintomatología grave y la expresión de *TLR4* en el total de pacientes. La expresión del gen *TLR4* se amplificó significativamente en los pacientes con COVID-19 con sintomatología grave en comparación con los casos de COVID-19 no graves. Nuestros datos sugieren que el sistema inmune innato mediado por *TLRs* podría responder de manera diferente a infecciones respiratorias aun con sintomatología similar. Confirmamos que *TLR3* y *TLR4* podrían estar involucrados en la respuesta a la patogénesis respiratoria, y particularmente *TLR4* en infecciones por COVID-19 con síntomas graves. Concluimos que es importante evaluar la expresión de los genes *TLR3* y *TLR4* para comprender el desarrollo de síntomas graves en enfermedades respiratorias; esto ayudará a identificar moduladores o antagonistas de estos genes para futuros tratamientos.

Palabras clave: expresión génica, amplificación isotérmica, diagnóstico de SARS-CoV-2, PCR semi-cuantitativa de retro-transcripción, genes de receptores tipo Toll.

INTRODUCTION

Toll-like receptors (*TLR*s) are a family of receptors comprising 10 members (*TLR1-TLR10*), which are expressed in innate immune cells such as macrophages, as well as in epithelial and fibroblast cells. These have been reported as a cause in the initial failure of viral clearance (O'Neill et al., 2013) and it was suggested that they are involved in the subsequent development of fatal clinical manifestations in severe COVID-19 patients, related to fatal respiratory failure in acute respiratory distress syndrome (ARDS) (Onofrio et al., 2020). *TLR*s activation can be induced by a multitude of pathogen associated molecular patterns (PAMPs) present in bacteria, viruses, and other microorganisms (Duran et al., 2014). Hence, it was suggested that *TLR*s play an important role in priming innate immune responses, with production of inflammatory cytokines, like interleukins 4/6, (IL-4, IL6), IL-1β and other mediators (Bortolotti et al., 2021).

*TLR*s are expressed on the cell surface (*TLR* 1, 2, 4, 5, 6 and 10) or in the endosomal compartment (*TLR* 3, 7, 8 and 9) (O'Neill et al., 2013; Onofrio et al., 2020). While *TLR*3 in the endosome recognizes viral double-stranded RNA (dsRNA), *TLR*7 recognizes viral single-stranded RNA and is therefore suggested to be involved in the clearance of SARS-CoV-2 and similar viruses (Imai et al., 2008; Onofrio et al., 2020). Although COVID-19 viral entry is mainly via the interaction of spike glycoprotein with the angiotensin-converting enzyme 2 (ACE2) of the host cell, other interactions have been described. It is now well known that the spike protein can interact with immune ligands like TLRs and C-lectin-like receptors (CLR) as well as with non-immune receptors as neuropilin-1 (NRP1) and glucose regulated protein 78 (GRP78) for the viral entry (Gadanec et al., 2021).

An intact SARS-CoV-2 virus is surrounded by a lipid enclosure that contains the envelope protein (E), the membrane protein (M), and the spike glycoprotein (S). The genome of SARS-CoV-2 consists of large, single-stranded positive RNA (from 29.8 to 29.9 kb) that contains fourteen (14) open-reading frames (ORFs) encoding twenty-seven (27) proteins; it is possible to suggest that before packaging, COVID-19 RNA virions could be detected by TLR7 that is localized in the intracellular membranes of endosomes (Nishiya et al., 2005). The genome sequence of SARS-CoV-2 displays 79.0% homology with SARS-CoV and 51.8% with MERS-CoV. Nucleocapsid (N) proteins form complexes with genomic RNA for genome packaging. In consequence, it is plausible that *TLR7* and *TLR3* are molecules that could recognize single- and double-stranded viral RNA structures before packaging (Nishiya et al., 2005).

During viral entry into host cells, the surface trimeric S glycoprotein mediates receptor recognition and virus-host cell membrane fusion. The host protease furin cleaves the S protein into S1 and S2 subunits for pre-activation, and the receptor-binding domain (RBD) of S1 binds to ACE2 expressed on the surfaces of host cells. Then, SARS-CoV-2 enters host cells by either direct fusion or endocytosis (Jung and Lee, 2021).

In case of TLR4 expressed on the cell surface, it has been shown that it binds SARS-CoV-2 spike glycoprotein and this activates TLR4 signaling to increase cell surface expression of ACE2, then facilitating SARS-CoV-2 entry. This is in part the mechanism that destroys the type II alveolar cells, the ones that secrete pulmonary surfactants. As a result, all these factors that otherwise contribute to a physiological lung equilibrium, during COVID-19 infection promote ARDS and inflammation (Manik and Singh, 2022). Thus, we suggest that an available antagonist against TLR4 could prevent the onset of severe COVID-19 or other viral induced ARDS in symptomatic patients, and synergize with active antiviral therapy.

While TLR3 recognizes viral double-stranded RNA (dsRNA), TLR7 recognizes viral single stranded RNA and is therefore involved in SARS-CoV-2 clearance, as evidenced by its loss of function in some cases of severe COVID-19 (van der Made et al., 2020). On the other hand, TLR4 at the surface of cells plays a role in the induction of damaging inflammatory responses during acute viral infections as it functions as a sensor for damage-associated molecular patterns (DAMPs). These patterns include a wide variety of molecules released from injured or dying tissues as well as molecules actively released in response to cellular stress from intact cells. Besides these functions in viral infections, the paramount role of TLR4 is the recognition of lipopolysaccharides (LPS) in Gram-negative bacteria (Poltorak et al., 1998) which could explain why TLR4 is not only associated to COVID-19 infection or ARDS.

It is well known that *TLR*s family members play also an important role in cancer progression. A comparison with cancer treatments is appropriate herein, as TLR stimulation of cancer cells can lead to tumor progression or inhibition. Stimulation of *TLR* 2, 4, and 7/8 can lead to tumor progression through production of immunosuppressive cytokines, increased cell proliferation, and resistance to apoptosis. On the other hand, stimulation of *TLR* 2, 3, 4, 5, 7/8 and 9 gene expression, often combined with chemo or immunotherapy, can lead to tumor inhibition through different pathways (Urban-Wojciuk et al., 2019). In addition, TLR stimulation in natural killer (NK) cells and APCs (antigen-presenting DC-dendritic cells and macrophages) can induce CTL (cytotoxic T lymphocytes) to further inhibit tumor growth (Urban-Wojciuk et al., 2019). In consequence, using cancer *TLR* insight could be an alternative approach to selectively activate some of these pathways in patients suffering a severe COVID-19 infection, for whom it could be a disadvantage to develop

the wrong cytokine storm (Chahal et al., 2013; Bezemer and Garssen, 2021). Thus, it is plausible to postulate that to decrease hyper-inflammation and thrombotic complications in vulnerable population (severe stages of COVID-19), a control of the expression of *TLR3*, *TLR4* and *TLR7* genes could be instrumental. However, there is a need of more evidence concerning the importance of the relative expression level of *TLR3*, *TLR4* and *TLR7* (Bortolotti et al., 2021; Manik and Singh, 2022) in ARDS and COVID-19 patients.

As the *TLR*s have been studied in the field of oncology, different anti-tumor treatments have been developed as activators, inhibitors or antagonists of these receptors (Urban-Wojciuk et al., 2019). However, there are few studies recently published showing the gene expression profile of some of these genes in COVID-19 infected patients (Chahal et al., 2013; Bezemer and Garssen, 2021) which were carried out on small cohorts of patients (with different symptomatology). Furthermore, there are also few studies relating treatment of COVID-19 by agonists of TLR7 and TLR8. For instance, one of these studies showed that *TLR7* stimulation may not only help viral clearance, but also exert an anti-inflammatory effect that decreases severe symptoms while synergizing with treatments (Khalifa and Ghoneim, 2021).

Here, our goal was to evidence *TLR* 3, 4, and 7 gene expression by semi-quantitative retro-transcription PCR in nasopharyngeal total RNA samples from patients admitted to the service of public hospitals of five departmental jurisdictions of the province of Entre Ríos, Argentina, during 2021.

MATERIALS AND METHODS

Patient groups (cohorts)

This study included a group of patients admitted during 2021 to the service of public hospitals in Diamante departmental jurisdiction and from other four departmental jurisdictions of the Province of Entre Ríos, Argentina. All patients showed symptomatology related to respiratory infections and at least two of the twelve symptoms were attributed to SARS-CoV-2 infection, according to the Health Ministry of Argentina and the World Health Organization (WHO) interim guidance.

Patients were divided into two main groups: 1- patients with active SARS-CoV-2 infection confirmed by a new isothermal amplification kit (developed by Cassara and Neokit S.A., Argentina) from nasopharyngeal swabs (DET, n=150) and, 2- SARS-CoV-2 negative patients and/or with SARS-CoV-2 IgG antibody-negative serology (ND, n=152). Disease severity was classified into three clinical types (low, mild/moderate and severe), according to the Health Ministry of Argentina and the WHO interim guidance. We have considered the following symptoms: 1=headache, 2=myalgia, 3=sore throat, 4=fever (no higher than 38 ºC), 5=fever (38 ºC or higher), 6=cough, 7=rhinitis/nasal congestion, 8=vomiting, 9=diarrhea, 10=respiratory failure, 11=anosmia, and 12=dysgeusia and death.

Patients with low symptomatology showed one or two of the symptoms 2, 8 or 9 (n=182), patients with mild/moderate symptomatology showed symptoms 3, 4 or 5 (n=76) and patients with severe symptomatology had symptoms 5, 6, 7, 10, 11 and 12 (n=44) (Table 1).

In brief, patients with low symptomatology did not present evidence of viral pneumonia or hypoxia. Symptoms were non-specific and could include separately fatigue, headache, myalgia, abdominal pain, vomiting and diarrhea. Mild/moderate symptomatology patients presented symptoms and signs of non-severe pneumonia, fever, sore throat (cough or fast breathing) and included patients with comorbidities such as cardiac or respiratory disease and/or diabetes. The cohort with severe symptomatology contained patients under severe disease, the majority with fever over 38 ºC, nasal congestion, respiratory failure, anosmia and dysgeusia. The characteristics of participants with or without COVID-19 are shown in Table 1.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board CEYSTE - Committee of Ethical and Experimental Work Safety (CEYSTE, CONICET), whose members reviewed and approved the informed consent, the sample collection and the overall study protocol (Expedient reference: CEYSTE-CE0112/2020).

COVID-19 determination

We used isothermal amplification by the COVID-19 NEOKIT-PLUS TECNOAMI, developed by CONICET and

Table 1. Characteristics of the cohorts of patients admitted to public hospitals in five jurisdictions of the Province of Entre Ríos with COVID-19 and miscellaneous respiratory infections. n=sample size; DET=COVID-19 detected infection; ND= COVID-19 non detected infection.

Patients Characteristics		DET (n=150)	ND (n=152)
Age/years	<2 baby	9	17
	<7 child	21	21
	< 18 teen	17	21
	< 36 young adult	49	40
	< 65 adult	47	46
	>65 old adult	8	7
Sex	female	70	77
	male	80	75
Symptomatology	low	91	91
	mild/moderate	40	36
	severe	19	25

Cassara S.A., Argentina. This diagnostic test is used for the simplified molecular detection of the SARS-CoV-2 virus (the etiological agent of atypical pneumonia or severe acute respiratory syndrome). The NEOKIT-PLUS system allowed the purification of total mRNA from patients swabs according to the manufacturer protocol. We obtained 50 µl of total RNA and used 10 µl to determine the presence of viral RNA.

Human cDNA

We used 5 µl of each total RNA sample purified using the NEOKIT PLUS system, which were stored at -80 °C. A random hexamer retro-transcription PCR method was performed by 1 µM of hexamer primers (PB-L SRL, Argentina), dNTPs 500 µM and MilliQ water in a final volume of 15 µl. This was exposed to thermo-cycling (5 min at 65 °C and 2 min on ice), and finally, the reaction was brought to a final volume of 20 µl by adding 50 mM of $MgCl_2$, 100 U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, and 4 µl of MMLV buffer (PB-L, Argentina); thermo-cycling was performed at 25 °C for 10 min and 37 °C for 50 min.

TLR3, TLR4 and TLR7 gene expression determination

We characterized the expression of *B-actin*, *TLR* 3, 4, and 7 genes. The determination of gene expression was performed following the protocols of Allborn et al. (2008) and Ghasemi et al. (2014). We must underline that the selection of semi-quantitative retro-transcription PCR rather than quantitative PCR (qPCR), was because we focused on very high deltas in order to found more biological relevant or significant changes between cohorts.

We used 5 µl of cDNA in a semi-quantitative gene expression protocol. Human *B-actin* gene was used as a housekeeper gene and the amplification reaction was performed with 200nM of forward (GAGCACAGAGCCTCGCCTTT) and reverse (ACATGCCGGAGCCGTTGTC) primers, 1U of Taq polymerase (Pegasus-PB-L SA Argentina), 3 mM of MgCl2 and 200 uM of dNTPs; the thermocycling conditions were: 2 min at 94 °C; 40 cycles of 45 s at 92 °C, 45 s at 60 °C, and 45 s at 72 °C; and an end step of 5 min at 72 °C. In Table 2 we show the *TLRs* primers. The conditions of end point PCR used for *TLR* 3 and 4 were the following: 2 mM of MgCl2, 200 uM of dNTPs, 200 nM of primers and 1 U of Taq polymerase (Pegasus –PB-l SA, Argentina). The PCR conditions for *TLR7* were the following: 2.6 mM of MgCl2, 200 uM of dNTPs, 200 nM of primers and 1 U Taq polymerase. The thermocycling conditions for all the genes were the following: 2 min at 94 °C; 40 cycles of 30 s at 95 °C, 1 min at 62 °C, and 1 min at 72 °C; and an end step of 5 min at 72 °C. PCR products (10 µl of each sample) were separated by electrophoresis in a 2% agarose gel (Sigma) with 1x TAE buffer (Invitrogen) and a voltage of 95 V for 30-40 min. The bands were visualized by using an ultraviolet trans-illumination device and digital images were captured by Gel documentary machine (Care stream, Berlin, Germany).

Statistical analysis

For groups (DET vs. ND) and subgroups (sex, age, severity, gene expression) comparisons we used a nested analysis, non-paired t-Test and Spearman's Correlation Coefficient to evidence association between level of symptomatology and the expression of the three *TLRs* genes. We also used a Point-Biserial Correlation Calculator to evidence any correlation between two variables in the special circumstance of dichotomous variables (DET/ND; female/masculine); for this calculator there are only two possible values, 1 or 0. We used Pearson's correlation to measure the strength and direction of the relationship between two paired variables (age or severity vs. gene expression)

Table 2. List of primers used in the experiment for the amplification of human *B-actin, TLR* 3, 4 and 7 genes by semi-quantitative RT-PCR. Forward and reverse primer sequences, annealing temperature and product size (bp= base pairs).

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (bp)
TLR3	GTATTGCCTGGTTTGTTAATTGG	AAGAGTTCAAAGGGGGCACT	62	112
TLR4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC	62	114
TLR7	CCTTGAGGCCAACAACATCT	GTAGGGACGGCTGTGACATT	62	285
B-actin	GAGCACAGAGCCTCGCCTTT	ACATGCCGGAGCCGTTGTC	60	108

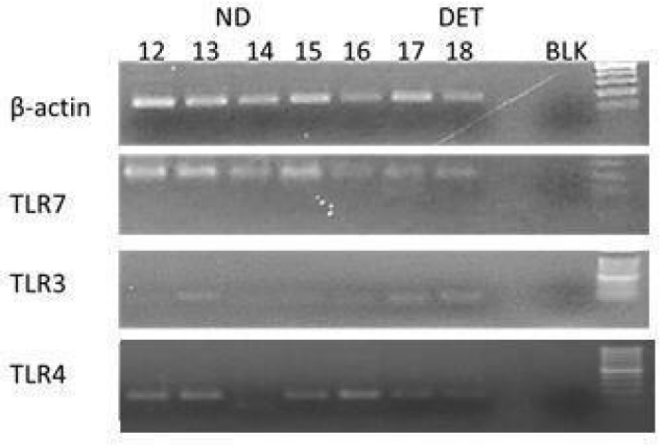


Figure 1. Characteristic results of the semi-quantitative retro-transcription PCR for *B-actin, TLR3, TLR4* and *TLR7* human RNAs. Agarose 2% gels showing characteristic results, BLK is retro-transcription and semi-quantitative PCR blank, the samples correspond to typical results from ND which are COVID-19 non detected samples and DET are COVID-19 detected samples.

and Spearman's Rho (RS) coefficient of correlation to compare the range of symptoms and other ordinal as well as continuous variables data, since ranks were used instead of assumptions of normality, especially for severity, sex and age. We used also a McNemar's test to evidence the strength of representation of each cohort for further analysis. We used a Welch's ANOVA for age and sex differences and gene expression. We further tested differences on sex and age by the Games-Howell post-hoc test from ANOVA as a nonparametric approach to compare combinations of cohorts without assuming equal variances and sample sizes.

We considered significant a p-value of 0.05 or lower. All statistical analyses were performed by the free access websites https://www.socscistatistics.com/tests/, https://www.graphpad.com/quickcalcs/ and https://www.biostathandbook.com/spearman.html.

RESULTS

By a low cost and efficient semi-quantitative retro-transcription and end point PCR, we analyzed on 2% agarose gels the gene expression of *B-actin*, *TLR3*, *TLR4* and *TLR7* (Figure 1).

From the collected nasopharyngeal swabs samples, we extracted RNA, and purified it using NeoKit Plus (SAS Argentina, SA). Only samples for which it was feasible to obtain very good retro-transcription and PCR product of *B-actin* gene were used for further analysis. By analyzing this important RNA collection of patients with respiratory disease symptoms sampled during 2021 and 2022, we evidenced that the expression of *TLR3*, *TLR4* and *TLR7* genes have no correlation with the presence of COVID-19 viral genome. This applies to the total cohort of patients by using the Point-Biserial Correlation calculator (Table 3, Figure 2). By applying the McNemar's test we did not evidence significant association between severe cases and COVID-19 presence in the patients, neither a bias in the representation of each used cohort for diverse comparisons (p>0.5). However, by applying a

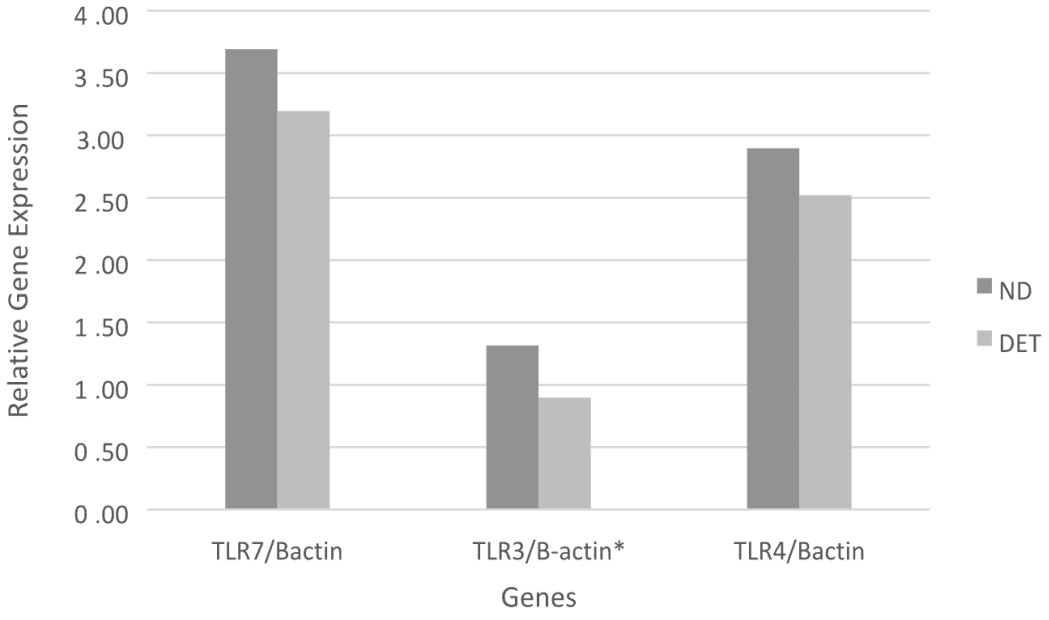


Figure 2. Gene expression by comparing COVID-19 Non-detected (ND) and Detected (DET) patients admitted to public hospitals in five jurisdictions of the Province of Entre Ríos with COVID-19 and miscellaneous respiratory infections.

Table 3. Semi-quantitative expression of *TLR3, TLR4* and *TLR7* normalized by housekeeping gene *B-actin* in two cohorts of patients: infected and not infected with COVID-19. SE = standard error, taking in account the sample size.

		TLR7/B-actin	*TLR3/B-actin*	*TLR4/B-actin*
COVID-19 non-detected	Average	3.69	1.32	2.90
	SE (±)	0.22	0.15	0.21
COVID-19 detected	Average	3.20	0.90	2.52
	SE (±)	0.19	0.10	0.17

non-paired t-Test, we found significant differences of expression for *TLR3*, in the 302 studied patients. Noteworthy, those who were infected with COVID-19 (n=150) had a significantly lower expression of *TLR3* (t=2.2, p=0.025) than the non infected ones (Figure 2).

When we took into account the severity of the disease, we observed non-significant differences between the cohorts and its *TLR3* or *TLR7* expression by ANOVA. For *TLR4*, we observed by ANOVA (followed by a Tukey's test) a low but significant difference between the gene expression of the cohorts (p=0.046).

When we analyzed the relationship between the severity of the symptoms and *TLR3* expression in the total sample (Table 4, Figure 3), we did not evidence a correlation between expression and level of symptomatology. On the other hand, *TLR4* expression showed a moderate positive and significant correlation in all patients with severe symptoms when we did not discriminate by other variables (Sperman's r_s=0.15054, *p* 2-tailed =0.00925). However, the differences in gene expression between low/mild and severe symptoms were non-significant (t-Test, p=0.054). The comparison of the four cohorts, DET low/mild/moderate, DET severe, NT low/mild/moderate and NT severe, revealed that *TLR4* expression was 1.5-fold higher in patients with severe symptoms and COVID-19 infection, although the difference was not significant (t-Test, p=0.06). Nevertheless, our results show a significant positive correlation when we took into account only infected patients (DET) and *TLR4* expression, which resulted to be significantly increased in patients with severe confirmed COVID-19 disease (t-Test, p<0.05). We also analyzed the gene expression according to sex and age of patients by a nested ANOVA. Only COVID-19 DET samples, by Game-Howell's test showed significant differences of *TLR7*, *TLR3* and *TLR4* expression when discriminated by sex. The chi-square test of independence showed that there was no significant association between sex and been infected or not, X^2 (2, n=302, p>0.5) as well as between severe symptoms and be female or male, X^2 (2, n=302, p>0.07). Briefly, the differences in expression were not significant associated with symptomatology or been in DET or ND groups.

When analyzing by age of patient, DET and ND displayed the same patient's age distribution. The *TLR3*, *TLR4* and *TLR7* gene expression displayed a non-significant and low negative correlation with age, as determined by a nested and correlation analysis. When analyzed separately by Spearman's correlation, *TLR4* expression level showed a significant negative correlation with age of ND or DET cohorts (r_s = -0.14216, *p* 2-tailed = 0.01372). In conclusion, of all genes analyzed, only *TLR4* was significantly correlated in its expression with age. On the other hand, when analyzed by the Spearman's correlation, severity and age resulted in a significant positive correlation (r(302)=0.2676, p<0.01). These results were confirmed by Point-Biserial Correlation taking into account ranks of ages (6) and scoring symptoms low/mild/moderate as 0 and severe as 1. A significant correlation was also found (r=-0.143, p=0.012), in both non-detected and detected COVID-19 patients, meaning that older patients of these cohorts would develop high severity of symptoms (of a respiratory disease) disregarding if infected or not with COVID-19 and their *TLRs* gene expression.

DISCUSSION

Our data partially contradict previous findings, showing here now in a larger cohort, a non-significant difference between *TLR4* and *TLR7* expression in patients with non-detected and detected COVID-19. We also show here that *TLR3* gene expression was increased in non-detected COVID-19 patients independently of the form of the clinical symptoms of the disease (low, mild and severe). Finally, when the severity of symptoms and gene expression were together taken into account,

TLR4 expression was positively correlated with severe symptomatology/deaths in COVID-19 positive patients.

A previous work of Menezes et al. (2021) was carried out in 79 patients with severe COVID-19 detected at admission, that according to the WHO classification, were divided into two groups: patients who needed mechanical ventilation and/or deceased (severe, n=50) and patients who used supplementary oxygen but not mechanical ventilation and survived (mild/moderate, n=29). A control group (control, n=17) of healthy people was enrolled too. Instead of nasopharyngeal tissue, they use the peripheral blood (which will account for systemic immunity, not mucosal) to characterize gene expression (mRNA) of Toll-like receptors (*TLRs*) 3, 4, 7, 8, and 9, as well as other immune response genes (*RIGI*, *NLRP3*, *IFN-α*, *IFN-β*, *IFN-γ*, *IFN-λ*, *pro-interleukin(IL)-1β (pro-IL-1β)*, and *IL-18*), all determination according to the authors were done on admission, between 5–9 days, and between 10–15 days (Menezes et al., 2021). Circulating cytokines in plasma were also measured. When they compared the COVID-19 mild/moderate group with the COVID-19 severe group, the former had lower expression of *TLR3* and overexpression of *TLR4*. In the present work, we informed the expression level of three *TLRs* at the moment of infection determination, between 5-9 days of symptoms, using nasopharyngeal swaps. Importantly, our samples not only corresponded to a larger cohort but also focused on mucosal immunity rather than systemic immunity. Furthermore, gene expression was analyzed over a shorter timeframe following symptom onset compared to the study by Menezes et al. (2021) and different severity levels, age and sex of subjects presenting some of the twelve WHO recognized COVID-19 symptoms and with SARS-CoV-2 viral genome determined were taken into account. In concordance with the findings of Menezes et al. (2021), we confirmed, in a much larger cohort of patients, that *TLR4* expression was increased in patients with severe symptomatology; on the contrary, in COVID-19 positive infected patients, we observed a non-significant tendency to higher expression.

In the case of *TLR3*, there were no difference in expression within the positive COVID-19 patients when contrasted according the severity of symptoms. *TLR3* was found to be increased in non-positive COVID-19 patients disregarding their disease severity. Since *TLR3* recognizes viral double stranded RNA (dsRNA), this increased expression is therefore likely to be related to other viral infections present in the ND cohort with low to severe symptomatology.

Other authors, such as Bagheri-Hosseinabadi et al. (2022) used the same type of biological samples though in a smaller set of individuals (90 COVID-19 patients and 50 controls) with symptomatology of COVID-19 infection. However, they were compared with controls with low or no symptoms, healthy and not infected or suffering from a respiratory illness. Samples were classified into those without symptoms, with symptoms but not hospitalized, and with symptoms and hospitalized. They showed that *TLR3*, *TLR7*, *TLR8*, and *TLR9* were over expressed in the COVID-19 patients with clinical symptoms and in need of hospitalization. By the contrary, we did not find differences between the expressions of *TLR3*, *TLR4* and *TLR7* in those hospitalized non COVID-19 (n=8) or hospitalized COVID-19 patients (n=5) (data not shown). Furthermore, we found no differences in the

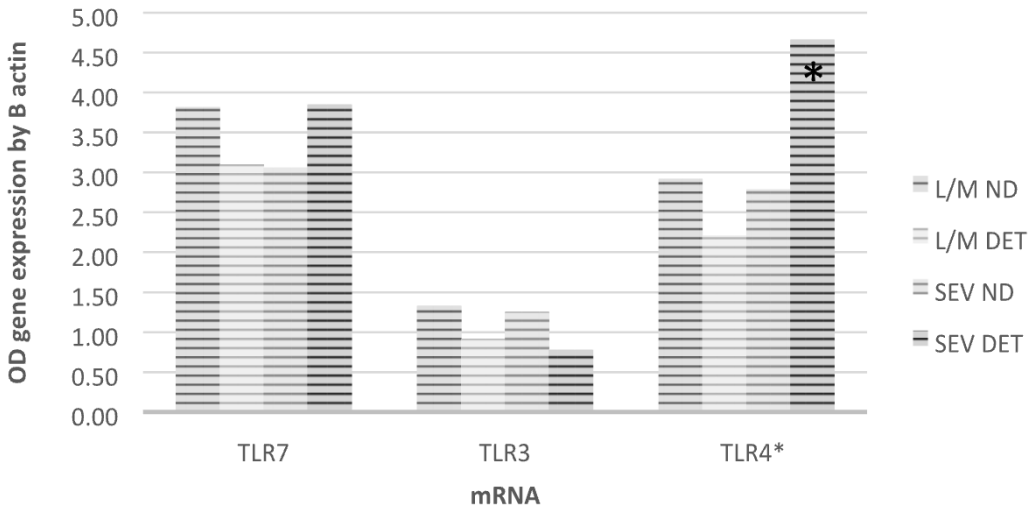


Figure 3. *TLRs* expression in the four studied cohorts: L/M ND: low or mild symptomatology, COVID-19 non-detected; SEV ND: severe symptomatology, COVID-19 non-detected; L/M DET: low or mild symptomatology, COVID-19 detected; SEV DET: severe symptomatology, COVID-19 detected. *Significant statistical differences ($p<0.05$).

Table 4. Semi-quantitative expression of *TLR3, TLR4* and *TLR7* normalized by housekeeping gene *B-actin* in four cohorts of patients: non-detected COVID-19 with low/mild symptoms, non-detected COVID-19 with severe symptoms, detected COVID-19 with low/mild and detected COVID-19 with severe symptoms. n= sample size; SE= standard error taking into account sample size. *Significant statistical differences ($p<0.05$).

	COVID-19 detected		**COVID-19 non-detected**	
	Low/Mild (n=131)	Severe (n=19)	Low/Mild (n=127)	Severe (n=25)
TLR7/B-actin	3.1	3.85	3.82	3.06
SE	0.2	0.53	0.24	0.37
TLR3/B-actin	0.91*	0.78*	1.33	1.25
SE	0.11	0.20	0.17	0.31
TLR4/B-actin	2.21	4.66*	2.92	2.79
SE	0.16	0.61	0.23	0.33

expression for *TLR3* and *TLR7* on COVID-19 patients vs. those without this infection (n=302). As expected, they concluded that the upregulation of *TLRs* was associated with clinical presentations of severity of symptoms on COVID-19 positive patients. They also concluded that the modulation of *TLR3*, *TLR7*, *TLR8* and *TLR9* in the epithelial cells of COVID-19 cases may estimate the disease severity and requirement for hospitalization. On the contrary, the analysis of our data demonstrated that *TLR3* is overexpressed in ND cohorts, while *TLR7* showed no significant differences between the samples. Our results showed that increased *TLR4* expression was correlated with the severity of infections.

When the severity is taken into account, the *TLR4* expression corroborates previous results of the higher level of expression in COVID-19 patients with high or severe symptomatology and hospitalization. Accordingly, *TLR4* expression is suggested as the only factor correlated to unfavorable outcome for COVID-19 infection (Bagheri-Hosseinabadi et al., 2022). On the other hand, the higher expression of *TLR3* correlates positively with severe symptoms in those patients without SARS-Cov-2 infection, a discrepancy with previous results, as those from Menezes et al. (2021). Noteworthy, the results are not comparable, since these authors set as controls patients without respiratory syndrome and analyzed a smaller sample size.

To the best of our knowledge, none of the previous studies (as well as this work) took into account the mRNA expressions in vaccinated patients. More likely because vaccinated patients will rarely develop severity symptoms like those common in non-vaccinated patients. As shown in mice (Do et al., 2023), the effects of co-administration of *TLR* agonists (*TLR* 3, 4, 9) as SARS-CoV vaccine adjuvants, fail to boost mucosal humoral immunity (i.e.: anti-spike neutralizing antibodies) underlining the effect of vaccination on *TLR*s expression and other immunity respondent. These effects are similarly reproduced in humans where the primary innate response to *TLR*s can be used to predict SARS-CoV vaccine protective humoral responses, as it was shown in a small clinical trial when using AstraZeneca ChAdOx1 nCoV-19(AZD1222) (Shen et al., 2022). Consequently, vaccination would introduce a plausible bias by modulating the immune response of patients towards more effective (less exaggerated) responses, in most cases, which can account for the differences observed here for *TLR3* and *TLR4*. Although we cannot completely discard vaccination effect for our study, we can assume the assayed population as naive for COVID-19 immunizations, since the first COVID-19 vaccination campaign in Argentina started only for vulnerable population (such as older adults, adults, younger adults) by late May 2021, meanwhile massive vaccination for all ages started at the end of 2021 in province of Entre Ríos. Another indication of the naive immune status of the analyzed cohorts in this study, is that we found a significant positive correlation between severity and age, in both non detected and detected COVID-19 patients, meaning that older patients of this cohort tend to develop severe symptoms (an almost opposite scenario than the attained upon immunization).

If we take into account the conclusion of a previous work carried out in our laboratory (Eberhardt et al., 2022) we can support that the vast majority of our samples are from non-vaccinated patients. In this sense, according with our results for *TLR3* and *TLR7* expressions, there were a non-significant decrease in expression by age, while, *TLR4* was found to be significantly increased in children, young adults and adults. By three-nested ANOVA analyses, we found that age and severity in DET cohorts only have a significant impact on *TLR4* expression. Repeating, *TLR4* is an innate immune response effector and it has the role of

pattern recognition receptors (PRRs) in the immune system. PRRs are crucial for detecting signs of infection or damage, helping to initiate appropriate immune responses. Specially it recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). *TRL4* intrinsic relationship with aging, could be part of its normal response in young and adult cohorts, overexpressing *TLR4* during infection in a significant correlation with severe disease (Wong et al., 2020; Khanmohammadi and Rezaei, 2021; Seery et al., 2021; Dai et al., 2022; Kim et al., 2023).

Overall, we found that these genes are expressed in a different manner between women and men. This can be supported specially from reports showing that *TLR7* expression depends on the methylation pattern of the X chromosome (Gomez-Carballa et al., 2022), however, in contrast to other reports there were no association between been infected or not by COVID-19, nor the symptomatology by sex, on the expression levels of *TLR7*. Importantly, regarding *TLR7*, it is known that its expression may be different in females and males due to the altered methylation patterns in the X-linked *TLR7* gene and consequential down-regulation of its expression in males (a difference that is not disease or infection related) (Gomez-Carballa et al., 2022).

Finally, the expression of *TLR4* in detected patients with severe symptoms was significantly higher than in detected patients with low or moderate symptoms, while the expression of *TLR3* in those patients without the infection was significantly higher than in detected patients. Therefore, we confirmed that the immune response of *TLR4* and *TLR3*, as measured by its gene expression, can be associated with harm (damage) rather than host protection, due to persistent inflammation and tissue destruction in the pathogenesis of COVID-19. This applies also to the other severe respiratory diseases. We suggest that research efforts should focus on therapies based on the regulation of *TLR4* and *TLR3* expression, to diminish inflammation in severe respiratory diseases, with or without COVID-19 infection. At the same time, the role of *TLR3* in different respiratory pathologies needs to be evaluated as it probably mitigates symptoms in other respiratory viral infections. Nonetheless, *TLR4* high expression could be monitored as a marker or cause of severe symptoms in COVID-19 infections.

In agreement with Onofrio et al. (2020), we suggest that *TLRs* are involved in the development of fatal clinical manifestations in severe COVID-19 patients, essentially in ARDS, and therefore related to the progression towards fatal respiratory failure. Our data also suggest that even though our innate immune system contributes to the elimination of viruses by *TLRs* sensing and signaling, the maintenance of inflammation and tissue destruction in the host could be linked with *TLR4* expression in COVID-19 infected patients. We confirmed that *TLR3*, *TLR4* and *TLR7* could be involved in the pathogenesis of other respiratory diseases and the severity in aged populations. We suggest that treatments focused on the expression of these genes during the progression of inflammatory respiratory diseases, could be an interesting starting point for the development of therapies that avoid unfavorable outcomes and severe symptoms in younger patients. We do not need to underline the importance of sex in the infection outcome, because both genders showed similar symptomatology (although with differences in gene expression).

We suggest to analyze the repositioning of TLR antagonist and agonist drugs, developed as consequence of anti-tumoral and cancer studies (Chahal et al., 2013; Urban-Wojciuk et al., 2019; Khalifa and Ghoneim, 2021; Dai et al., 2022), for its use in ARDS and severe COVID-19 infected patients.

We found a low positive correlation between severity and age. Taking into account these findings and those from previous studies showing cytokine storms and age-related disease development (Khanmohammadi and Rezaei, 2021; Dai et al., 2022), we can conclude that acute lung injury is a feature of severe respiratory disease in adults, disregarding the gene expression here studied.

As a consequence of the differences found in *TLR3* and *TLR4*, we suggest conducting a much larger study, with patients with symptoms of respiratory disease during the Delta and Omicron waves in Argentina (2020-2021), which would decrease biases by number and allow analysis from different detection systems, including the vaccination status. A thorough knowledge of the status of *TLR3*, *TLR4* and *TLR7* expression in infected patients with ARDS or SARS- Cov-2, can help in repositioning cancer drugs for ARDS treatment for those patients with increased *TLR4* expression.

Advances in the development of TLR agonists and antagonists as immunomodulators, including their roles as vaccine adjuvants and treatments for hyperinflammatory responses, hold promise for future interventions against SARS-CoV-2 or similar infections (Ghasemi et al., 2014; Eberhardt et al., 2022).

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AUTHOR CONTRIBUTION STATEMENT

Conceptualization: Martinez Marignac V.L. and Lirussi D. Data collection and curation: Martinez Marignac V.L., Gazali Z., Oertlin G., Salinas M. and Fleischmann E. Formal analysis: Martinez Marignac V.L. and Lirussi D. Funding acquisition and Resources: Marchetti G. and Martinez Marignac V.L. Methodology: Richard S., Martinez Marignac V.L., Oertlin G., Fleishmann E. and Lirussi D. Writing and revision: Richard S., Martinez Marignac V.L. and Lirussi D. Final editing: Martinez Marignac V.L. and Lirussi D.

CONFLICTS OF INTEREST

The authors inform that they are all public employees belonging to CONICET or to the Ministry of Health of the Province of Entre Ríos. The authors declare that they have no conflict of interest.