UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

FACULTADE DE MEDICINA

DEPARTAMENTO DE PEDIATRÍA

IDENTIFICATION AND ANALYSIS OF THE IMPACT OF CO-INFECTIONS IN PEDIATRIC PATIENTS HOSPITALIZED WITH LOWER TRACT ACUTE RESPIRATORY INFECTIONS

Memoria que presenta, para optar al grado de doctor,

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CERTIFICAN:

Que la presente memoria que lleva por título **"Identification and analysis of the impact of co-infections in pediatric patients hospitalized with lower tract acute respiratory infections",** de la licenciada en Farmacia por la Universidad de Santiago de Compostela *Miriam Cebey López*, ha sido realizada bajo nuestra dirección, considerándola en condiciones para optar al Grado de Doctor y autorizándola para su presentación ante el Tribunal correspondiente.

Y para que así conste, firmamos la presente en Santiago de Compostela, a 18 de septiembre de 2015.

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A Jorge y a Claudia.

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Agradecimientos

Aunque no lo parece, es cierto que ésta es una de las partes más difíciles de escribir de la tesis, a pesar de que la mayoría de las personas que aquí menciono ya saben cuánto les agradezco el apoyo que me han dado de una u otra forma.

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LIST OF ABBREVIATIONS

%: percentage

95CI: confidence Interval 95%

AdV: adenovirus

AOM: acute otitis media

ARI: acute respiratory infection

DNA: deoxyribonucleic acid

hBoV: human bocavirus

hCoV: human coronavirus

hMPV: human metapneumovirus

hPIV: human parainfluenza virus

hRV: rhinovirus

IV: influenza virus

LT-ARI: lower tract acute respiratory infection

NGS: next generation sequencing

OR: Odds Ratio

PICU: pediatric intensive care unit.

RNA: ribonucleic acid

RSV: respiratory syncytial virus

UK: United Kingdom

URTI: upper respiratory tract infection

WES: whole exome sequencing

WHO: World Health Organization

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ABSTRACT

Background: Respiratory infections are a well-established child morbidity and mortality cause, which are estimated to cause 75% of all acute illness and are the leading cause of hospitalization for infants and young children worldwide. There are no methods of treatment or prevention through vaccination, except for specific agents (seasonal flu and H1N1) and in specific children with risk factors. However, the majority of respiratory infections occur in apparently healthy children without identifiable medical history, in which also the susceptibility, clinical course and prognosis vary widely even being affected by the same virus. Within this spectrum, respiratory syncytial virus (RSV) specifically is one of the paradigms of pediatric respiratory infection, frequency, morbidity and the absence of demonstrably effective preventive or therapeutic measures. While the clinical

features of ARIs are easily recognized, the etiological agent responsible for disease is often not detected, as typically it is used direct immunofluorescence to detect RSV, influenza virus, parainfluenza virus and adenovirus. In this regard, the etiology of most lower respiratory tract infection is thought to be viral, but a virus is identified in approximately 40% of cases with this approach. Since the introduction of molecular diagnostic techniques, the identification of pathogens that escape from conventional modalities has increased. These molecular techniques frequently reveal the presence of more than one microorganism in the samples. The importance of these co-infections in the pathogenesis, severity or course of these respiratory infections is not well established. In the other hand, bacteremia risk is considered low in children with acute bronchiolitis and fever. However the concrete rates of occult bacteremia in infants with respiratory syncytial virus infection is not well established.

Objectives: The main aims of this study were: 1) to assess using molecular diagnosis the epidemiology of viral co-infection in hospitalized children with ARI and to evaluate its eventual influence in the clinical manifestations and disease course; 2) to determine the actual rate and predictive factors of bacteraemia assessed by conventional cultures and molecular techniques in children admitted to hospital due to confirmed RVS acute respiratory illness.

Methods: A prospective observational multicenter study was designed using the GENDRES research network (www.gendres.org). The GENDRES network was created with research purposes in 2010 for the study of the influence of the genetics and vitamin D in respiratory infections, leading from Genetic, Vaccines, Infections and Pediatrics group (GENVIP). It includes

thirteen Spanish tertiary hospitals and more than fifty multidisciplinary collaborators. An independent cohort was collected in parallel in the UK for comparison purposes. Children admitted to any of the network's hospitals with acute respiratory infection between 2011-2013 were eligible for the study. On the top of the conventional diagnostic work-up performed in the referring hospital, a real time nested polymerase chain reaction (PCR) approach designed to detect Influenza (A and B), metapneumovirus, RSV, parainfluenza (1-4), rhinovirus, adenovirus (A-F), bocavirus and coronaviruses (NL63, 229E, OC43) was applied to all recruited patients.

Additionally, in children admitted to hospital because of an acute respiratory infection caused by RSV, bacterial presence in blood was assessed using PCR for *Meningococcus, Streptococcus pneumoniae, Haemophilus influenzae, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* in addition to conventional cultures.

Results: A total of 301 subjects were recruited, 204 in GENDRES and 97 in the UK with a median age of 6.4 months (first quartile: 2.2, third quartile: 17.0) in the GENDRES cohort and 20.0 months (first quartile: 7.0, third quartile: 48.7) in the UK cohort. In both cohorts, RSV was the most frequent pathogen (52.9% and 36.1% samples, respectively). Co-infection with multiple viruses was found in 92 samples (45.1%), and 29 samples (29.9%) respectively; this was most frequent in the 12-48 months age group. The most frequently observed co-infection pattern was RSV-Rhinovirus (23 patients, 11.3%, GENDRES cohort) and RSV-bocavirus/bocavirus-influenza (5 patients, 5.2%, UK cohort).

The pattern of co-infection did not correlate with any markers of severity. However, bacterial superinfection was associated with increased severity (OR: 4.356; P-value = 0.005), PICU admission (OR: 3.342; P-value = 0.006), higher clinical score (1.988; P-value = 0.002) respiratory support requirement (OR: 7.484; P-value < 0.001) and longer hospital length of stay (OR: 1.468; P-value<0.001). In addition, pneumococcal vaccination was found to be a protective factor in terms of degree of respiratory distress (OR: 2.917; P-value = 0.035), PICU admission (OR: 0.301; P-value=0.011), lower clinical score (-1.499; P-value = 0.021) respiratory support requirement (OR: 0.324; P-value = 0.016) and oxygen necessity (OR: 0.328; P-value = 0.001). All these findings were replicated in the UK cohort.

A total of 66 previous healthy children with a positive RSV respiratory illness were included for bacterial detection in blood. In 10.6 % patients bacterial presence was detected in the blood, predominantly H. influenzae (n = 4); S. pneumoniae (n = 2). In those patients with bacteremia there was a previous suspicion of bacterial superinfection in 6 out of 7 patients (85.7%). There were also significant differences in terms of severity between children with positive or negative bacterial PCR: PICU admission (100 % vs. 50 %, P-value = 0.015); respiratory support necessity (100 % vs. 18.6 %, P-value < 0.001); Wood-Downes score (mean = 4.8 vs. 8.7 points; P-value < 0.001); the GENVIP scale (mean = 10.1 vs. 17.0 P-value <0.001); and longer length of hospitalization (mean = 12.1 vs 7.5 days; P-value = 0.007).

Conclusion: The presence of more than one virus in children admitted to hospital with LT-ARI ranged from one third to two thirds of these patients, depending on the age, and being particularly frequent in the second year of

age, but the clinical significance of this finding remains unclear. The presence of more than one virus in hospitalized children with ARI is very frequent but it does not seem to have a major clinical impact in terms of severity. However bacterial superinfection increases the severity of the disease course. On the contrary, pneumococcal vaccination plays a protective role. With regards to bacteremia in infants hospitalized with RSV respiratory infection, it is not frequently found, even in the presence of fever; however, the possibility of bacteremia has to be considered in the most severe respiratory diseases.

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RESUMEN

Introducción: Las infecciones respiratorias constituyen una causa bien establecida de la morbilidad y mortalidad infantil, las cuales se estima que causan el 75% de las enfermedades agudas y son la causa principal de hospitalizaciones en lactantes y niños a nivel mundial. No hay formas de tratamiento y prevención a través de la vacunación, salvo para agentes específicos (gripe estacional y H1N1) y en individuos concretos con factores de riesgo. Sin embargo, la gran mayoría de las infecciones respiratorias ocurren en niños aparentemente sanos y sin antecedentes patológicos identificables, en los que además la susceptibilidad, el curso clínico y el pronóstico son muy variables aun estando afectados por el mismo virus. Dentro de este espectro, la infección por virus respiratorio sincitial (VRS) constituye específicamente uno de los paradigmas de la infección

respiratoria pediátrica, por su frecuencia, morbimortalidad y la ausencia de medidas preventivas o terapéuticas demostradamente eficaces. Mientras que las características clínicas de las IRAs son fácilmente reconocidas, el agente etiológico responsable de la enfermedad es a menudo no detectado, ya que habitualmente se usa la inmunofluorescencia como método de detección para VRS, virus influenza, parainfluenza y adenovirus. En este sentido, la etiología de la mayoría de las infecciones respiratorias se cree que son virales, pero con este enfoque, solo se identifica el virus en aproximadamente un 40% de los casos. Desde la introducción de las técnicas de diagnóstico molecular, la identificación de los patógenos que se escapan de las modalidades convencionales han aumentado. Estas técnicas moleculares con frecuencia revelan la presencia de más de un microorganismo en las muestras. La importancia de estas co-infecciones en la patogénesis, la gravedad o el curso de estas infecciones respiratorias no está bien establecida. Por otro lado, el riesgo de bacteremia en niños con infección respiratoria viral y fiebre se considera tradicionalmente baja. Sin embargo, la tasa concreta de bacteremia oculta en lactantes con infecciones por VRS no está bien establecida.

Objetivos: Los principales objetivos de nuestro trabajo fueron: 1) analizar utilizando técnicas de diagnóstico molecular la epidemiología de las co-infecciones virales en niños hospitalizados con IRA y evaluar su eventual influencia en las manifestaciones clínicas y curso de la enfermedad y 2) determinar la tasa actual y los factores predictivos de bacteremia evaluada por cultivos convencionales y por técnicas moleculares en niños ingresados en el hospital a causa de una infección respiratoria aguda por VRS.

Métodos: Se diseñó un estudio multicéntrico, prospectivo observacional usando la red GENDRES (www.gendres.org). La red clínica GENDRES fue creada en el año 2010 con fines investigadores para el estudio de la influencia de la genética y la vitamina D en las infecciones respiratorias, coordinado desde el grupo de Genética, Vacunas, Infecciones y Pediatría (GENVIP). Está formada por trece centros hospitalarios distribuidos por toda la península y más de cincuenta investigadores colaboradores multidisciplinares. Además, los resultados se compararán con una cohorte de réplica de Reino Unido. Los niños ingresados por infecciones agudas respiratorias en cualquiera de los hospitales de ambas redes entre los años 2011-2013 eran elegibles para el estudio. Conjuntamente al estudio diagnóstico con técnicas convencionales aplicado en el hospital de referencia, se realizó una reacción en cadena de la polimerasa (PCR) anidada y a tiempo real para detectar el virus influenza (A and B), metapneumovirus, VRS, parainfluenza (1-4), rinovirus, adenovirus (A-F), bocavirus y coronavirus (NL63, 229E, OC43) a todos los pacientes reclutados para el estudio.

Adicionalmente, en niños que fueran ingresados en el hospital debido a una IRA por VRS, se evaluó la presencia bacteriana en sangre para los organismos *Meningococcus, Streptococcus pneumoniae, Haemophilus influenzae, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* además del uso de cultivos convencionales.

Resultados: Se reclutaron un total de 301 sujetos; 204 en GENDRES y 97 en UK con una mediana de edad de 6.4 meses (primer cuartil: 2.2, tercer

cuartil: 17.0) en la cohorte GENDRES y 20.0 meses (primer cuartil: 7.0, tercer cuartil: 48.7) en UK. En ambas cohortes, el patógeno que se encontró más frecuentemente fue el VRS (52.9% y 36.1% muestras, respectivamente). Se encontró co-infección con múltiples virus en 92 muestras (45.1%), y 29 muestras (29.9%) respectivamente; fue más frecuente en el grupo de edad de 12-48 meses. El patrón de co-infección observado más frecuente fue VRS-rinovirus (23 pacientes, 11.3%, cohorte GENDRES) y VRS-bocavirus/bocavirus-influenza (5 pacientes, 5.2%, cohorte UK).

El patrón de co-infección no se correlacionó con ningún marcador de gravedad. Sin embargo, la sobreinfección bacteriana se asoció con un aumento en la gravedad (OR-"odds ratio": 4.356; P-valor = 0.005), ingreso en UCIP (OR: 3.342; *P*-valor = 0.006), mayor puntuación en escala clínica (1.988; *P*-valor = 0.002), necesidad de soporte respiratorio (OR: 7.484; *P*-valor < 0.001) y mayor estancia hospitalaria (OR: 1.468; *P*-valor < 0.001). Además, se encontró que la vacunación antineumocócica es un factor protector en términos del grado de dificultad respiratoria (OR: 2.917; *P*-valor = 0.035), ingreso en UCIP (OR: 0.301; *P*-valor = 0.011), menor valores en escalas clínicas (-1.499; *P*-valor = 0.021) necesidad de soporte respiratorio (OR: 0.324; *P*-valor = 0.016) y necesidad de oxígeno (OR: 0.328; *P*-valor = 0.001). Todos los hallazgos se corroboraron en la cohorte de UK.

Se incluyó un total de 66 niños que padecían infecciones respiratorias con VRS positivo. En un 10.6% de los pacientes se detectó presencia bacteriana en sangre, predominantemente *H. Influenzae* (*n*=4); *S. pneumoniae* (*n*=2). En estos pacientes con bacteremia existía una sospecha previa de

sobreinfección bacteriana en 6 de los 7 pacientes (85.7%). Se encontraron diferencias significativas en la gravedad de la enfermedad entre los niños con PCR bacteriana positiva o negativa: ingreso en UCIP (100% vs. 50%, *P*-valor = 0.015); necesidad de soporte respiratorio (100% vs. 18.6%, *P*-valor < 0.001); Score Wood-Downes (media=8.7 vs. 4.8 puntos; *P*-valor < 0.001); en la escala GENVIP (media = 17 vs. 10.1 *P*-valor < 0.001); y mayor estancia hospitalaria (media = 12.1 vs. 7.5 días; *P*-valor = 0.007).

Conclusiones: Entre un tercio y dos tercios de los niños ingresados por IRAs de vías bajas están afectados por más de un virus, siendo particularmente frecuente en el segundo año de edad. La significancia clínica de estos hechos aun no está clara, pero no parecen tener un impacto clínico importante en términos de gravedad. Sin embargo, la sobreinfección bacteriana aumenta la gravedad del curso de la enfermedad. Por el contrario, la vacunación antineumocócica juega un papel protector. Por otro lado, la bacteremia no es frecuente en lactantes hospitalizados con una infección respiratoria por VRS, sin embargo, debe considerarse la posibilidad de una bacteremia oculta en los casos más graves.

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RESUMO

Introducción: As infeccións respiratorias son unha causa ben establecida da morbilidade e mortalidade infantil a nivel mundial, as cales estímase que causan o 75% das enfermidades agudas e son a causa principal de hospitalizacións en lactantes e nenos a nivel mundial. Non hai formas de tratamento e prevención a través da vacunación, agás para axentes específicos (gripe estacional e H1N1) e en individuos concretos con factores de risco. Con todo, a gran maioría das infeccións respiratorias ocorren en nenos aparentemente sans, sen antecedentes patolóxicos identificables, no que tamén a susceptibilidade, o curso clínico e prognóstico varían moito, mesmo sendo afectados polo mesmo virus. Dentro de este espectro, a infección por virus respiratorio sincitial (VRS) constitúe específicamente un dos paradigmas da infección respiratoria pediátrica, pola súa frecuencia,

morbimortalidade e a ausencia de medidas preventivas ou terapéuticas demostradamente eficaces. Mentras que as características clínicas das IRAs son fácilmente recoñecidas, o agente etiológico responsable da enfermidade é a menudo non detectado, xa que habitualmente usase a inmunofluorescencia como método de detección para VRS, virus influenza, parainfluenza e adenovirus. Neste sentido, a etioloxía da maioría das infeccións respiratorias crese que son virales, pero con este enfoque, só se identifica o virus en aproximadamente un 40% dos casos.

Dende a introdución das técnicas de diagnóstico molecular a identificación de patóxenos que escapan a métodos convencionais aumentaron. Estas técnicas moleculares con frecuencia revelan a presenza de máis dun microorganismo nas mostras. A importancia destas co-infeccións na patoxenese, a gravidade ou o curso destas infeccións respiratorias non está ben establecida. Ademais, o risco de bacteremia en nenos con infección respiratoria viral e febre considerase tradicionalmente baixa. Nembergantes, a taxa concreta de bacteremia oculta en lactantes con infeccións por VRS non está ben establecida.

Obxectivos: Os principais obxectivos do noso traballo foron: 1) analizar utilizando técnicas de diagnóstico molecular a epidemioloxía das co-infeccións virais en nenos hospitalizados con IRA e evaluar a sua eventual influencia nas manifestacións clínicas e curso da enfermidade e 2) determinar a taxa actual e os factores predictivos de bacteremia evaluada por cultivos convencionais e por técnicas moleculares en nenos ingresados no hospital a causa dunha infección respiratoria aguda por VRS.

Métodos: Aplicouse un estudio multicéntrico, prospectivo, observacional usando a rede GENDRES (www.gendres.org). A rede clínica GENDRES foi creada no ano 2010 con fines investigadores para o estudio da influencia da xenética e da vitamina D nas infeccións respiratorias, coordinado desde o grupo de Xenética, Vacunas, Infeccións e Pediatría (GENVIP). Está formada por trece centros hospitalarios distribuidos por toda a península e máis de cincuenta investigadores colaboradores multidisciplinares. Además, os resultados compararanse con unha cohorte de réplica de Reino Unido. Os nenos ingresados por infeccións agudas respiratorias en calquera dos hospitais de ambalas dúas redes entre os anos 2011-2013 foron elixibles para o estudio. Xunto ó estudio diagnóstico con técnicas convencionais aplicado no hospital de referencia, realizouse unha reacción en cadena da polimerasa (PCR) anidada e a tempo real para detectar o virus influenza (A and B), metapneumovirus, VRS, parainfluenza (1-4), rinovirus, adenovirus (A-F), bocavirus y coronavirus (NL63, 229E, OC43) a tódolos pacientes reclutados para o estudio.

Además, nos nenos que foran ingresados no hospital debido a unha IRA por VRS, evaluouse a presencia bacteriana en sangue para os organismos *Meningococcus, Streptococcus pneumoniae, Haemophilus influenzae, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* además do uso de cultivos convencionais.

Resultados: Reclutáronse un total de 301 suxeitos; 204 en GENDRES e 97 en UK con unha mediana de idade de 6.4 meses (primeiro cuartil: 2.2, terceiro cuartil: 17.0) na cohorte GENDRES e 20.0 meses (primeiro cuartil:

7.0, terceiro cuartil: 48.7) en UK. Nas dúas cohortes, o patóxeno que se encontrou máis frecuentemente foi o VRS (52.9% e 36.1% mostras, respectivamente). Encontrouse co-infección con múltiples virus en 92 mostras (45.1%), e 29 mostras (29.9%) respectivamente; foi máis frecuente no grupo de idade de 12-48 meses. O patrón de co-infección observado máis frecuente foi VRS-rinovirus (23 pacientes, 11.3%, cohorte GENDRES) e VRS-bocavirus/bocavirus-influenza (5 pacientes, 5.2%, cohorte UK).

O patrón de co-infección non se correlacionou con ningún marcador de gravidade. Nembargantes, a sobreinfección bacteriana asociouse con un aumento na gravidade (OR-"odds ratio": 4.356; P-valor = 0.005), ingreso en UCIP (OR: 3.342; P-valor = 0.006), maior puntuación na escala clínica (1.988; P-valor = 0.002), necesidade de soporte respiratorio (OR: 7.484; P-valor < 0.001) e mayor estancia hospitalaria (OR: 1.468; P-valor < 0.001). Ademáis, encontrouse que a vacunación antineumocócica é un factor protector en términos do grado de dificultad respiratoria (OR: 2.917; P-valor = 0.035), ingreso en UCIP (OR: 0.301; P-valor = 0.011), menor valor na escala clínica (-1.499; P-valor = 0.021) necesidade de soporte respiratorio (OR: 0.324; P-valor = 0.016) e necesidade de oxíxeno (OR: 0.328; P-valor = 0.001). Todos os achamentos corroboraronse na cohorte de UK.

Incluíronse 66 nenos que padecían infeccións respiratorias con VRS positivo. Nun 10.6% dos pacientes detectouse presencia bacteriana en sangue, predominantemente H. Influenzae (n = 4); S.pneumoniae (n = 2). Nestos pacientes con bacteremia existía unha sospeita previa de sobreinfección bacteriana en 6 dos 7 pacientes (85.7%). Encontraronse diferencias significativas na gravidade da enfermidade entre os nenos con

PCR bacteriana positiva ou negativa: ingreso en UCIP (100% vs. 50%, P-valor = 0.015); necesidade de soporte respiratorio (100% vs. 18.6%, P-valor < 0.001); Score Wood-Downes (media = 8.7 vs. 4.8 puntos; P-valor < 0.001); na escala GENVIP (media = 17 vs. 10.1 P-valor < 0.001); e maior estancia hospitalaria (media = 12.1 vs. 7.5 días; P-valor = 0.007).

Conclusiones: Entre un tercio e dous tercios dos nenos ingresados por IRAs de vías baixas están afectos por máis dun virus, sendo particularmente frecuente no segundo ano de idade. A significancia clínica destos feitos ainda non está clara, pero non parece ter un impacto clínico importante en términos de gravidade. Poren, a sobreinfección bacteriana aumenta a gravidade do curso da enfermidade. Polo contrario, a vacunación antineumocócica xoga un papel protector. Por outro lado, a bacteremia non é frecuente en lactantes hospitalizados con unha infección respiratoria por VRS, nembargantes, débese considerar a posibilidade dunha bacteremia oculta nos casos máis graves.

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1 INTRODUCTION

1.1 Acute Respiratory Infection

Definition

Acute respiratory infection (ARI) includes all types of infection of the respiratory tract with multitude of signs and symptoms. It is usually characterised by cough or wheeze, with or without the presence of fever, acute rhinitis and rhinorrhea, pharyngitis and respiratory distress.

Burden of the disease

ARIs are estimated to cause 75% of all acute illness and are the leading cause of hospitalization for infants and young children worldwide [1,2]. The burden of the diseases for ARIs is estimated at 3.9 million deaths (WHO, 2002) [3] and are among the leading causes of death in children under five years old [2,4,5]. In addition to producing significant morbidity in the short term, some viral ARIs acquired early in life have been related to increased risk of illness as asthma years after the infection [6].

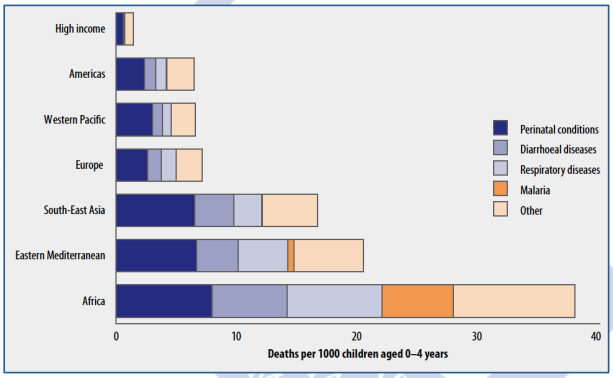

Figure 1. *Child mortality rates by cause and region, 2004 (WHO)*

Etiology

Viruses are responsible for most infections in children and adults [1,7] including respiratory syncytial virus (RSV), influenza virus (IV), human parainfluenza virus (hPIV), adenovirus (AdV) and rhinovirus (hRV). In the past decade, several new respiratory viruses, including human metapneumovirus (hMPV) [8], new subtypes of human coronaviruses (hCoV)

[1] and bocavirus (hBoV) [9], have been associated with ARI, though their clinical importance requires clarification.

While the clinical features of ARIs are easily recognized, the etiological agent responsible for disease is not often detected with non-molecular diagnostic techniques, which typically use direct immunofluorescence to detect RSV, IV, hPIV and AdV. Although the etiology of most lower respiratory tract infection is thought to be viral, specially in younger children, a virus is identified in approximately 40% of cases with this approach [10].

Epidemiology

- *Age groups*

All age groups are affected by ARIs, although the infants or young children are most likely to suffer severe disease and require hospital admission. In this regard, infections of the respiratory tract are a common problem in the first decade of life. The yearly prevalence of ARI in an otherwise healthy 3-year old child is about three to ten infections [11] and among the causes of death, only respiratory tract infections are a leading cause of death in newborn and older children [12].

- *Seasonal Distribution*

ARIs arise throughout the year, however they are more prevalent in the autumn and especially in winter months. This is because the respiratory viruses have a strong seasonality, peaking in the winter months in temperate

climates, and in the wetter months in tropical climates, with very few infections being detected in the summer months. In pediatric patients and particularly in children under one year of age this strong peak incidence in the winter months is particularly remarkable due to the high risk of hospitalization.

Risk Factors

Risk factors for ARI can be divided into clinical and demographic factors. Clinical risk factors include: prematurity; low birth weight; co-existing cardiac or respiratory problems; immunodeficiency and birth during the first half of the season. Demographic risk factors include the male sex, a lack of breastfeeding; multiple siblings/crowded living conditions; a low socioeconomic status and smokers in the household [13,14].

Clinical Manifestations and Management of ARI

The term ‘acute respiratory infection’ includes all infections of the respiratory tract, from a mild upper respiratory tract infection (URTI) to bronchiolitis or pneumonia. Diagnoses are usually made on clinical grounds and the majority of children with ARI will have a mild, self-limiting illness and do not require any treatment. However, ARI can cause very severe infections, especially in the younger children and those with other co-morbidities, leading to many hospital admissions especially during winter season. The core treatment is supportive, and includes the use of supplemental fluids and oxygen.

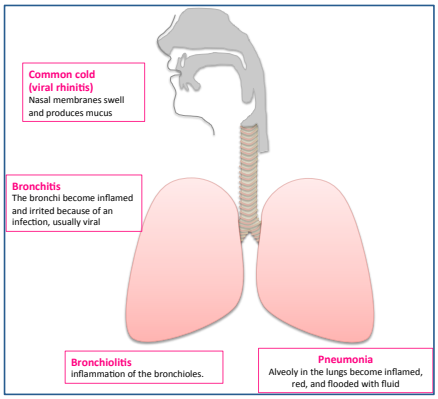

Figure 2. *Respiratory system and main disorders*

- *Upper Respiratory Tract Infection*

URTI are very common and usually mild and self-limiting, with the common cold being the most common manifestation. The common cold requires supportive treatment, making sure that the child is kept well hydrated, and the use of anti-pyretics in the presence of fever. Other manifestations classed as URTI include acute otitis media (AOM), croup, whooping cough and epiglottitis.

- *Lower Tract Respiratory Infection*

Lower tract acute respiratory infections (LT-ARI) are those diseases below the level of the larynx, so it affects to the trachea and lungs. According to the part of the respiratory tract that is affected include: bronchiolitis or bronchitis, pneumonia and bronchospasm.

- *Bronchiolitis*

The term bronchiolitis refers to the inflammation of the bronchioles, although these findings are rarely observed directly and inferred in a young child who presents respiratory distress in association with signs of viral infection. In 1983 clinical criteria for defining bronchiolitis were established by McConnochie as the first acute episode of wheezing in children younger than 24 months, expiratory dyspnoea and existence of prodromal catarrhal [15]. However, definitions of bronchiolitis may vary in the different studies, and in recent years researchers have attempted to homogenize the population of children with bronchiolitis by limiting to infants under twelve months with a first-time episode of wheezing [16].

Acute bronchiolitis is the most frequent low respiratory tract infection in infants and is a significant health care demand, not only in primary care, where it generates a sizeable number of medical visits, but also in hospitals with large attendance requirements in the emergency room and large number of admissions in epidemic periods. Bronchiolitis is the most common cause of hospitalization, and 90% of these cases requiring hospitalization occur in children under twelve months of age [13,17] with incidence peaks at age three to six months [18]. Its annual incidence is 10% and the admission rate between 2 – 5% [19], with an important increment in the last few years. Between 5 – 16% of the admissions will require pediatric intensive care unit (PICU) attendance. Although the bronchiolitis morbidity is high, deaths in industrialized countries from respiratory syncytial virus infection have been estimated generally as less than 500 per year, and most of these fatal

infections occur among children with co-morbid cardiopulmonary conditions [18]. It was estimated that in Spain the annual average cost for National Health Care System for bronchiolitis requiring hospitalization was €47 M with a mean hospitalization costs of €2162 in children up to five years old [20].

The course of bronchiolitis is variable and ranges from temporary events, to progressive respiratory distress from lower airway obstruction. The viral infection occurs through the upper respiratory tract and spreads to the lower within a few days, resulting in inflammation of the bronchiolar ephitelium, with peribronchial infiltration of white cells (mononuclear cells mostly) and oedema of the submucosa and adventia. The cause of the partial or total obstruction to airflow is the necrotic epithelium and fibrin in the airways.

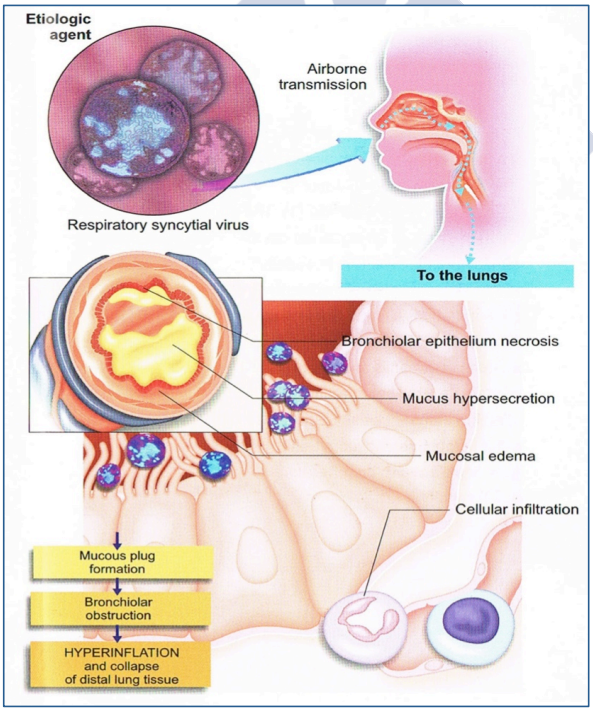

Figure 3. *Bronchiolitis physiopathology (medicsindex.ning.com)*

The main clinical features are initial rhinitis and coryza, developing into cough, and may progress to wheezing and/or crackles and signs of respiratory distress, manifested as tachypnea, nasal flaring, hyperinflation and intercostal and/or subcostal retraction [21,22].

The core of treatment for bronchiolitis, as it is a viral infection, remains supportive. In the clinical practice, oxygen is usually given if oxyhemoglobin saturations are below 90%, if the child has severe respiratory distress or if the child is cyanosed. Additionally, adequate nutrition and hydration should be maintained, and if this is not possible through oral feeding, nasogastric feeding or the use of intravenous fluids should be considered [21].

Many studies have investigated the use of corticosteroids and bronchodilators in bronchiolitis, and although both may improve short-term clinical parameters, there is little evidence and a lot of controversy supporting the use of both of them [21].

- *Pneumonia*

Pneumonia describes an infectious process resulting from the invasion and overgrowth of microorganisms in lung parenchyma, breaking down defenses and provoking intra-alveolar exudates.

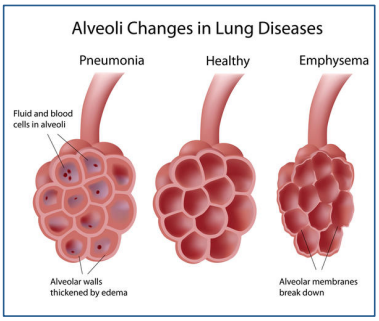

Figure 4. *Alveoli changes in pneumonia (www.vtherm.com)*

Despite significant reductions in child mortality over the last decade, pneumonia remains the leading cause of childhood mortality worldwide, accounting for 18% of deaths in children under the age of five. In this range of age, an estimated 1.1 million children are fatality cases every year due to pneumonia, more than acquired immune deficiency syndrome, malaria and tuberculosis cases combined [23,24].

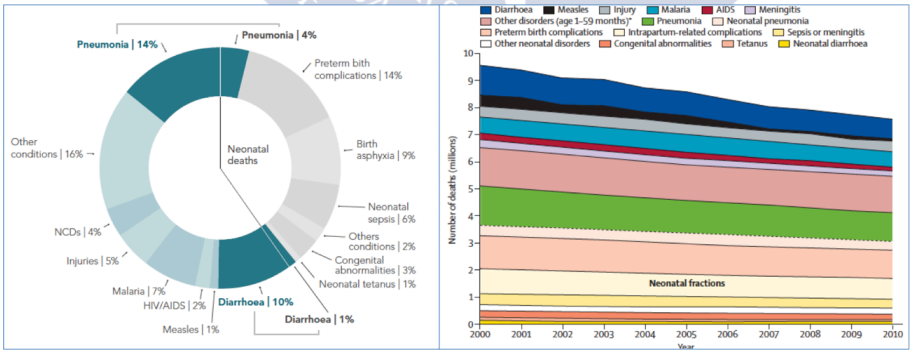

Figure 5. a) *Deaths in children less than five years of age (Source: WHO/UNICEF: End preventable deaths: Global Action Plan for Prevention and Control of Pneumonia and Diarrhoea.* ***b)*** *Global trends in burden of childhood deaths in 2000–2010 (Source: Liu et al. 2012)*

The clinical symptoms in children diagnosed by pneumonia include fever, tachypnoea and respiratory distress, often with focal or diffuse crackles on auscultation. Chest X-ray are usually performed to support clinical grounds.

The etiology of pneumonia varies significantly depending on the age of the patient [25]. Respiratory viruses are the most frequent pathogens in children aged between four months and five years (with RSV and rhinovirus the main viruses), and are responsible for approximately 40% of the community-acquired pneumonia episodes in hospitalised children. *S. pneumoniae* is reported in one-third of the cases of all ages, and *Mycoplasma pneumoniae* is the main pathogen in children aged 5 - 15 years [26].

Treatment of pneumonia is empirical, due to the absence of reliable markers capable of distinguishing viral and bacterial pneumonia [25]. In viral pneumonia, only supportive treatment is recommended.

- *Bronchospasm*

Bronchospasm or bronchial spasm is the abnormal and abrupt contraction of bronchial smooth muscles. It is considered to be mediated by release of substances from mast cells or basophils as inflammatory mediators, chemokines and cytokines and alterations in mechanical load [27]. Typical symptoms associated with acute bronchospasm include cough, wheezing, and chest tight-ness and it causes difficulty in breathing which can be very mild to severe. Bronchospasm are caused by a number of reasons. Triggers can include environmental exposures and allergens or lower respiratory tract

diseases such as pneumonia, asthma, chronic obstructive pulmonary disease (COPD), and even physical activity [28].

Indicators of disease severity

Perhaps, more important than labelling a condition is the ability to recognise the more severe disease, or the prognostic to more severe disease. The reasons for hospitalization in illnesses as bronchiolitis are often subjective so it is important to recognise the more severe diseases or factors that have the ability to increase the disease severity. On this way, and although there is controversy on its utility, oxygen saturations derived from the non-invasive method pulse oximetry are the single predictor of severe disease in bronchiolitis [29]. In patients with pneumonia, as we mention previously, a chest X-ray is also performed to see if there is evidence of consolidation.

Special attention should be paid to patients with other comorbidities or with recurrent wheezing, as they are at high risk of complications.

Although the immediate morbidity and mortality associated with LT-ARI comes from the acute illness, some investigators postulated the association between viral infection in infancy or childhood with respiratory problems later in life, such as recurrent wheeze and asthma.

1.2 Viral infection

Respiratory tract infections are caused clinically by a multitude of pathogens, but viruses are the main causative agents [10]. Moreover, pathogen-specific clinical symptoms are often lacking. The respiratory agents described below

is not intended as a complete list of all agents that cause acute respiratory tract infections, and some of the viruses described may cause symptoms outside of the respiratory tract. The description of each virus below focus on the respiratory illness they cause.

Virus: definition and general characteristics

A virus is an infectious agent that only replicates inside the living cells of other organisms. Viruses can infect all types of life forms, from animals and plants to bacteria and archaea.

A complete virus particle is known as a virion. Consist of two or three parts:

- Genetic material: either DNA or RNA
- Protein coat creating a capsid that protects the genes.
- In some cases, an envelope of lipids encloses the protein coat. This is derived from the host cell membrane.

The shapes of viruses range from simple helical and icosahedral forms to more complex structures (Figure 6).

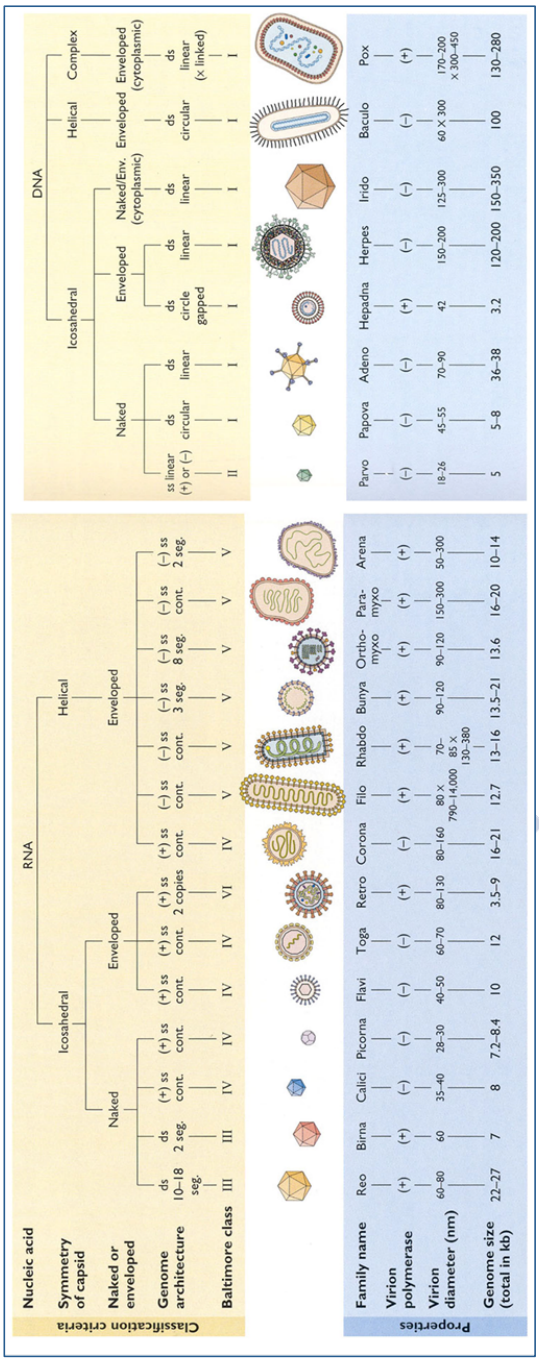

Figure 6. *Basic characteristics of RNA and DNA virus (www.nlv.ch).*

- *Virological characteristics*

Taxonomic characteristics of the main virus found in ARI are described in the following table:

Virus	Family	Genome	Sub-/serotypes
RSV	Paramyxoviridae	(–) ssRNA linear	Subtypes A and B
Rhinovirus	Picornaviridae	(+) ssRNA linear	Species A to C, > 100 serotypes
Influenza	Orthomyxoviridae	(–) ssRNA linear/segmented	A, B, C
Adenovirus	Adenoviridae	dsDNA linear	> 50 serotypes
Metapneumovirus	Paramyxoviridae	(–)ssRNA linear	Subtypes A and B
Parainfluenza	Paramyxoviridae	(–)ssRNA linear	Serotypes 1–4
Coronavirus	Coronaviridae	(+)ssRNA linear	229E, OC43, NL63, HKU1, MERS, SARS
Bocavirus	Parvoviridae	(+) and (–) ssDNA linear	Serotypes 1–4

Table 1. *Taxonomic characteristics of respiratory virus*

Respiratory Syncytial Virus

- *History of RSV*

The respiratory syncytial virus (RSV) gets its name from its ability to cause fusion between the membranes of nearby syncytia cells. It was first isolated in 1956 by Morris et al. [30] in a group of chimpanzees who had coryza, and they named the virus Chimpancee Coryza Agent. The following year, in 1957, Chanock and his team [31] in Baltimore (USA), isolated the same agent in two children diagnosed with pneumonia and stridulus laryngitis, being called from that time RSV. In Spain the first isolate was reported by Pumarola in 1967.

- *Virology of RSV*

Based on the virological classification, RSV is categorized within the family *Paramyxoviridae* with such well-known viruses such as measles, mumps and the paramyxovirus and is classified in the genus *Pneumovirus* [32]. RSV has a single negative strand of non-segmented RNA (Figure 7), which is important because the virus does not reassort with other viruses as influenza or rotavirus do [33].

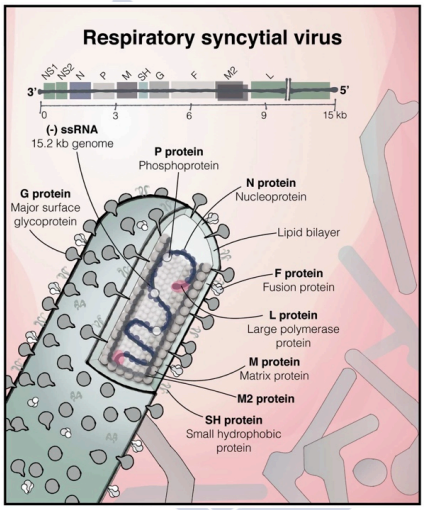

Figure 7. *Schematic diagram of RSV [34]*

It is an enveloped virus medium size (120-300 nm) with glycoprotein projections of 12 nm in length (15222 nucleotides) which encodes the synthesis of 11 viral proteins: three transmembrane glycoproteins known as the attachment glycoprotein (G), the fusion protein (F), and the small hydrophobic protein (SH); one matrix protein (M); two transcription factors (M1 and M2); three proteins associated with the nucleocapsid (N, P, and L);

and two non-structural proteins (NS1 and NS2) [32]. The F and G glycoproteins are the main surface antigenic determinants and they stimulate the production of protective host immune responses (Figure 8).

- **G protein:** It is a type II transmembrane protein with an N-terminal cytoplasmic domain, a hydrophobic fix region and a highly variable ectodomain. Many of the epitopes recognized by the host antibody response lie in the C terminal variable regional [35].
- **F protein:** It is a highly conserved protein among the *Paramyxoviridae* family and is a very important protein for RSV because of the fusion of the viral envelope or infected cell membranes with uninfected cell membranes. The part of the F protein that enters the cell membrane is situated at the N terminal region [35].

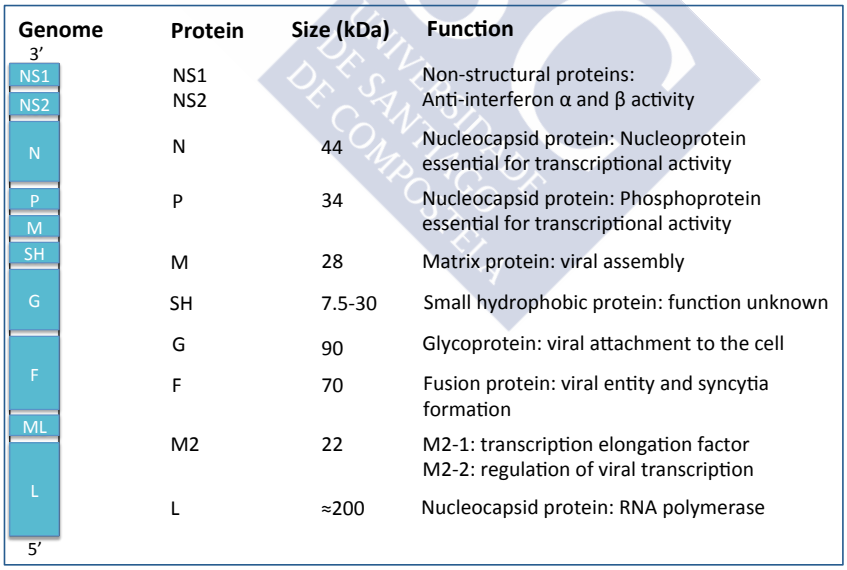

Figure 8. *Schematic genome and proteins of the RSV.*

- *Subtypes of RSV*

Human RSV isolates can be classified into two major groups, A and B, each containing several distinct subgroups. This classification is based upon antigenic and genomic differences found in several viral proteins, but specially the G protein [33]. The two major strain groups circulate simultaneously during an outbreak, although group A viruses are more prevalent (Table 2). The dynamics of annual epidemics appear to be local rather than national or global [36].

Protein	Nucleotide	Amino acid sequence
F	79%	89%
G	67%	53%

Table 2. *Nucleotid and amino acid sequence homology between group A and B.*

- *Genetic diversity of RSV*

RSV has a non-segmented RNA genome. Thus, it does not have the capacity for reassortment of genome segments, the process by which influenza viruses undergo antigenic shifts leading to influenza virus pandemics. However, as with other RNA viruses, RSV has a quite mutable genome by virtue of its dependence on an RNA polymerase that lacks the capacity of RNA proofreading and editing. The main antigenic and genetic differences between RSV groups A and B were found in the attachment glycoprotein G. Variability in this protein is greater than that in the other proteins, and consequently contributes to the ability of the virus to cause re-infections and annual epidemics [37].

- *Epidemiology of RSV*

RSV infection is a very prevalent illness. The WHO-estimated global annual infection and mortality data for human RSV are 64 million and 160.000, respectively. Furthermore, about 90% of infants and young children by the age of two years are affected and peak rates occur in infants aged 6 weeks to 6 months.

RSV is responsible for approximately 50% of all pneumonia and up to 90% of the reported cases of bronchiolitis in infancy. In industrial countries, infants and young children RSV infection is recognized as the leading cause of hospitalization and in hospitalized infants with RSV bronchiolitis, mechanical ventilation is required in 7 - 21% of the cases [38]. One large study found RSV to be responsible for 20% of hospitalizations, 18% of emergency department visits and 15% of general practitioner visits in children under five with ARI in the United States. It is also thought to be responsible for 50 - 90% of hospitalisations for bronchiolitis.

- *Immunopathogenic aspects of RSV*

The incubation period of RSV is estimated to be five days. Respiratory tract inflammation in RSV bronchiolitis is a multicellular process in which epithelial cells, macrophages, cytotoxic T cells and eosinophils are implicated [33].

At the beginning of the infection, the virus replicates in the nasopharynx epithelial cells and cytokines are secreted by macrophagues [33]. During bronchiolitis, ciliated epithelial cells are destroyed. In its severe form, the

disease involves peribronchiolar mononuclear cell infiltrates accompanied by submucosal edema and bronchorrhea [39] (Figure 3).

- *Spread of the RSV*

The virus is primarily spread through large particle aerosols (due to coughing or sneezing) or by fomites followed by self-inoculation. RSV can survive on non-porous surfaces for 6 – 7 hours, porous surfaces for 2 hours, and the skin for 20 – 30 minutes. These characteristics make transmission relatively probable among children and infants in close contact with each other [40].

- *Clinical manifestations of RSV*

Clinical manifestations vary based on patient age and on whether the infection is primary or secondary [41]. In infants, lower respiratory tract signs, such tachypnea, wheezing, or rales, usually appear 1 to 3 days after the onset of rhinorrhea, representing viral spread into the bronchi and bronchioles. RSV infection starts with a short course of upper respiratory symptoms. Increased respiratory rate, intercostal and subcostal retractions, and difficulty in feeding characterize the lower airway disease. Prolonged expiration, with or without wheezing, and audible crackles during inspiration are characteristic of bronchiolitis. The typical radiographic features are air trapping, peribronchial patchy infiltrates, and segmental atelectases. Thus, bronchiolitis shares common features with viral pneumonia and in fact only represent different phases of the same disease [42].

- *RSV Risk Factors*

Some predisposing factors for severe RSV disease have been identified (Table 3). However, risk factors for severe infection cannot completely explain differences in disease severity, and the majority of patients hospitalized for severe RSV disease do not fit the profile of high-risk patient [43].

Children at risk for severe RSV infection
Young age < 2 months (term infants)
Premature birth ≤ 34 weeks
Lack of breastfeeding [19]
Chronic disorders: bronchopulmonary dysplasia, congenital heart disease, cystic fibrosis and immunosupressed patients.
Environmental factors [44]: Tobacco smoke exposure, outdoor air pollution, low socioeconomic status, crowded living conditions, malnutrition.

Table 3. *Children at risk for severe RSV infection*

Rhinoviruses

Human rhinovirus (hRV) was first discovered in the 1950s studying the etiology of the common cold, and at the beginning it was thought to be not a very severe infectious agent just causing URTI. Nowadays, studies have correlated the hRV with bronchiolitis or asthma exacerbations and wheezing

hospitalizations [45].

hRV is a member of the family *Picornaviridae* and the genus *Enterovirus*. It is a positive-sense, single-stranded-RNA virus of approximately 7,200 bp and is classified into three species, hRV-A (74 serotypes); hRV-B (25 serotypes) and hRV-C. The hRV structure is composed by a capsid, which protects the RNA, is composed of 60 copies of each of 4 structural proteins (Figure 9).

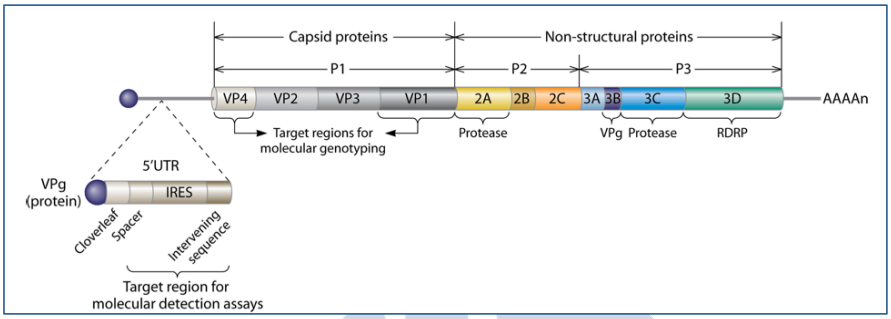

Figure 9. *Schematic genome and proteins of hRV [46].*

Virus protein 1 (VP1), VP2, and VP3 are located on the capsid surface and are responsible for its antigenic diversity while VP4 is located inside the capsid and attaches the RNA core to the viral capsid.

- *Epidemiology of hRV*

An initial work has shown hRV have optimum replication temperatures of 33ºC – the temperature in the nasal passages, as contrasting to the higher temperatures in the lower respiratory tract, another factor in support of hRV being associated with URTI and the common cold. However, further research has shown that although the optimum temperature for hRV replication is

33ºC, they are still able to replicate effectively at 37ºC, proving it is feasible that they are a cause of LT-ARI [47].

The prevalence of hRV bronchiolitis was described in approximately 20 – 40% in emergency department and hospitalized patients. Rhinoviruses have been found to cause about two-thirds of common colds and asthma exacerbations in adults and older children.

Since the introduction of molecular methods of viral detection, hRV has frequently been found in asymptomatic children. This detection has established controversy to the clinical utility of the molecular techniques and it was postulated that these findings might be due to:

i) A past and resolved respiratory illness with prolonged virus shedding; hRV was detected in children even 15 days after the respiratory illness.
ii) Mild or diffuse symptoms.
iii) The incubation period prior to the onset of symptoms. Rates of asymptomatic infection described range from 12 to 32% in children under four years old.

Adenovirus

Adenovirus was first isolated in 1953 in cells culture of human adenoids [48]. It is a member of the family *Adenoviridae* and is a linear double stranded DNA virus with a terminal protein, medium-sized (90 – 100 nm), with an icosahedral nucleocapsid and nonenveloped virus (without an outer lipid

bilayer). Adenovirus (AdV) infection can be classified according to over 50 different serotypes, which are included in seven species (A - G) [49].

AdV typically cause mild and self-limited infections involving the upper or lower respiratory tract, gastrointestinal tract, or conjunctiva [49,50]. AdV infection of the respiratory tract can lead to a wide spectrum of disease from mild upper respiratory tract symptoms to severe pneumonia, with one particular serotype (serotype 14) being associated with particularly severe disease and a high mortality rate. Up to 10% of LT-ARIs in pediatric population are caused by AdVs and infections are more common in young children, aproximately an 80% of AdV infections occur in children <4 years old [51].

- *Structure of AdV*

Adenoviruses are nonenveloped, icosahedral particles about 90 nm in diameter with fibers projecting from the vertices of the icosahedrons. The DNA is linear, double-stranded and nonsegmented. The outer structure of the virus is comprised of 240 hexons and 12 pentons at vertices of the icosahedron. Adenovirus fibers of species-specific lengths extend from the penton and are associated with hemagglutination properties

Parainfluenza Viruses

Parainfluenza viruses (hPIV) are large (150 - 200nm in diameter) enveloped RNA viruses with a genome encompassing ≈ 15,000 nucleotides belonging to the *Paramyxoviridae* family. hPIV have been designated into five subtypes

(type 1-3, 4a and 4b) that cause human disease, and hPIV 1-3 are the most significant in humans. All known human hPIV were isolated in the second half of the 1950s [52].

hPIV are common causes of ARI, especially in children, with studies estimating that most children will have evidence of infection with multiple serotypes by the age of five. Most hPIV infections are limited to the upper respiratory tract, with up to 50% complicated by AOM, and only 15% involving the lower respiratory tract [52].

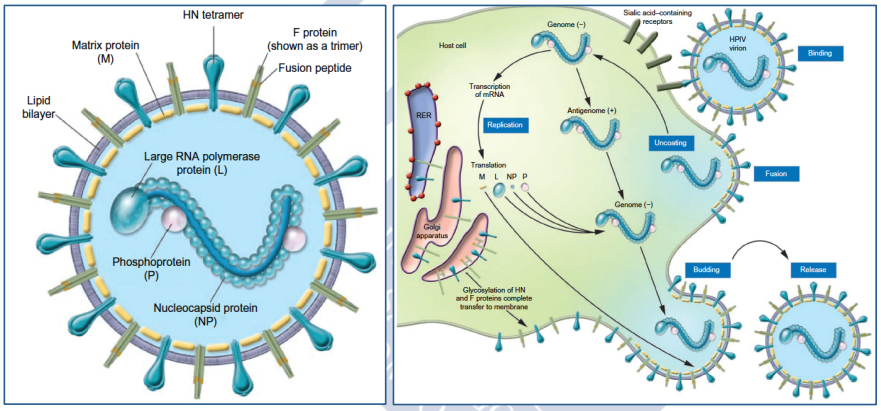

Figure 10: a) *Schematic parainfluenza virion* ***b)*** *Schematic illustration of the parainfluenza life cycle [53].*

Abbreviations: HN, Hemagglutinin-neuraminidase protein, RER, rough endoplasmic reticulum; hPIV, human parainfluenza virus; L, large RNA polymerase protein; M, matrix protein; NP, nucleocapsid protein; P, phosphoprotein

The clinical diseases caused by hPIVs include rhinorrhea, cough, croup (laryngotracheobronchitis), bronchiolitis, and pneumonia. hPIV-3 is the most common type, as seen in studies of children. Clinical manifestations are broad, but most result in an URTI, although a significant number (30 – 50%) are associated with AOM [52,54]. About 15% of hPIV infections cause LRTIs;

hPIV-1 being associated with croup, and hPIV-2 and hPIV-3 with bronchiolitis. As with many other respiratory viruses, hPIV can cause severe disease in immunocompromised hosts [52].

Influenza Virus

Influenza (IV) is a RNA virus classified in the family *Orthomyxoviridae* and three antigenic types of IV have been identified: A, B and C. All of them are causative agents for respiratory infections, but the main differences regarding the clinical course of the illness caused between them is the severity of the disease and the prevalence. Influenza A frequently causes more severe and pandemic illness, while influenza B and C have caused illness of epidemic proportion. The principal reason is that Influenza A has the ability of being high mutagenic while Influenza B does not experience as much antigenic changes so it causes only a minority of seasonal influenza cases each year. Influenza C is usually associated with minor symptoms.

- *Epidemiology of IV*

Influenza is an infection that spreads easily from person to person and the WHO estimated an annual attack rate at 5% – 10% in adults and 20% – 30% in children. Symptoms can be mild to severe, and on this regards, the 2014 annual epidemics were estimated to result worldwide in about 3 to 5 million cases of severe illness, and about 250 000 to 500 000 deaths.

- *IV Risk factors*

Influenza illnesses can result in hospitalization and death mainly among high-risk groups:

- Age: children younger than two years of age and elderly
- pregnant women
- certain medical condition such as chronic heart, lung, kidney, liver, blood or metabolic diseases (such as diabetes), or debilitated immune systems.

- *Influenza A pandemics*

For Influenza pandemics occur (Table 4), two conditions have to take place: 1) to emerge in the humans an influenza virus with a hemagglutinin against which there is weak or no existing immunity; 2) spreads easily from human-to-human.

Name of pandemic	Date	Subtype involved
1889–1890 flu pandemic (Asiatic/Russian Flu)	1889–1890	possibly H3N8 or H2N2
1918 flu pandemic (Spanish flu)	1918–1920	H1N1
Asian Flu	1957–1958	H2N2
Hong Kong Flu	1968–1969	H3N2
Russian flu	1977–1978	H1N1
2009 flu pandemic	2009–2010	H1N1

Table 4. *Influenza pandemics throughout the history.*

Since 2009, a new reassorted A/H1N1 virus had circulated worldwide among humans, causing morbidity and mortality, and was referred to as a pandemic H1N1.

Coronaviruses

Tyrell and Bynoe identified the first human coronaviruses in the 1960s. Phylogenetic analysis grouped the Coronaviruses into the order *Nidovirales*, family *Coraviridae*, belonging to one of two subfamilies: *Coronavirinae* and *Torovirinae*. They are enveloped viruses with a positive-sense RNA genome (27.000 a 30.000 bases) and with a nucleocapsid of helical symmetry. The genomic size of coronaviruses ranges from approximately 26 to 32 kilobases.

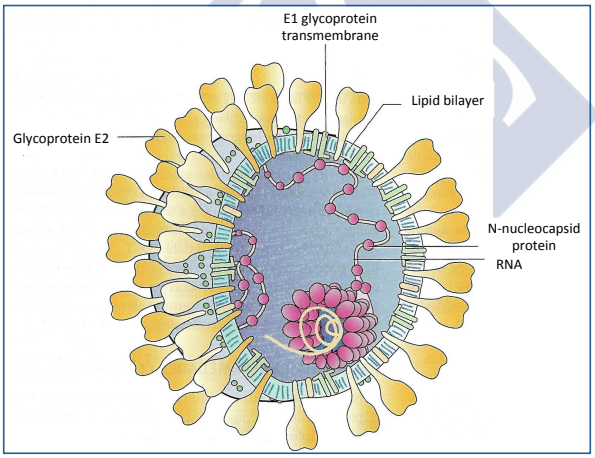

Figure 11: *Schematic representation of the hCoV*

Four human coronaviruses (HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1) are endemic in the human population and are mainly associated with mild, self-limiting respiratory illnesses. In this regards, hCoV

is the second cause of common cold (the first one is Rhinovirus, as said previously) and mainly affects toddlers and children. hCoV infects epithelial cells and generally the infection remains localized in the upper respiratory tract due to the optimal temperature to virus proliferation is 33 - 35ºC (see replication of hCoV in figure 12). However, this four hCoV can also present with high morbidity outcomes of the lower respiratory tract including bronchiolitis and pneumonia [55,56].

Another two human coronaviruses, namely SARS-CoV and MERS-CoV cause severe respiratory syndromes and present a significant risk with their high fatality rates. Severe associated respiratory syndrome (SARS)-associated coronavirus was identified in 2002 firstly in the south part of China, which had particularly severe clinical manifestations, with some patients developing respiratory distress syndrome, requiring intensive care and ventilation. In June 2012 the most recent emergence of a completely novel strain of human coronavirus was identified (MERS-CoV) [57].

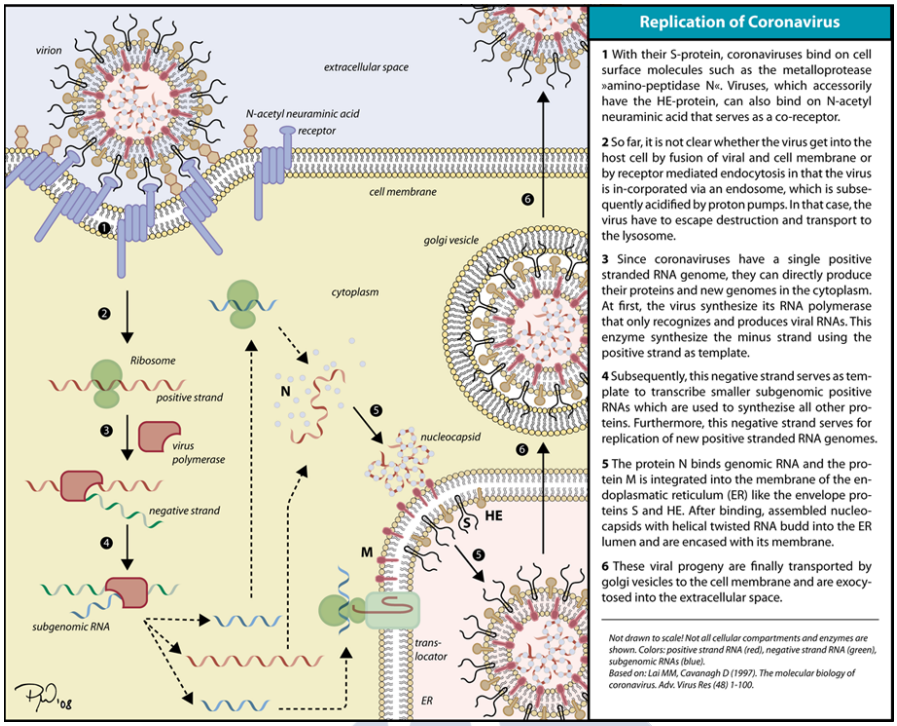

***Figure 12**: Schematic illustration of the coronavirus life cycle (www.wikipedia.org)*

Human metapneumovirus

The team of Proffesor Osterhaus discovered the human metapneumovirus (hMPV) in 2001 in Netherlands [8]. It is a RNA virus belonging to the *Paramyxoviridae* family, subfamily *Pneumovirinae* and two main groups, A and B, have been identified. Both antigenic subtypes usually circulate concurrently every year being humans the single source of infection. The incubation period is estimated in 3 - 5 days and the duration of viral clearance in otherwise healthy children is about 1 - 2 weeks.

- *Structure of hMPV*

hMPV virions were visualized by electron microscopy as pleomorphic spheres and filaments that were reported to have general similarity to those of RSV. hMPV is an enveloped, single stranded negative sense RNA virus that consists of 13,350 nucleotides, and nine proteins, comprising the N (nucleoprotein), P (phosphoprotein), M (matrix protein), F (fusion protein), M2 (matrix proteins M2-1 and M2-2), SH (small hydrophobic protein), G (glycoprotein) and L (RNA-dependent RNA polymerase) genes. The M2 gene contains two open reading frames and encodes the M2-1 and M2-2 proteins [58] (Figure 13).

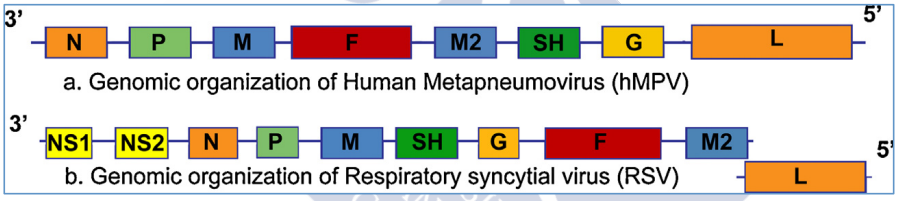

Figure 13: *A schematic representation of the differences between Metapneumovirus and RSV is represented. Genes are represented as boxes with the corresponding encoded protein [58].*

- *Epidemiology of hMPV*

hMPV is a main cause of LT-ARI in infants and children worldwide. The most common presentation of hMPV in children includes complications of the upper respiratory tract but hMPV causes a clinical spectrum of illness from upper airway infection to severe lower respiratory tract infections. Bronchiolitis, pneumonia, croup and asthmatic exacerbations are the most frequently associated lower respiratory tract complications [59]. hMPV and has been shown to play a major role in respiratory infections, being found in

7 - 19% of samples from children with ARI [59]. It is a very prevalent virus, serological studies indicate that all children at the age of five were infected at least once by hMPV and recurrent infections may occur during all ages. This may be due to insufficient immunity acquired during the initial infection and/or due to infection by different viral genotypes. The incubation period varies from individual to individual, but is commonly between 3 and 5 days [58].

Human Bocavirus

Allander et al. [9] reported in 2005 the discovery of a previously undescribed human parvovirus in respiratory secretions from children with respiratory tract disease with unknown etiology in Sweden. It was discovered by molecular virus screening which is based on random cloning and bioinformatical analysis. Phylogenetic analysis grouped the hBoV into the family *Parvoviridae*, subfamily *Parvovirinae*, and genus Bocavirus. Up to the date, four subtypes have been identified, hBoV 1 - 4. Of these, hBoV1 is most frequently detected in clinical samples of the respiratory tract and the remaining isolates are more frequently associated with gastrointestinal infections and symptoms [60]. Its genome is a linear, single-stranded DNA 5.2 - 5.3 kilobases in length with terminal hairpin structures at both ends.

- *Epidemiology of hBoV*

hBoV was detected in 1.5% – 18.3% of respiratory samples from individuals with ARIs, especially those from young children and infants [61]. In Spanish children, Garcia-Garcia et al. [62] found applying a PCR technique that a

17.1% of the patients hospitalized for respiratory infections were positive in hBoV. This virus can be detected not only in respiratory samples but also in blood, urine, and stools [61]. The seroprevalence of HBoV is strongly dependent on the age of the investigated patient cohort and ranges from 40% in children between 18 and 23 months of age up to virtually 100% in children older than two years. In contrast to other viruses, hBoV has been co-detected with other pathogens than any other respiratory virus [63].

1.3 Bacteremia

Definition

Bacteremia is the presence of viable bacteria in the circulating blood. The detection of bacteria in the blood is always abnormal as blood is a sterile environment. It is most commonly accomplished by blood cultures.

Bacteremia is different from sepsis, which is a condition where bacteremia is associated with an inflammatory response from the body (causing systemic inflammatory response syndrome, characterised by rapid breathing, low blood pressure, fever, etc.)

Bacteremia in respiratory infections

The main complication of viral respiratory infections is bacterial co-infection and the synergism established between virus and bacteria have been widely discussed in the literature, particularly for respiratory viruses and secondary bacterial pneumonia [64]. Bacteremia rates reported in children with

respiratory illness are low, with rates <1.2% in RSV confirmed cases [65-69]. In those requiring mechanical ventilation bacterial co-infection rates may vary, with much higher rates, ranging from 21.0% to 43.9%. However, the risk of bacteremia with pathogenic bacteria in the setting of acute RSV infection in infants with no other risk factors as well as the impact of this bacteremia in the severity of the respiratory disease is still unclear.

1.4 Techniques

For the viral diagnosis in respiratory illness, different methods can be used either by the detection of the virus or parts of the virus or identifying the immune response developed by the infected individual: virus isolation, antigen detection, genome detection and serology. The differences between them are the cost, time-requirement and sensitivity and specificity.

In this regard, the wider availability of molecular diagnosis techniques has allowed the identification of pathogens otherwise missed using conventional modalities, frequently detecting more than one microorganism but the importance of co-infections in the pathogenesis, severity or course of respiratory infections is not well established. In children with ARI, the confirmation frequency of pathogens may exceed 80% [70]. Contrary, the high sensitivity of molecular techniques raises questions about the clinical relevance of positive test results.

Direct immunofluorescence

Immunofluorescence is a common laboratory technique used both in research and clinical diagnostics, developed by Coons in the early 1940s. This technique uses an antibody chemically linked to a fluorophore that recognizes the virus antigen. The signal emitted by the fluorophore can then be quantified by different techniques: flow cytometer, array scanner or automated imaging instrument, or visualized using fluorescence or confocal microscopy [71].

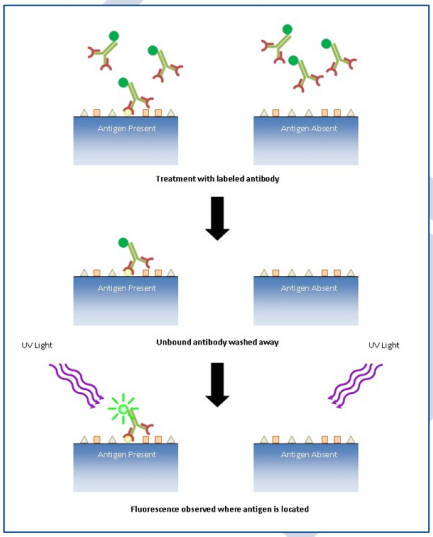

Figure 14: *Schematic of the direct immunofluorescence technique (http://www.di.uq.edu.au/sparqcbeifbackground)*

The advantage comparing with viral culture is that in antigen detection the non-viable viruses can be analyzed, which may be important when samples need a long transportation to the laboratory, but is not as sensible as the genome analysis.

- *Rapid antigen test*

Rapid point-of-care tests can be done in 15 – 30 minutes and were designed for use where a preliminary screening test result is required, so, therefore can be performed in a doctor's office, in an outpatient clinic or a hospital ward. These so called "point-of-care" tests have been in clinical use for over 20 years for the detection of IV and RSV [72,73]. They have the advantage of being a rapid bed-side test. The diagnostic sensitivity and reliability of such rapid tests remains to be a topic of discussion. The main characteristics are that they are quick and easy to perform and require little or no additional equipment, but in the other hand, they are lack in sensitivity compared to virus isolation and genome detection.

Polymerase Chain Reaction

Kary Mullis and cols developed the polymerase chain reaction (PCR) technique in 1986 [71]. It is an enzymatic DNA amplification process divided in series of cycles based in the DNA replication. Ideally, every cycle of the PCR process doubles the amount of the desired DNA fragment available, resulting in exponential product accumulation. The new molecules synthesized (amplicons) can be visualized by fluorescence.

In brief the PCR process includes the following steps:

1. Extraction of genetic material from the sample.
2. Transformation of RNA to complimentary DNA (if the virus is an RNA virus), by the enzyme reverse transcriptase.

3. Repeated amplification cycles.

Even though the PCR has been known for more than 25 years, it is not wide used in diagnosing viral respiratory infections. One reason is that more than one virus was detected in the same sample, and consequences of this co-infection are not fully understood.

- *PCR phases:*

A PCR reaction can be divided into three distinct phases: exponential, linear, and plateau (Figure 15).

1. Exponential phase:

It is the first phase in a PCR reaction in which, considering a reaction with a 100% of efficiency, in each cycle the amount of product is doubled. At the end of this phase, as the amplicon exponentially accumulates in quantity and the PCR components decreases, the primer starts competing with amplicon, and the reaction efficiency decreases.

2. Linear phase:

In this phase, the reaction reduces the quantity of amplicon, so there is no longer near doubling of the amplicon. The product formed is highly variable due to many factors, including differences in the rate at which specific components are depleted and the accumulation of products.

3. Plateau phase:

Finally the reaction will slow down and stop due to depletion of substrates and product inhibition. Each replicate reaction can plateau at different points due to different reaction kinetics unique to each sample.

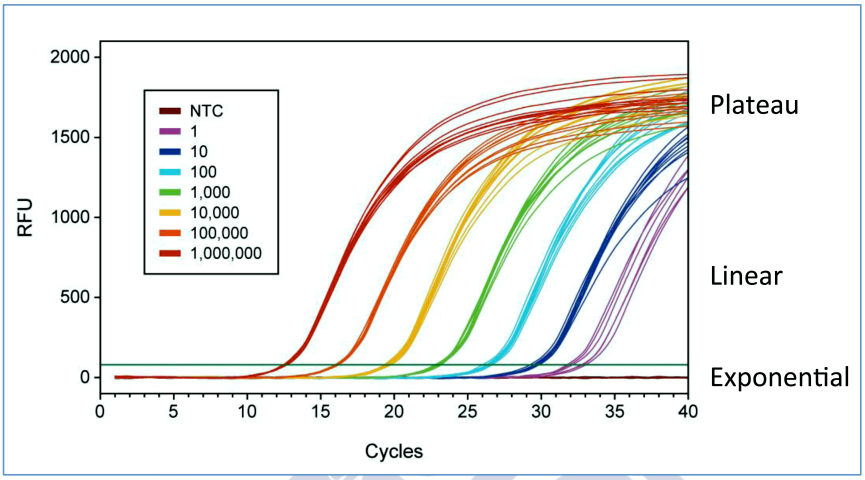

***Figure 15**: Example plot of an experimental PCR reaction.*

x axis: cycle number; y axis: amount of DNA (RFU: relative fluorescence units). Colors represent number of input DNA molecules. (http://www.5prime.com)

- *Types of PCR*

Since its development, the original method has experienced different modifications or adaptations.

1) Real time PCR

It is a modification to PCR first introduced in 1992 by Higuchi et al. and it has realized a rapid increase in its use since then.

Real-time PCR is a sensitive and reliable method for detection of nucleic acids (DNA and RNA-cDNA) levels. It is based on detection of fluorescence emitted from a reporter molecule and this signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. How quickly the amplified target reaches a threshold detection level correlates with the amount of starting material present. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product.

a) Quantitative PCR

Real-time qPCR is based on detection and quantification of fluorescence emitted from a reporter molecule at real time. This detection occurs during the accumulation of the PCR product with each cycle of amplification, thus allows monitoring the PCR reaction during early and exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template.

2) Nested PCR

Nested polymerase chain reaction is a modification of PCR aimed to reduce non-specific binding or contamination in products due to the amplification of unexpected or unintended primer binding sites (mispriming). Conventional PCR requires primers complementary to the termini of the DNA target but

primers could bind to incorrect regions of the DNA, giving unexpected products. Nested PCR can increase the yield and specificity of amplification of the target DNA. Nested PCR involves two sets of primers in two successive rounds of PCR. This involves taking an aliquot of the product from the primary PCR, and using it as a template for a secondary round of PCR amplification:

- The first primer set binds to sequences outside the target DNA, as expected in standard PCR. In this first step fewer nonspecific amplification products are produced.
- The second set of primers will bind and amplify target DNA within the products of the first reaction.

3) Reverse transcription PCR

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. The use of RT-PCR for the detection of RNA transcript has revolutionalized the study of gene expression in the following important ways:

- Made it theoretically possible to detect the transcripts of practically any gene.
- Enabled sample amplification and eliminated the need for abundant starting material that one faces when using northern blot analysis.

- Provided tolerance for RNA degradation as long as the RNA spanning the primer is intact.

4) Multiplex PCR

Multiplex PCR refers to a process whereby several agents can be analyzed in the same test run, by using multiple primer sets within a single PCR mixture. Amplicons (amplification products sized ≈ 80-150 bp) of varying sizes, specific to different DNA sequences, are produced. Even though 20 or more different pathogens are analyzed simultaneously. Multiplex PCR uses multiple pairs of primers in the same reaction to amplify multiple sequences of DNA. This allows the detection and identification of multiple viruses within one reaction.

1.5 Co-infection and superinfection

Co-infection and superinfection describe both a secondary infection of a previously infected patient. The time when the second infection occurs is the main difference between the two concepts (Figure 16).

Co-infection is the simultaneous infection of a host by multiple pathogens, that is infection occur at the same time. Global prevalence of co-infection is unknown, but it is thought to be usual and more common than single infection in respiratory illness.

In the other hand, superinfection is defined as a new infection occurring in a patient having a pre-existing infection, such as bacterial superinfection in

viral respiratory disease. The second infection superimposed is resistant to the treatment used against the first infection.

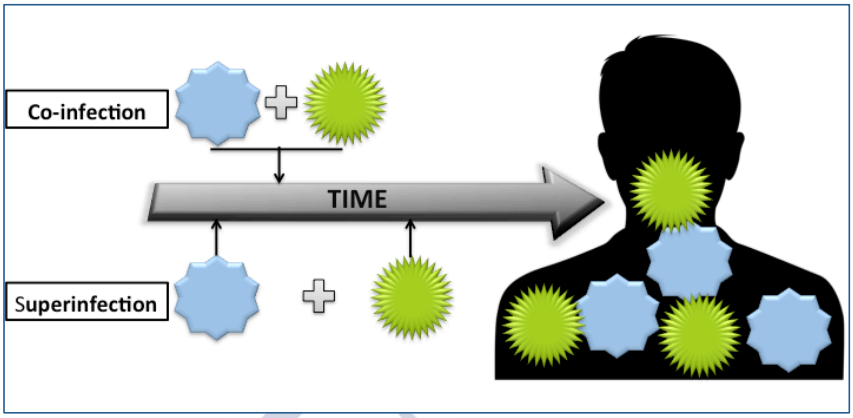

***Figure 16**: Differences between co-infection and superinfection.*

Co-infection and superinfection are important concepts on human health because different pathogen species can interact within the host and the resulting effect is not clear in all the cases, but some of the interactions are thought to have negative effects.

The importance of co-infections in respiratory infections

The arrival of molecular methods in the biomedical sciences has given investigators the ability to detect co-infections with increasing facility. However, little is known about the clinical significance of these multiple infections compared to single pathogen infections.

- *Viral co-infection*

There is controversy in the importance and impact of viral co-infection in respiratory diseases. The elucidation of the epidemiologic and clinical importance of mixed respiratory infections has become an area of active research in recent years. Co-infection rates vary widely among these studies and are estimated to account for 47% and 95% [7,74,75] of ARI for which at least one virus was detected in children.

- *Virus-bacteria disease*

Co-infections could be acute and chronic infections caused by various combinations of viruses, bacteria, fungi, and parasites [76]. It is set that for virus-bacteria infection, for example, the infection arise when the virus creates a niche for or predisposes the host to colonization by other pathogens (bacteria). The majority of deaths in the 1918-1919 influenza pandemic likely resulted from secondary bacterial pneumonia [77]. Many factors are involved in the phenomenon of bacterial superinfection during viral respiratory disease as it is represented in Figure 17. Virus could predispose bacterial infection in respiratory tract by different mechanisms [78]:

- Physical damage to the local respiratory physical barriers: Viruses may render the epithelium more susceptible to bacterial colonization by altering the mucosal surfaces. Cilia may be damaged, leading to decreased mucociliar function of the respiratory epithelium. Additionally, due to viral-induced damage and loss of integrity of the

epithelium layer, bacterial colonization may be enhanced and translocation may be increased.

- Virus-infected cells may decrease the expression of antimicrobial peptides, as shown for b-defensins, thereby affecting the natural defense of the host epithelium.
- Viral neuraminidase activity is able to cleave sialic acids residues, thereby giving access to bacterial receptors that were covered by these residues.
- Viruses may induce bacterial colonization and replication both directly and indirectly, the latter by inducing up-regulation of various receptors required for bacterial adherence.

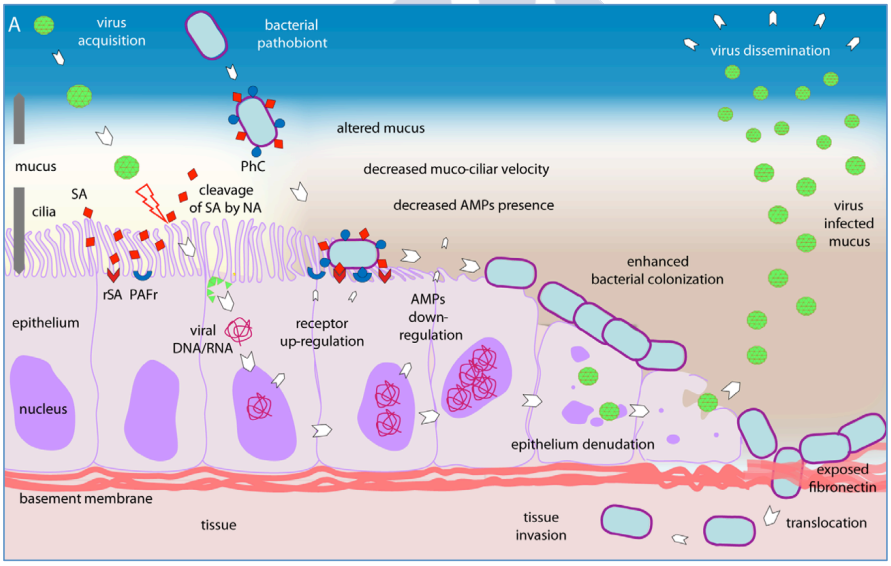

***Figure 17**. Mechanism of the viral–bacterial interaction on the respiratory epithelial surface. Viral presence is thought to predispose the respiratory niche to bacterial colonization by different mechanisms [78].*

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2 BACKGROUND & OBJECTIVES

Viral co-infections in the nasopharynx

While the clinical features of ARIs are easily recognized, the etiological agent responsible for disease is often not detected with non-molecular diagnostic techniques, which typically use direct immunofluorescence to detect RSV, IV, hPIV and AdV. In this regard, the etiology of most lower respiratory tract infection is thought to be viral, but a virus is identified in approximately 40% of cases with this approach [10]. Molecular techniques, including PCR, increase the sensitivity of detection for common and emerging respiratory viruses [79], and often reveal the presence of more than one pathogen.

Laboratory diagnosis and surveillance of respiratory infections are important due to rapid identification of the causing agent can help to take decisions concerning optimal antimicrobial or symptomatic treatment strategies. Also, the recognition of the causative agent may help prevent further spread of the pathogen by reducing social contacts. The importance of co-infections in the pathogenesis, severity or course of respiratory infections is not well established.

Viral-bacterial interaction

The main complication of viral respiratory infections is bacterial co-infection and the synergism established between viral and bacterial infections, which have been widely discussed in the literature, particularly for respiratory viruses and secondary bacterial pneumonia [64]. Bacterial superinfection is the most important and frequent complication in infants and children with viral ARI. In the setting of acute bronchiolitis, the risk of bacterial infection is low in children with respiratory illness [65-68]. However, these studies focused on fever as a predictive value of bacterial infections diagnosed by culture.

Objectives

With this background, the overall purposes of the current study were:

i) To assess the epidemiology of viral co-infection in hospitalized children with ARI using molecular diagnosis and to evaluate its eventual influence in the clinical phenotype and disease course.

ii) To determine the actual rate of bacteraemia assessed by molecular techniques in children admitted to hospital due to confirmed RSV acute respiratory illness.

The specific aims were:

- To examine the rate and pattern of viral co-infection in children with lower respiratory tract infections using a multiplex PCR assay.
- To analyze the impact of the viral pattern on the clinical presentation and the duration of symptoms in children with lower respiratory tract infections in an inpatient setting.
- To investigate if patient's age could affect the viral pattern and clinical significance.
- To describe the seasonal distribution of respiratory pathogens as detected using multiplex PCR in children hospitalized with ARI.
- To investigate the actual rate of bacteremia in RSV infected infants using molecular methods and its impact on the clinical phenotype and antibiotic prescription rates.

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3 MATERIAL & METHODS

3.1 Study design and recruitment criteria

Viral co-infection

Two independent observational, prospective patient groups were collected in Spain (main group) and in the United Kingdom (replication group). Spanish children were recruited between January 2011 and January 2013 through a national hospital based research network: GENDRES (Genetic, vitamin D and Respiratory infections research network – www.gendres.org), which includes 13 Spanish tertiary hospitals (Figure 18). UK children were recruited

between October 2009 and May 2010 at St Mary's hospital (UK). In both cohorts, eligible study participants were previously healthy children under 14 years of age with respiratory illness of sufficient severity to warrant admission to hospital.

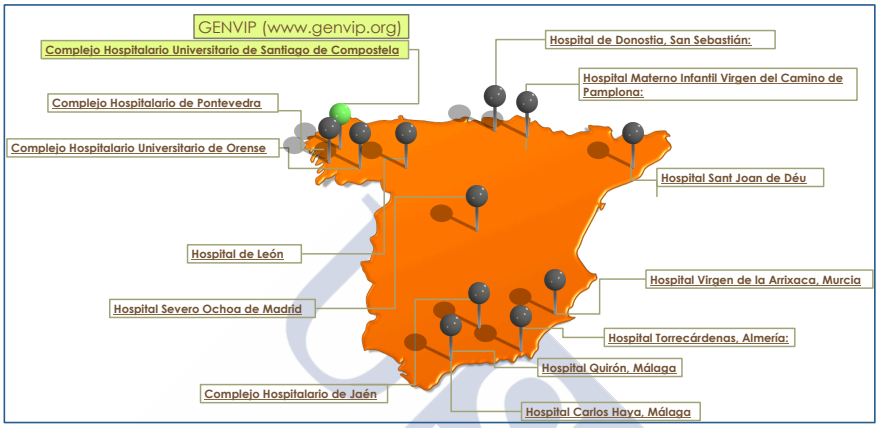

Figure 18: *GENDRES network. Participant hospitals are shown.*

Viral-bacterial interaction

Previously healthy infants admitted to any of the participant hospitals in GENDRES network with confirmed RSV infection were included. Positive RSV results for perform the blood PCR was considered when in both immunofluorescence (hospital) and PCR techniques the virus was detected.

3.2 Ethical clearance

All investigators were trained in the study protocol for patient recruitment, sample processing and sample storage. The study was performed according to Good Clinical Practice. Written informed consent was obtained from a parent or legal guardian for each subject before study inclusion (Appendix Figure 1). The study was approved by the Ethical Committee of Clinical Investigation of Galicia (CEIC ref. 2010/015). The UK cohort study was approved by the St Mary's Research Ethics Committee (REC 09/H0712/58).

3.3 Laboratory methods

During hospitalization a nasopharyngeal sample was obtained using a sterile feeding tube and injector for nasopharyngeal aspirate/wash or a sterile nylon swab (FLOQSwabs™ by Copan Diagnostics, Brescia, Italy) without culture medium.

The swab was inserted into one nostril straight back (not upwards) to the nasopharynx and was leaved in place for 2-3 seconds with small rotating motion to absorb secretions. Finally the swab was slowly removed (Figure 19).

For the nasopharyngeal aspirate a Mucus extractor (Poly medicure limited, Brussels, Belgium) was used. The coned section was placed in the infant's nasopharynx area. Suction was applied via the green cone end using either a pump system or manual suction to retrieve mucus sample (Figure 19). When

sufficient sample was obtained, the cap and tube assembly was removed and replaced with spare container cap supplied.

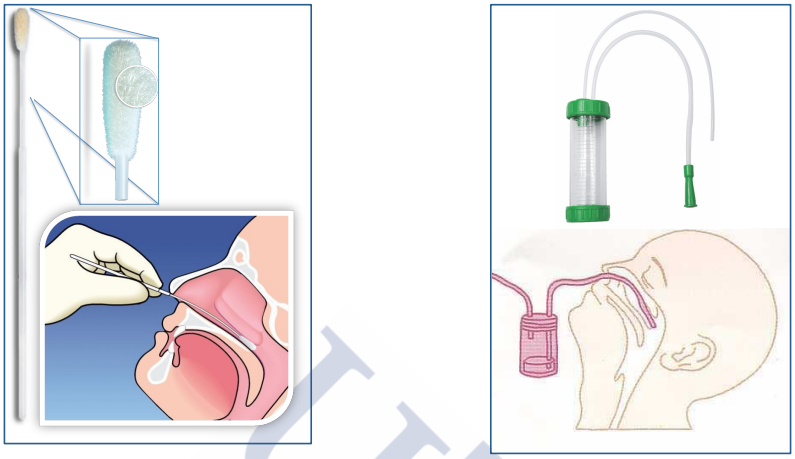

***Figure 19**: Collection methods of the nasopharyngeal samples in the study. Swab and nasopharyngeal aspirate collection is shown.*

A blood sample was also collected by venipuncture from each patient at the same time point for analysis *BD Vacutainer® K2E 5.4mg* tube (Becton Dickinson, Franklin Lakes, Plymouth, UK). Samples were stored at 4ºC for up to 24 hours before being stored at -80ºC. Samples were transported on dry ice to the Micropathology Laboratory (Coventry, United Kingdom) for viral and bacterial nucleic acid amplification. This PCR was an additional determination to the diagnostic work-up performed at physician discretion at the hospital of origin, which usually included direct immunofluorescent assays to detect influenza A and B, RSV, metapneumovirus, parainfluenza and adenovirus.

Viral PCR in nasopharyngeal samples

A panel of 19 viruses was investigated in nasopharyngeal samples by real time nested PCR:

- Respiratory syncytial virus
- Influenza (A, B)
- Parainfluenza types (1-4)
- Adenovirus (A-F)
- Rhinovirus
- Metapneumovirus
- Coronavirus (NL63, 229E, OC43)
- Bocavirus

The real-time PCR procedure used in the study is based on automated specimen extraction and multiplex amplification. Nasopharyngeal samples were tested in Micropathology lab. Nucleic acid extracts were prepared from 200 µl sample using a QiagenMDx BioRobot. In the RNA virus (RSV, hRV, hPIV, Flu, hCoV, hMPV) a reverse transcription process was made in which a single-stranded genomic RNA was converted into double-stranded cDNA. First round amplification was performed using 20 µl nucleic acid extract. Second round PCR was performed using 1 µl amplicon from first round PCR as template material. Reactions were run using a Lightcycler®480 with melt curve analysis for the detection of PCR products. The LightCycler®480 System is a high-performance, flexible throughput PCR platform from Roche Diagnostics.

Second round PCR products of AdV were analyzed using standard Sanger sequencing procedures. An ABI 3130xl genetic analyzer (Applied Biosystems®) in combination with ABI BigDye 3.1 technology to perform an in-house sequencing was used. The BigDye Terminator v3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. Sequences were analyzed using the BLAST algorithm.

Virus	Gene targets
Influenza A [80]	Gene N
Influenza B [81]	Gene M
Metapneumovirus [82]	Gene N
RSV [83]	Gene N
Parainfluenza 1 [80]	Gene HN
Parainfluenza 2 [84]	Gene HN
Parainfluenza 3 [84]	Gene HN
Parainfluenza 4 [85]	Gene P
Rhinovirus [86]	5' UTR
Adenovirus (A-F) [87,88]	Hexon
Bocavirus [89]	NS encoding region
Coronavirus NL63 [90]	Gene N
Coronavirus 229E [91]	Gene M
Coronavirus OC43 [92]	Gene N

Table 5. *PCR gene targets and sources from which the primers were obtained.*

- *Sequencing: Sanger method*

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. Fred Sanger developed the first method of sequencing the genetic code in 1977. Before the DNA can be sequenced, it has to be denatured into single strands using heat. A sequencing reaction mix containing amplified template DNA, oligonucleotide primers and a mixture of dNTPs and fluorescently tagged terminator dNTPs is heated to

95^{o}C to denature the double stranded DNA template. The primers are specifically constructed so that its 3' end is located next to the DNA sequence of interest.

The temperature is reduced and short, complementary primers bind to the now single-stranded template. A subsequent extension step at an enzyme-specific temperature allows DNA polymerase to replicate the template. The inclusion of chain-terminating fluorescently tagged nucleotide analogues leads to the random termination of growing DNA molecules.

Next a primer is annealed to the single stranded DNA. This primer is specifically constructed so that its 3' end is located next to the DNA sequence of interest. Then reagents are added to the primer and template, including: DNA polymerase, dNTPs, and a small amount of all four dideoxynucleotides (ddNTPs) labeled with fluorophores. During primer elongation, the random insertion of a ddNTP instead of a dNTP terminates synthesis of the chain because DNA polymerase cannot react with the missing hydroxyl. This produces all possible lengths of chains. Then the products are separated on a single lane capillary gel, where the resulting bands are read by an imaging system.

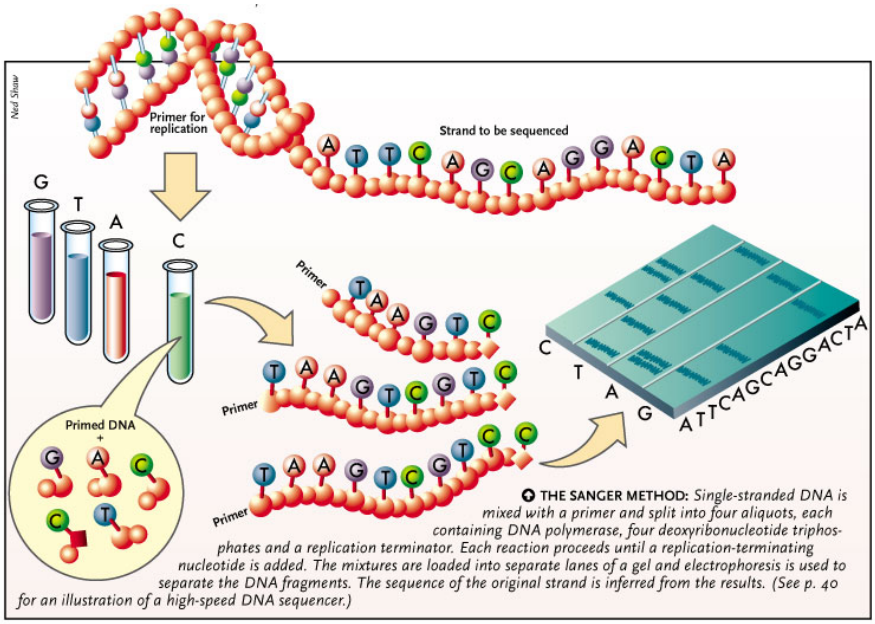

***Figure 20**: DNA sequencing. (www.the-scientist.com)*

- *Blast algorithm*

We used the Basic Local Alignment Search Tool (BLAST), which is one of the most widely used bioinformatics programs for sequence searching. It is an algorithm for comparing primary biological sequence information as the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. The BLAST finds regions of local similarity between sequences. The program compares nucleotide to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and

evolutionary relationships between sequences as well as help identify members of gene families.

Bacterial PCR in blood samples

In the positive RSV samples, an eight bacteria PCR was performed in blood, searching for:

- *Meningococcus*
- *Streptococcus pneumonia*
- *Haemophilus influenza*
- *Streptococcus pyogenes*
- *Klebsiella pneumonia*
- *Pseudomonas aeruginosa*
- *Escherichia coli*
- *Staphylococcus aureus*

In the same way as for virus detection, for bacterial identification a real-time PCR procedure was used based on automated specimen extraction and multiplex amplification. Blood samples collected in the same timepoint than the nasopharyngeal samples were tested in Micropathology lab. Nucleic acid extracts were prepared from 200 µl sample using a QiagenMDx BioRobot.

3.4 Clinical data collection

Detailed clinical data on each patient were collected using a secured web-based platform (www.gendres.org)

Figure 21: *GENDRES secure web page is presented (www.gendres.org).*

This included risk factors for ARI (ethnicity, prematurity, immunization status, obesity, diabetes, asthma and previous admissions to hospital), current medications, and family history of asthma or other respiratory conditions.

Severity of the episode

Clinical information of the episode of the illness during admission was documented (Appendix Figure 2). A specific questionnaire had to be answered and recorded searching information as hospital stay of length, if the patient was admitted to PICU, treatments during hospitalization or discharge diagnosis. Supplemental oxygen and / or mechanical ventilation requirement during admission were also recorded. Respiratory support was considered as either invasive (mechanical ventilation) or non-invasive ventilation (CPAP, BiPAP). The few patients using Optiflow® were included under the non-invasive ventilation category, although this could be debatable as the inadvertent PEEP generated by this technology cannot be strictly considered a non-invasive mode. The referring physician, with reference to clinical data, inflammatory markers, radiological findings and/or appropriate cultures, assessed the possibility of bacterial co-infection

The severity of each respiratory episode was ranked as follows:

1. Physician criteria (mild, moderate or severe). The respiratory distress was rated in the worst moment of the illness.

2. Modified Wood-Downes scale (0 to 10 points; mild <3, moderate 4 - 7, severe >8). In children with bronchiolitis and asthma,r severity assessment can be performed using the Wood-Downes scale which analyzes the following variables:

	0	0.5	1	2
Oxygenation	$SatO_2$ ≥95% ambient	95%>$SatO_2$≥90% ambient	$SatO_2$ ≥90% with FiO_2 >21	$SatO_2$ <90% with FiO_2 >21
Inspiratory breath sounds	Normal	Discreetly unequal	Highly unequal	Decreased or absent
Expiratory wheezing	None	Mild	Moderate	Maximal
Accessory muscles	None	Mild	Moderate	Marked
Cerebral function	Normal	Agitated when stimulated	Depressed/agitated	Coma

***Table 6**. Modified Wood-Downes Score.*

3. A newly developed scale -named GENVIP score- (0 to 20 points) that assesses food tolerance, degree of medical intervention needed, respiratory distress, respiratory frequency, apnea, malaise and fever. For each of the 6 items, the clinician had to choose the option that better described the situation of the child. The worst condition anytime during the whole course of the patient illness was considered.

		0 points	1 points	2 points	3 points
1	**Feeding intolerance**	**No**	**Mild** Decreased appetite and/or isolated vomits with cough.	**Partial** Frequent vomits with cough, rejected feed but able to tolerate fluids sufficiently to ensure hydration	**Total** Oral intolerance or absolute rejection of oral feed, not able to guarantee adequate hydration orally. Required nasogastric and/or intravenous fluids
2	**Medical intervention**	**No**	**Basic** Nasal secretions aspiration, physical examination, trial of nebulised bronchodilators, antipyretics.	**Intermediate** Oxygen therapy required. Complementary exams were needed (chest X-ray, blood gases, hematimetry…). Maintained nebulised therapy with bronchodilators	**High** Required respiratory support with positive pressure (either non-invasive in CPAP, BiPAP or high-flow O2; or invasive through endotracheal tube).
3	**Respiratory difficulty**	**No**	**Mild** Not in basal situation but do not impress of severity. Wheezing only audible with stethoscope, good air entrance. If modified Wood Downes, Wang score or any other respiratory distress score is applied, punctuation reveals mild severity.	**Moderate** Makes some extra respiratory effort (intercostal and/or tracheosternal retraction). Presented expiratory wheezing audible even without stethoscope, and air entrance may be localized decreased. If modified Wood Downes, Wang score or any other respiratory distress score is applied, punctuation reveals mild severity.	**Severe** Respiratory effort is obvious. Inspiratory and expiratory wheezing and/or clearly decreased air entry. If modified Wood Downes, Wang score or any other respiratory distress score is applied, punctuation reveals mild severity.
4	**Respiratory frequency**	**Normal**	**Mild/occasional tachypnea** Presented episodes of tachypnea, well tolerated, limited in time by self-resolution or response to secretion aspiration or nebulisation.	**Prolongued/ recurrent tachypnea** Tachypnea persisted or recurred despite secretion aspiration and/or nebulisation with bronchodilators.	**Severe alteration** Severe and maintained tachypnea. Very superficial and quick breath rate. Normal/low breath rate with obvious increased respiratory effort and/or mental status affected. Orientative rates of severe tachypnea: < 2 m: > 70 bpm 2-6 m: > 60 bpm 6-12m: >55 bpm 12-24m: >50 bpm 24-36m: >40 bpm
5	**Apnea**	**No**			**Yes** At least one episode of respiratory pause medically documented or strongly suggested through anamnesis.
6	**General Condition**	**Normal**	**Mild** Not in basal situation, child was mildly uncomfortable but did not impress of severity. Parent are not alarmed. Could wait in the waiting room or even stay at home.	**Moderate** Patient looks ill, and will need medical exam and eventually further complementary exams and/or therapy. Parent are concern. Not to be waiting in the waiting room.	**Severe** Agitated, apathetic, lethargic. No need to be physician to be worried. Parent are very concern. Immediate medical evaluation and/or intervention was required
7	**Fever**	**No**	**Yes, mild** Central Tª <38,5ºC	**Yes, moderate** Central Tª 38,5-39C	**Yes, severe** Central >39ºC

Table 7. *GENVIP Score. Clinical severity score for healthy infants with respiratory infections*

3.5 Data analysis

In the data analysis, we compared clinical data to the results of pathogen identification in respiratory and blood samples. General data are shown as percentages or means with 95% confidence intervals (CI). The level of statistical significance was set to 0.05. Statistical tests and Figures were carried out using R software v. 3.0.2 (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/http://www.r-project.org).

Different statistical models were used to assess the bivariate association between the variables depending on the dependent variable.

Odds ratio calculation

An odds ratio (OR) is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure.

$$OR = \frac{a \times d}{b \times c}$$

$$OR \times \exp\ (\pm 1{,}96 \times \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}})$$

The odds ratio is always a positive value, and can also be used to determine whether a particular exposure is a risk factor for a particular outcome, and to compare the magnitude of various risk factors for that outcome.

- OR = 1 Exposure does not affect odds of outcome
- OR > 1 Exposure associated with higher odds of outcome
- OR < 1 Exposure associated with lower odds of outcome

The 95% CI is used to estimate the precision of the OR. A large CI indicates a low level of precision of the OR, whereas a small CI indicates a higher precision of the OR.

Simple regression models

The logistic regression models are statistical models in which you want to know the relationship between a dichotomous qualitative dependent variable (binary logistic regression) or with more than two values (multinomial logistic regression) and one or more independent explanatory variables (covariates), whether qualitative (dicotomic) or quantitative. In simple linear regression, we predict scores on one variable from the scores on a second variable. Simple linear regression fits a straight line through the set of *n* points in such a way that makes the sum of squared residuals of the model (that is, vertical distances between the points of the data set and the fitted line) as small as possible.

Depending on the characteristics of the variables (binary, continuous, etc) the simple regression models can be categorized in:

- Binary logistic model
- Linear model
- Multinomial logistic model.

Multiple regression models

Multiple regression is an extension of simple linear regression. Regression analysis is a statistical process for estimating the relationships among variables. It includes many techniques for modelling and analysing several variables, when the focus is on the relationship between a dependent variable and one or more independent variables. It is used when we want to predict the value of a variable based on the value of two or more other variables. The variable we want to predict is called the dependent variable (or sometimes, the outcome, target or criterion variable). The variables we are using to predict the value of the dependent variable are called the independent variables (or sometimes, the predictor, explanatory or regressor variables).

X^2 test

A chi square (X^2) statistic is used to investigate whether distributions of categorical variables differ from one another.

2 x 2 Contingency Table: There are several types of chi square tests depending on the way the data was collected and the hypothesis being tested. The simplest case is a 2 x 2 contingency table. If we set the 2 x 2 table to the general notation shown below in Table 1, using the letters a, b, c,

and d to denote the contents of the cells, then we would have the following table:

Variable 2	Data type 1	Data type 2	Totals
Category 1	a	b	a + b
Category 2	c	d	c + d
Total	a + c	b + d	a + b + c + d = N

Table 8. *General notation for a 2 x 2 contingency table for Variable 1.*

Fisher's exact test

It is a statistical significance test used in the analysis of contingency tables. The test is useful for categorical data that result from classifying objects in two different ways; it is used to examine the significance of the association (contingency) between the two kinds of classification.

Wilcoxon test

The Wilcoxon test is a non-parametric statistical hypothesis test used when comparing two related samples, matched samples, or repeated measurements on a single sample to assess whether their population mean ranks differ.

Bonferroni correction

The Bonferroni correction is an adjustment made to P values when several dependent or independent statistical tests are being performed simultaneously on a single data set. To perform a Bonferroni correction, a

division of the critical *P* value (*α*) by the number of comparisons being made. For example, if 10 hypotheses are being tested, the new critical *P* value would be α/10. The statistical power of the study is then calculated based on this modified *P* value.

The Bonferroni correction is used to reduce the chances of obtaining false-positive results (type I errors) when multiple pair wise tests are performed on a single set of data.

False Discovery Rate

The False discovery rate (FDR) is one way of conceptualizing the rate of type I errors in null hypothesis testing when conducting multiple comparisons. FDR-controlling procedures are designed to control the expected proportion of rejected null hypotheses that were incorrect rejections.

Data analysis applied in our study

- The relationship between demographic and clinical variables with mono-infection and co-infection was analyzed using *simple logistic regression*. A binary logistic model was used for the binary variables (co-infection status, oxygen requirements, respiratory support needed and PICU admission), linear model for continuous variables (Wood-Downes Score and GENVIP Score), negative binomial regression model for counted data (hospital stay length) and logistic multinomial model for the multinomial variable (respiratory distress status).

- *Multiple regression models* were considered using the significant risk factors obtained in the bivariate analysis and sex and age variables. In order to reduce the likelihood of false significant results due to too many statistical comparisons, the *Bonferroni multiple test correction* and *False Discovery Rate* were considered.

- A *χ2 test* was performed to evaluate the correlation between bacterial superinfection and pneumococcal vaccine.

- For viral-bacterial interaction the *Fisher's exact test* for discrete variables and *Wilcoxon test* for continuous variables were used.

- *Fisher's exact test* was used to study the association between the viruses and PICU admission.

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4 RESULTS

4.1 General characteristics of the GENDRES and UK cohort

The GENDRES cohort had a median age of 6.4 (first quartile: 2.2, third quartile: 17.0) months and a male-to-female sex ratio of 1.7. One patient was excluded due to incomplete clinical data (Figure 22). The cohort included nasopharyngeal samples from 204 patients: 23 (11.3%) nasopharyngeal swabs and 181 (88.7%) nasopharyngeal aspirates/wash. No differences in findings were observed in relation to the method used for sample collection (data not shown).

The UK cohort included samples from 97 patients, with a median age of 20.0 (first quartile: 7.0, third quartile: 48.7) months and a male-to-female ratio of 0.94. (Figure 22).

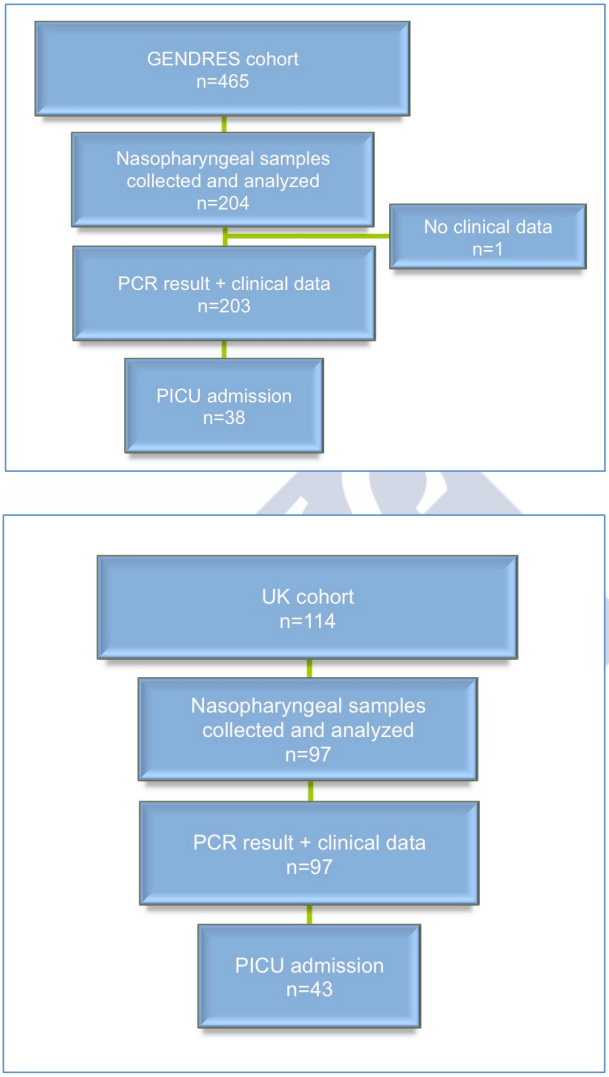

***Figure 22**: Flow chart of study population of the main cohort (GENDRES cohort) and replication cohort (UK cohort)*

Comparison between both cohorts is shown in Table 9.

Variable	GENDRES cohort n (%)	UK cohort n (%)	P-value
Sex (female proportion) [1]	75 (36.9)	50 (51.5)	0.018
Age (months) [1]			<0.001[β]
0-12	136 (66.7)	39 (40.2)	
13-24	25 (12.3)	17 (17.5)	
25-48	26 (12.8)	17 (17.5)	
<48	16 (7.9)	24 (24.7)	
Pneumoccocal vaccine[1]	110 (53.9)	57 (64.0)	0.124
Bacterial superinfection[1]	56 (29.5)	53 (54.6)	<0.001[β]
PICU admission[1]	38 (29.0)	43 (44.3)	0.024
Respiratory support[1]	30 (14.8)	36 (38.3)	<0.001[β]
Oxygen needed[1]	56 (29.5)	55 (57.9)	<0.001[β]
Hospital stay length[2*]	15.4 (8.8)	7.3 (9.1)	<0.001[β]

Table 9. *Description of the characteristics of the two cohorts analyzed: the GENDRES cohort and the UK cohort.*

P-value results from the comparison between both cohorts. A P-value < 0.005 was considered significant. (1) Fisher Exact Test. (2) Wilcoxon test. (*) Mean and Standard deviation in days. (β) Significant under Bonferroni correction.

4.2 Molecular diagnostics

Molecular diagnostics in the GENDRES cohort

Molecular diagnostics identified at least one pathogen in 187 samples. Of these PCR positive samples, 73 had previously yielded negative results using conventional methodology – immunufluorescence assays and/or rapid techniques. Five samples (2.5%) were negative for both PCR and the initial diagnostic work-up. In 12 cases (5.9%) where the referring hospital had established a diagnosis, PCR was negative (RSV, *n* = 8; IV H1N1, *n* = 2; Mycoplasma, *n* = 1; and Influenza C, *n* = 1). By PCR multiplex assay, a

single pathogen was detected in 95 (46.6%) children and two or more pathogens were detected in 92 (45.1%) patients, giving an overall detection rate of 91.7%. The most commonly detected virus was RSV (*n* = 108), followed by hRV (*n* = 68), hBoV (*n* = 48), AdV (*n* = 39), HMPV (*n* = 27), IV (*n* = 12), hPIV and hCoV (both *n* = 5) (Table 10). In co-infected samples, the most frequent combination of pathogens was RSV + hRV (*n* = 23) followed by RSV + hBoV (*n* = 10) and RSV + AdV (*n* = 7) (see Table 11). The virus most frequently found in dual infection was RSV (*n* = 42), followed by hRV (*n* = 35) (Table 10). RSV was observed with the same frequency as a single infection (*n* = 53) and as a co-infection agent (*n* = 55) (see Figure 23). However, hRV, IV, hBoV, AdV and hMPV were more frequently found in co-infections (Figure 23).

Table 10. *Distribution of viral agents according to age in the GENDRES cohort (GEN) and UK cohort (UK). Data are presented as number of positive samples (percentage of samples evaluated) or the mean (standard deviation). Age is expressed in months.*

	Total n (%)		Single infection n (%)		Co-infection n (%)		Mean age (SD)		Age (months) [GEN n=203; UK n=97]							
									0-12		12-24		24-48		>48	
	GEN	UK	GEN	UK	GEN	UK	GEN	UK	GEN	UK	GEN	UK	GEN	UK	GEN	UK
RSV	108 (52.9)	35 (36.1)	53 (55.8)	21 (33.3)	55 (59.8)	14 (43.8)	8.1 (14.9)	12.8 (14.6)	88 (64.7)	21 (53.8)	12 (48.0)	8 (47.1)	5 (19.2)	5 (29.4)	3 (18.8)	1 (4.2)
Rhinovirus	68 (33.3)	24 (24.7)	17 (17.9)	11 (17.5)	51 (55.4)	13 (40.6)	14.4 (17.4)	40.9 (46.3)	44 (32.4)	7 (17.9)	9 (36.0)	5 (29.4)	11 (42.3)	6 (35.3)	3 (18.8)	6 (25.0)
Bocavirus	48 (23.5)	19 (19.6)	6 (6.3)	3 (4.8)	42 (45.7)	16 (50.0)	22.5 (27.4)	31.9 (35.8)	21 (15.4)	4 (10.3)	10 (40.0)	8 (47.1)	13 (50.0)	4 (23.5)	4 (25.0)	3 (12.5)
Adenovirus	39 (19.1)	9 (9.3)	4 (4.2)	3 (4.8)	35 (38.0)	6 (18.8)	21.4 (20.1)	51.2 (48.9)	15 (11.0)	2 (5.1)	11 (44.0)	1 (5.9)	9 (34.6)	3 (17.6)	4 (25.0)	3 (12.5)
Metapneumovirus	27 (13.2)	4 (4.1)	9 (9.5)	2 (3.2)	18 (19.6)	2 (6.3)	16.8 (27.7)	48.5 (57.2)	18 (13.2)	1 (2.6)	3 (12.0)	1 (5.9)	5 (19.2)	1 (5.9)	1 (6.3)	1 (4.2)
Influenza virus	12 (5.9)	23 (23.7)	4 (4.2)	13 (20.6)	8 (8.7)	10 (31.3)	35.7 (43.2)	45.9 (44.0)	2 (1.5)	5 (12.8)	4 (16.0)	5 (29.4)	4 (15.4)	5 (29.4)	2 (12.5)	8 (33.3)
Parainfluenza	5 (2.5)	6 (6.2)	1 (1.1)	3 (4.8)	4 (4.3)	3 (9.3)	40.4 (53.5)	41.67 (55.8)	3 (2.2)	2 (5.1)	0 (0.0)	1 (5.9)	1 (3.8)	2 (11.8)	1 (6.3)	1 (4.2)
Coronavirus	5 (2.5)	–	1 (1.1)	–	4 (4.3)	–	25.3 (23.5)	–	2 (1.5)	–	0 (0.0)	–	1 (3.8)	–	1 (6.3)	–
Co-infection	92 (45.1)	29 (29.9)	–	–	–	–	15.1 (16.1)	25.6 (25.1)	53 (41.7)	8 (23.5)	16 (72.7)	10 (66.7)	18 (75.0)	6 (35.3)	4 (30.8)	4 (21.1)
Total samples	204	97	95 (46.1)	56 (57.7)	92 (45.1)	29 (29.9)	16.7 (28.5)	36.6 (44.6)	136 (67.0)	39 (40.2)	25 (12.3)	17 (17.5)	26 (12.8)	17 (17.5)	16 (7.9)	24 (24.7)

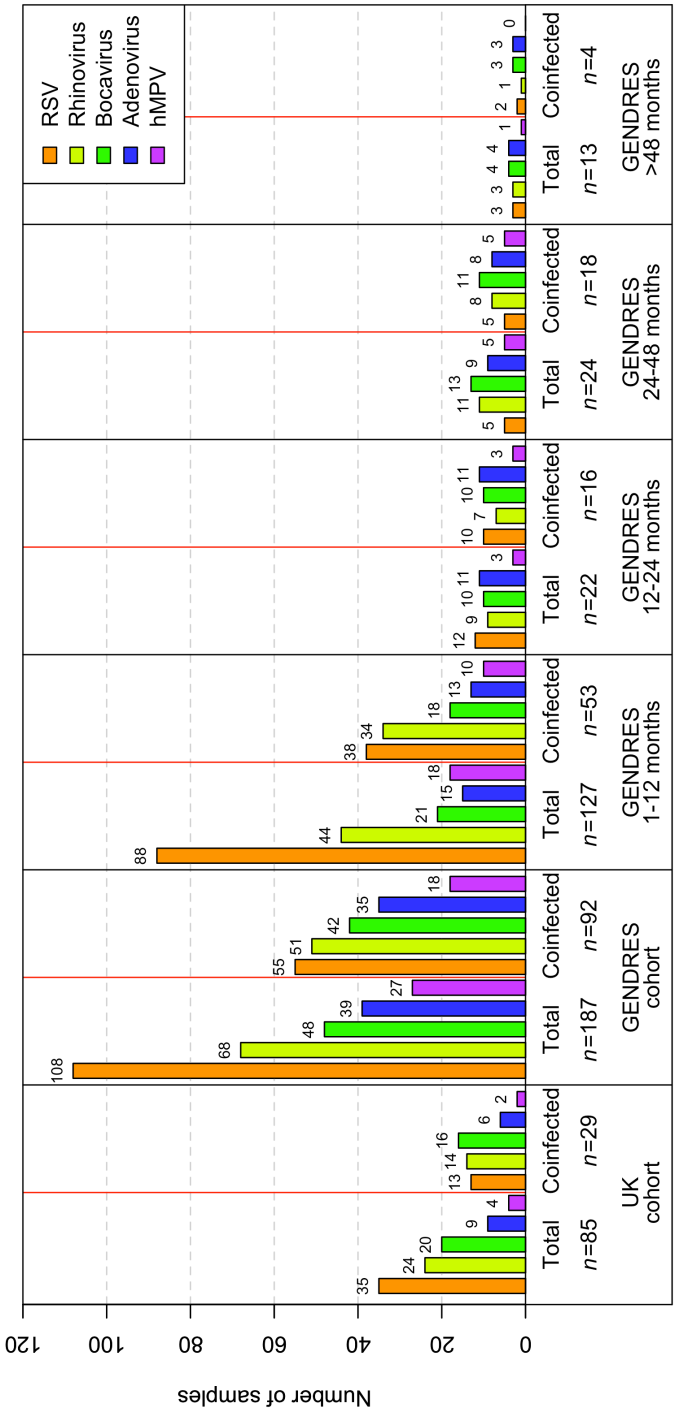

***Figure 23**. Pathogen prevalence in the main and replication cohorts showed as number in nasopharyngeal samples considering the age of the children. Only the more prevalent viruses are presented.*

Table 11. Associations among respiratory pathogens in hospitalized children in the GENDRES cohort and UK cohort

Pathogens detected	GENDRES cohort		UK cohort	
	Number	% of positive cases	Number	% of positive cases
Co-infection, two pathogens	65	34.2	24	28.2
RSV+hRV/hBov/AdV/MPV/IV	23/10/7/2/0	12.3/5.3/3.7/1.1/0.0	3/4/2/0/2	3.5/4.7/2.4/0/2.4
hRV+hBoV/hCoV/AdV/MPV/IV/hPIV	2/2/6/3/0/0	0.5/1.1/3.2/1.6/0.0/0.0	3/0/1/1/1/1	3.5/0.0/1.2/1.2/1.2/1.2
IV+hBoV/MPV	2/1	1.1/0.5	5/0	5.9/0.0
hBoV+AdV/hCoV/MPV	1/1/4	0.5/0.5/2.1	0/0/0	0.0/0.0/0.0
AdV+hPIV/MPV	1/1	0.5/0.5	1/0	1.2/0
Co-infection, three pathogens	21	11.3	4.0	4.7
RSV+hRV+AdV/hBoV/hCoV	3/3/1	1.6/1.6/0.5	0/0/0	0.0/0.0/0.0
RSV+hBoV+IV/AdV	2/4	0.5/1.1	0/1	0.0/1.2
hRV+hBoV+AdV/IV	4/0	2.1/0.0	1/1	1.2/1.2
IV+hBoV+AdV	2	1.1	1	1.2
hBoV+MPV+AdV/hPIV	2/2	1.1/1.1	0/0	0.0/0.0
Co-infection, four pathogens	6	3.2	1	1.2
hRV+hBoV+AdV+IV/MPV/hPIV	1/3/1	0.5/1.6/0.5	0/0/0	0.0/0.0/0.0
hRV+hBoV+MPV+hPIV/RSV	1/0	0.5/0.0	0/1	0.0/1.2

Molecular diagnostics in the UK cohort

We identified at least one virus in 85 (87.6%) samples; in 12 samples no virus was identified. A single virus was present in 56 (57.7%) patients, and two or more viruses in 29 (29.9%) children. The most commonly detected virus was RSV (*n* = 35), followed by hRV (*n* = 24), IV (*n* = 23), hBoV (*n* = 19), AdV (*n* = 9), HMPV (*n* = 4) and hPIV (*n* = 6) (Table 10). In the co-infected samples, the most frequent combinations were RSV + hBoV and IV + hBoV (both *n* = 5). The viruses most frequently found in dual infections were RSV and hBoV (both *n* = 12), followed by hRV (*n* = 9) and IV (*n* = 8) (Table 11). hRV and IV were observed with similar frequencies in single infection and co-infection (hRV *n* = 11 vs 13; IV *n* = 13 vs 10), while hBoV and AdV were more frequently found as co-infections, and RSV was more commonly present as a single infection.

Age differences in infection

We compared co-infection frequencies according to age groups. In both cohorts, co-infection was found in all age groups. In the GENDRES cohort, there is a significant association between age and co-infection: in children aged 12 - 24 months (72.7% of infected patients; see Figure 23) and those aged 24 - 48 months (75.0% of infected patients; *P*-value = 0.001). In the UK cohort, there was also a significant association age and co-infection in patients aged 12 - 24 months (73.3% of the infected patients; *P*-value = 0.005).

In the GENDRES cohort, RSV infection affected younger children more frequently (mean age: 8.1 months; SD: 14.9) and hPIV was principally found in older patients (mean age: 40.4 months; SD: 53.5) (Table 10). In the UK cohort, RSV infection also affected younger children more frequently (mean age: 12.8 months; SD: 14.6) and AdV predominated in older patients (mean age: 51.2 months; SD: 48.9).

PICU admission differences in infection

There was no significant difference in the number of infections between the PICU and non-PICU cohorts. Although modest differences were found for hBoV (PICU: 15.8% - non PICU: 22.6%) and hMPV (PICU: 2.6% - non PICU: 14.0%) in the GENDRES cohort, these were not statistically significant. These differences were only observed in the UK cohort for hBoV (PICU: 16.3% - non PICU: 22.2%) (Table 12).

Virus	GENDRES cohort			UK cohort		
	PICU (n=38) n (%)	*No PICU (n=93) n (%)*	*P*-value	*PICU (n=43) n (%)*	*No PICU (n=54) n (%)*	*P*-value
RSV	24 (63.2)	56 (60.2)	0.844	17 (39.5)	18 (33.3)	0.671
hRV	15 (39.5)	28 (30.1)	0.312	11 (25.6)	13 (24.1)	1.000
hBoV	6 (15.8)	21 (22.6)	0.479	7 (16.3)	12 (22.2)	0.451
AdV	5 (13.2)	14 (15.1)	1.000	3 (7.0)	6 (11.1)	0.727
hMPV	1 (2.6)	13 (14.0)	0.066	2 (4.7)	2 (3.7)	1.000
IV	1 (2.6)	4 (4.3)	1.000	8 (18.6)	15 (27.8)	0.342
hPIV	1 (2.6)	3 (3.2)	1.000	1 (2.3)	5 (9.3)	0.223
hCoV	1 (2.6)	1 (1.1)	0.498	-	-	-

Table 12. *Virus detection in patients admitted to pediatric intensive care unit (PICU) in both cohorts. No differences were found when compared to those children not requiring PICU admission.*

4.3 Severity of the illness

Mono-infection versus Multi-infection

Clinical data, family or past medical history, need of PICU admission or hospital length of stay, and oxygen or respiratory support need, were equivalent in the GENDRES and the UK cohort (Table 13). In the GENDRES cohort the presence of rhinovirus as co-pathogen was associated with a significantly increased Wood-Downes score by 1.289 points (95% CI: 0.387, 2.192; *P*-value = 0.006). RSV infection was associated with increased oxygen requirements [OR (95% CI): 3.154 (1.302, 7.966); *P*-value = 0.012] (Appendix Table 5). These isolated findings were not replicated in the UK cohort (Appendix Table 6).

Variable	GENDRES cohort (n = 203)					UK-cohort (n = 97)		
	GENDRES % (95% CI)	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P* -value	UK-cohort % (95% CI)	OR (95% CI)	*P* -value
Demographic characteristics								
Sex (female proportion)	36.9 (30.3, 43.6)	1.287 (0.711, 2.340)	0.405	1.069 (0.564, 2.014)	0.838	51.5 (41.1, 62.0)	1.228 (0.498, 3.086)	0.657
Age			β♯					
12 - 24 months	12.3 (7.8 16.8)	3.723 (1.428, 10.955)	0.010	3.173 (1.177, 9.569)	0.028	17.5 (9.4 , 25.6)	8.937 (2.377, 40.192)	0.002
24 - 48 months	12.8 (8.2, 17.4)	4.189 (1.635, 12.201)	0.005	3.463 (1.290, 10.447)	0.018	17.5 (9.4 , 25.6)	1.773 (0.484, 6.373)	0.378
> 48 months	7.9 (4.2, 11.6)	0.621 (0.161, 2.015)	0.447	0.544 (0.139, 1.800)	0.339	24.7 (15.6, 33.9)	0.867 (0.203, 3.261)	0.836
Family history								
Asthma	39.9 (33.2, 46.6)	0.753 (0.417, 1.352)	0.343			n.a.		
Respiratory conditions	15.8 (10.8, 20.9)	0.824 (0.375, 1.785)	0.623			n.a.		
Patient medical history								
Premature birth	8.5 (4.5, 12.5)	0.450 (0.118, 1.443)	0.199			n.a.		
Pneumococcal vaccine	53.9 (47.1, 60.8)	2.055 (1.151, 3.709)	0.016	1.550 (0.821,2.932)	0.176	64.0 (54.1, 74.0)	1.990 (0.734, 5.849)	0.189
Pulmonary conditions	3.5 (0.9, 6.0)	0.517 (0.070, 2.718)	0.453			n.a.		
Asthma	11.8 (7.4, 16.3)	0.943 (0.374, 2.354)	0.899			n.a.		
Clinical data								
Bacterial superinfection	29.5 (23.0, 36.0)	1.396 (0.736, 2.667)	0.308			54.6 (44.2, 65.1)	1.319 (0.536, 3.315)	0.549

Table 13. *Relationship between demographic and clinical variables with mono-infection and co-infection is shown. The correlation was analyzed using simple logistic regression. Data are presented as OR (95% confidence interval) and P-value. βSignificant under Bonferroni correction; ♯significant under FDR correction. n.a. not applicable.*

Bacterial superinfection

Children presenting a bacterial superinfection had more severe respiratory distress [OR (95% CI): 4.356 (1.564, 12.128); *P*-value = 0.005] and a higher severity score [2.124 (95% CI: 0.864, 3.385); *P*-value = 0.001]. They were more likely to be admitted to PICU in the GENDRES cohort [OR (95% CI): 2.851 (1.300, 6.252); *P*-value = 0.009] and the UK cohort [5.357 (2.081, 15.085); *P*-value = 0.001]. Children with bacterial co-infection required significantly more respiratory support in both cohorts: discovery cohort [OR (95% CI): 6.368 (2.724, 14.886); *P*-value < 0.001] and replication cohort [OR (95% CI): 3.432 (1.402, 8.404); *P*-value = 0.007], and they had a longer hospital stay in both cohorts: 1.48 days (*P*-value = 0.025) longer stay in GENDRES cohort and 1.87 days (*P*-value = 0.005) in UK cohort, respectively (Figure 24; Appendix Tables 1 - 8). In addition, 34.0% of the patients with bacterial infection in the GENDRES cohort received the pneumococcal vaccine and 24.7% did not receive it (*P*-value = 0.213).

Pneumococcal vaccine

In the GENDRES cohort the pneumococcal vaccine was given to 53.9% (46.9, 61.1) of the patients of whom 43.8% (33.3, 54.2) were mono-infected and 62.7% (52.2, 73.1) were viral co-infected patients [OR (95% CI): 1.550 (0.821, 2.932); *P*-value = 0.176]. Vaccinated patients had lower risk of being admitted to PICU in GENDRES cohort [OR (95% CI): 0.301 (0.116, 0.735); *P*-value = 0.011] and in the UK cohort [OR (95% CI): 0.208 (0.046, 0.776); *P*-value = 0.027] and had less risk of respiratory support requirement in the

main cohort [OR (95% CI): 0.324 (0., 0.790); *P*-value = 0.016] and in the replication one [OR (95% CI): 0.267 (0.070, 0.901); *P*-value = 0.040]. In the Spanish cohort, patients who received the pneumococcal vaccine received less oxygen support [OR (95% CI): 0.328 (0.162, 0.639); *P*-value = 0.001], and had a lower clinical severity score [-1.499 (95% CI: -2.768, -0.231) points; *P*-value = 0.021] and a lower respiratory distress score [OR (95% CI): 2.917 (1.078, 7.889); *P*-value = 0.035]. These findings were not replicated in the UK cohort (Figure 24; Appendix Tables 1 - 8).

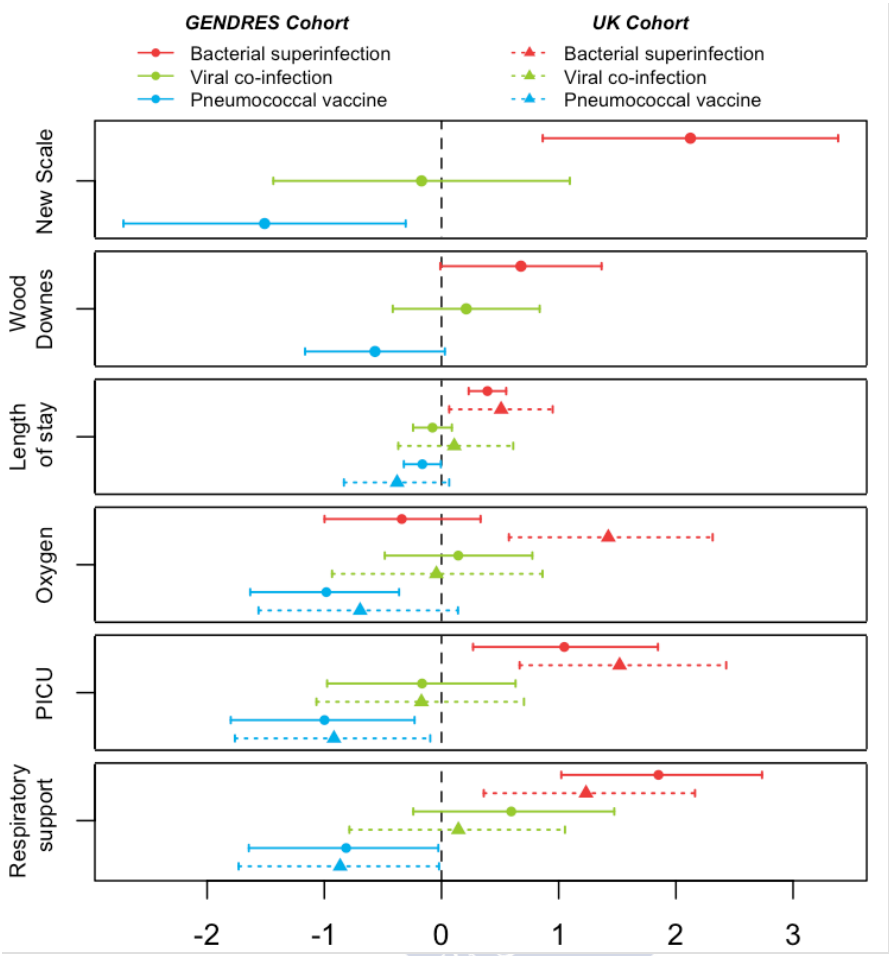

***Figure 24**. Influence of bacterial superinfection, pneumococcal vaccine and the presence of viral co-infection on disease severity of children with ARI, according to oxygen and respiratory support requirement, clinical scales, hospital stay length and PICU admission.*

Data are shown as OR (95% CI) for both main cohort and replication cohort. A binary logistic model was used for the binary variables (co-infection status, oxygen requirements, respiratory support needed and PICU admission), linear model for continuous variables (Wood-Downes Score and the GENVIP score) and negative binomial regression model for counted data (number of days since admission).

4.4 Seasonal and geographical distribution

A strong seasonal pattern was exhibited by most viruses, the majority of which were mainly detected in the cold seasons: 23 (11.4%) in autumn and 122 (60.4%) in winter, compared to 56 (27.7%) in spring and only 1 (0.5%) in

summer. Seasonal distribution varied according to the virus (Figure 25). AdV and hMPV were typically detected at the end of the winter season and during spring, while hRV were detected from the end of autumn to spring with a constant frequency during this period (plateau). RSV was mostly identified during winter with an incidence peak in January. hBoV respiratory infections occurred mainly in December and January with another epidemic peak in March. IV showed a peak during winter and hPIV and hCoV were infrequently observed throughout the year.

Viruses were not found with any geographical distribution. We detected IV in the centre and south regions but not in the north ones.

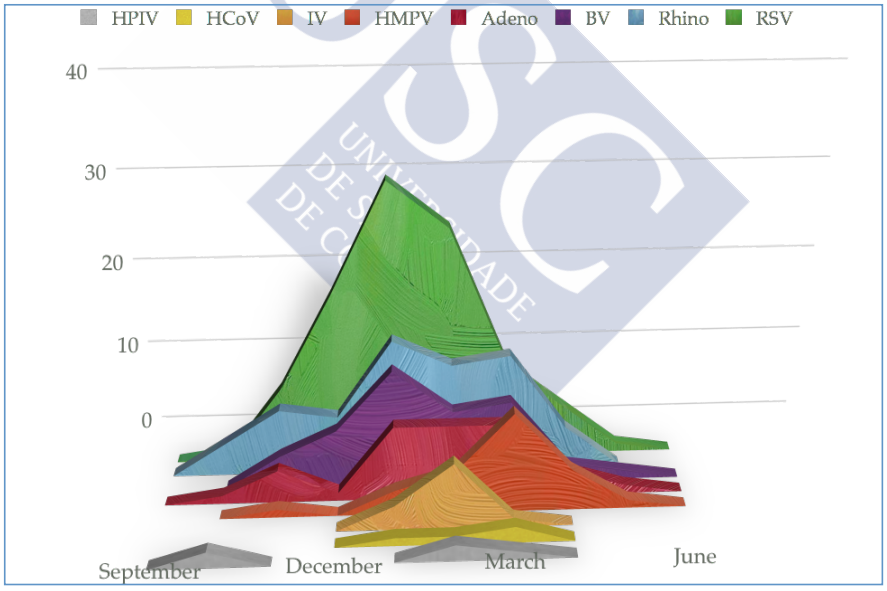

Figure 25. *Seasonal distribution of respiratory viral agents. Monthly distribution of respiratory samples analyzed in the study from 2011-2013.*

We can see the different patterns of the virus and in all of them, we can observe that in summer the prevalence had been reduced to the minimum number of samples in the years analyzed.

4.5 Virus-bacteria interaction

In 130 patients a nasopharyngeal sample PCR was performed, and of these, a total of 66 patients with a positive RSV by immunofluorescence technique in a nasopharyngeal sample/rapid test and confirmed by PCR were included in this study (Figure 26).

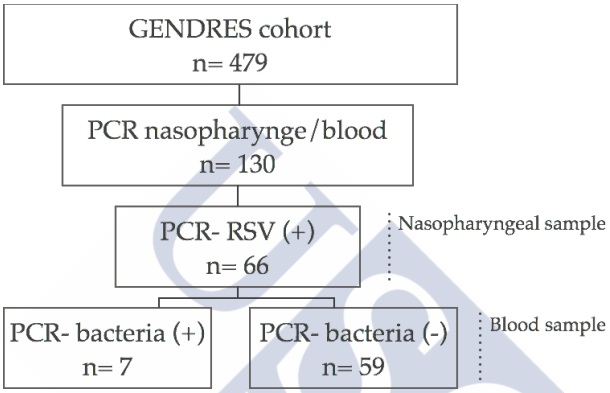

Figure 26. *Flow chart of study population of the GENDRES cohort for bacterial presence in blood analysis.*

Almost all of the children (92.4 %) were <12 months of age and the majority (66.7%) of the patients were boys. Although most of the patients were previously healthy children, 3.0% had diagnosed asthma and 4.8 % were premature. 23 of 66 (34.9%) of these patients had suspicion of bacterial superinfection according to the referring physician and in 5 of these 23 patients (21.7%) the bacterial superinfections were confirmed by microbiological methods in the referring hospital. In *n* = 7 (10.6%) patients molecular assessment revealed bacterial presence in the blood (Table 14).

Risk factor	Total cohort (*n* = 66)	Negative PCR (*n* = 59)	Positive PCR (*n* = 7)	*P*-value
Demographics				
Sex. Female[1]	33.3% (22/66)	35.6% (21/59)	14.3% (1/7)	0.409
Age[1]				1.000
< 12	92.4 (61/66)	91.5 (54/59)	100.0 (7/7)	
12 - 24	3.0% (2/66)	3.4% (2/59)	0.0% (0/7)	
24 - 48	4.5% (3/66)	5.1% (3/59)	0.0% (0/7)	
Family history				
Asthma[1]	30.3% (20/66)	71.2% (42/59)	57.1% (4/7)	0.425
Respiratory conditions[1]	30.3% (20/66)	28.8% (17/59)	42.9% (3/7)	0.425
Medical history				
Premature[1]	4.8% (3/63)	3.6% (2/56)	14.3% (1/7)	0.302
Pneumococcal vaccine	48.5 (32/66)	52.5% (31/59)	14.3% (1/7)	0.106
Clinical data				
Oxygen needed[1]	80.3% (53/66)	78.0% (46/59)	100.0% (7/7)	0.329
Respiratory support[1]	27.3% (18/66)	18.6% (11/59)	100.0% (7/7)	<0.001
Diagnosis[1]				0.739
Bronchiolitis	78.8% (52/66)	76.3% (45/59)	100.0% (7/7)	
Pneumonia	6.1% (4/66)	6.8% (4/59)	0.0% (0/7)	
Others	15.2% (10/66)	16.9% (10/59)	0.0% (0/7)	
Respiratory distress[1]				0.001
Mild	22.7% (15/66)	25.4% (15/59)	0.0% (0/7)	
Moderate	53.0% (35/66)	57.6% (34/59)	14.3% (1/7)	
Severe	21.2% (14/66)	13.6% (8/59)	85.7% (6/7)	
PICU[1]	57.1% (28/49)	50.0% (21/42)	100.0% (7/7)	0.015
Fever[1]				0.733
Febricula (< 38º)	37.5% (18/48)	36.6% (15/41)	42.9% (3/7)	
Fever (> 38º)	50.0% (24/48)	48.8% (20/41)	57.1% (4/7)	
Wood Downes Score (mean-SD)[2]	5.2 (2.4)	4.8 (2.2)	8.7 (1.1)	<0.001
GENVIP scale (mean-SD)[2]	11.1 (4.1)	10.1 (3.6)	17.0 (1.0)	<0.001
Hospital stay of length (mean-SD)[2]	8.0 (4.8)	7.5 (4.7)	12.1 (4.3)	0.007
Suspected bacterial superinfectión[1]	34.8% (23/66)	30.5% (18/59)	71.4% (5/7)	0.044
Blood culture				
Done. Yes[1]	60.0% (27/45)	55.3% (21/38)	85.7% (6/7)	0.215
Positive[1]	3.8% (1/26)	0.0% (0/21)	17.0% (1/6)	0.222
Antibiotic treatment[1]	66.7% (30/45)	63.2% (24/38)	85.7% (6/7)	0.395

Table 14. *Summary of the characteristics of RSV cohort and comparison between those with positive and negative blood PCR for bacteria.*

General data is presented as percentage or means with 95% confidence intervals. Different statistical models were used to assess the association between the variables: Fisher's exact test (1) for discrete variables and Wilcoxon test (2) for continuous variables.

The pathogen found were *H. influenzae* (*n* = 4), *S. pneumoniae* (*n* = 1) and both simltaneously (*n* = 2). The cycle thresholds were <20 except for *S. pneumoniae* in the co-infection cases, in which the cycle threshold was >25. Only one of these cases this bacteria had also been identified by conventional cultures.

In nasopharyngeal samples, more than one virus was detected in 35 out of 66 (53.0%) patients by molecular techniques. In bacterial PCR positive patients, viral co-infection was observed in 5 out of 7 (71.4%) subjects (Table 15). However, results of positive blood cultures performed at hospital and the later PCR were not in agreement. There was only one patient in whom a blood culture was performed at the referring hospital with a positive result (*H. influenzae* and *S. aureus*) and when the molecular technique was performed a similar result was obtained. (*H. influenzae*) (Table 15).

Antibiotic administration in patients with suspected bacterial superinfection was recorded in 50.0% (*n* = 33) of the RSV-infected patients and in 3.3% (*n* = 1) of these patients a bacterial superinfection was confirmed by conventional blood cultures.

There was no correlation between the patients suspected of superinfection and/or prescribed antibiotic by the referring physician, and those with positive blood PCR results (Cohen's kappa coefficient bacterial superinfection-PCR = 0.15). A total of 87.5% of the patients presented fever: > 38ºC in 50% of the included children (24 out of 48), and mild fever in 18 of them (37.5%). Fever frequency in children with bacteremia confirmed by PCR was similar to that in the rest of the cohort (*P*-value = 0.733).

Patient	Blood PCR - Bacteria	PCR - Virus	Respiratory affection	Wood-Downes Score	Oxygen	Respiratory support	Bronchiolitis diagnosis	Suspected bacterial superinfection
1	H. influenzae/ S. pneumoniae	RSV + Rhinovirus	Severe	9	Yes	NINV	Yes	Yes
2	H. influenzae	RSV	Severe	10	Yes	NINV	Yes	Yes
3	S. pneumoniae	RSV + Rhinovirus + Bocavirus	Severe	10	Yes	INV	Yes	Yes
4	H. influenzae	RSV + Bocavirus	Moderate	7	Yes	NINV	Yes	Yes
5	H. influenzae/ S. pneumoniae	RSV + Bocavirus	Severe	8	Yes	NINV	Yes	No
6	H. influenzae	RSV + Rhinovirus + Coronavirus	Severe	9	Yes	INV	Yes	Yes
7	H. influenzae	RSV	Severe	8	Yes	INV	Yes	Yes

Table 15. *Description of RSV infected patients with positive blood bacterial PCR. Abbreviatures: NINV: non-invasive ventilation, INV: invasive ventilation*

Severity of the illness

There were significant differences in terms of illness severity between children with positive bacterial PCR and those with negative results: PICU admission (100% vs. 50%, *P*-value = 0.015) and respiratory support necessity (100% vs. 18.6%, *P*-value < 0.001). Patients with confirmed bacteremia had a more severe respiratory affection than those with no bacteria identified in blood (Table 14). Both the Wood-Downes score and the GENVIP scale indicated a worse value in the blood PCR positive patients (mean = 8.7 points and 17.0 points, respectively) than in the blood PCR negative patients (mean = 4.8 points and 10.1 points) (*P*-value < 0.001 for both). Hospitalization was longer for children with PCR-confirmed bacteremia (mean = 12.1 vs. 7.5 days, *P*-value = 0.007) (Figure 27).

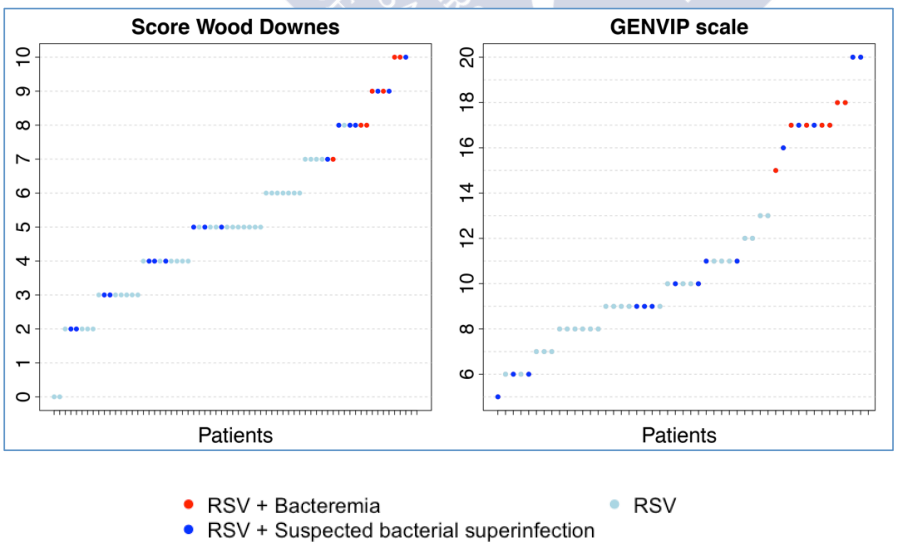

Figure 27. (continues). Severity parameters for the patients: Wood Downes score, GENVIP score, length of hospitalization, oxygen, respiratory support, respiratory distress and PICU admission

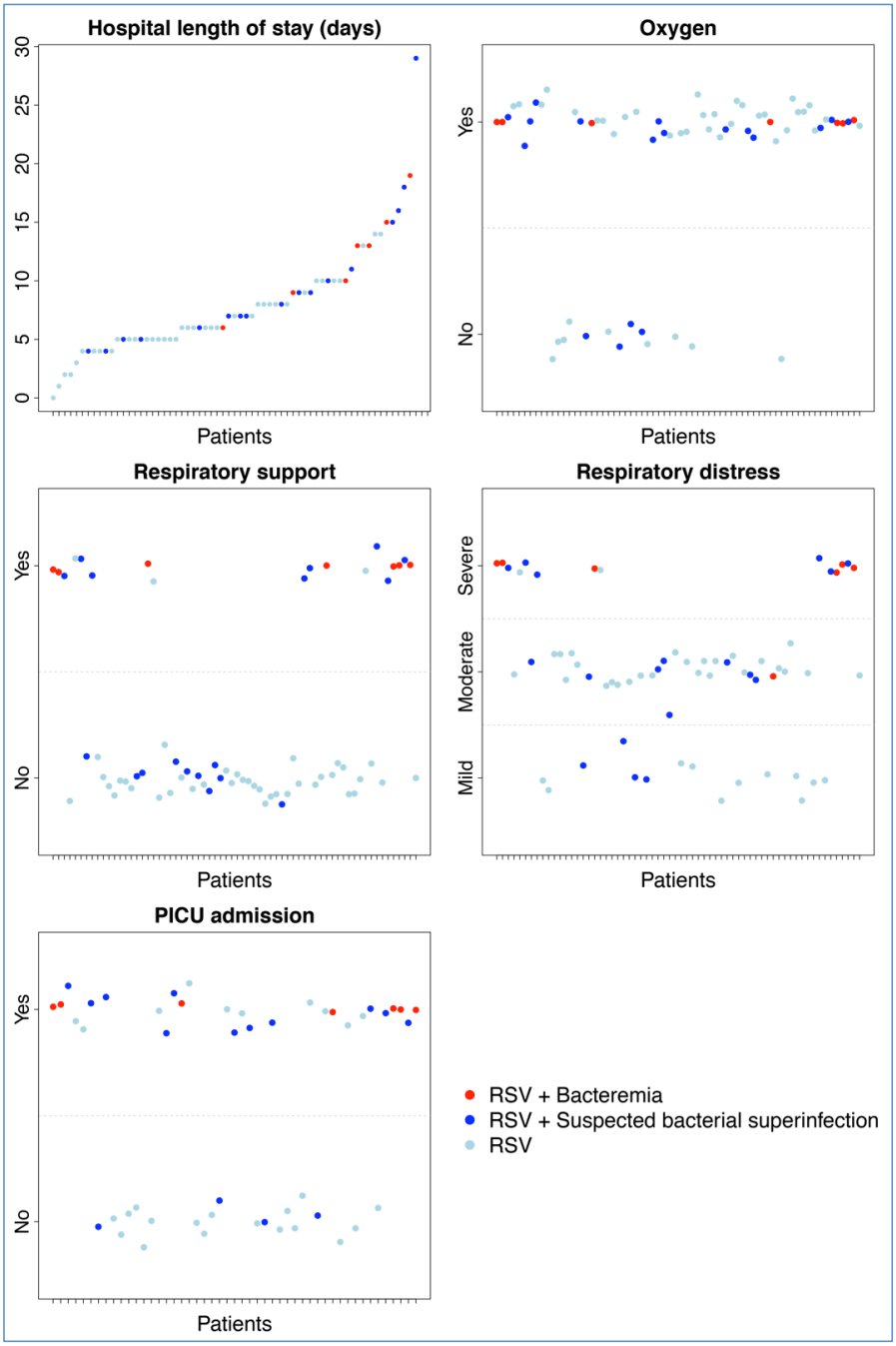

Figure 27. *Severity parameters for the patients: Wood Downes score, GENVIP score, length of hospitalization, oxygen, respiratory support, respiratory distress and PICU admission. Patients are classified as: positive RSV in nasopharyngeal sample, positive RSV with confirmed bacteremia, and positive RSV and suspected bacterial superinfection.*

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5 DISCUSSION

5.1 Molecular diagnostics

Multiple viruses are detected in at least one third of children hospitalized with LT-ARI. This rate reaches two thirds for patients in their second year of age. With the introduction of molecular techniques, the detection of multiple co-infecting viruses has become common [79], though the prevalence of each virus varies between studies. Our results show that viral co-infection is frequent, particularly in children above one year of age: children aged 12-24 months had the highest number of detected viruses, which may reflect slower clearance (and perhaps increased pathogenicity) following primary infection by a virus, and an immature immune system [7,93]. We observed that co-infection rates were lower in older children in both cohorts, despite this group

being prone to greater LT-ARI exposure through increased participation in shared childcare groups. This finding is inconsistent with two previous reports. In particular, Chorazy et al. [94] reported a non-significant increase in co-infection in children aged 6 - 12 months, and co-infection decreased after one year with increasing age. Peng et al. [95] reported that co-infection was more frequent in children between 3 - 6 years of age.

At least one respiratory pathogen was detected in 91.7% of the enrolled patients in the Spanish cohort and 87.2% in the UK cohort. This finding is in the upper end of the reported range in children (between 47% and 95%) [7,74,75]. Possible explanations for the wide differences in detection rates found in the literature include: (i) heterogeneity in studied populations (including genetic variability and predisposition), (ii) differences in respiratory symptoms at presentation (upper or lower respiratory symptoms), (iii) differences in the time of sampling, (iv) number of respiratory pathogens tested, and (v) the kind of diagnostic tests used [7,74,93]. Many patients had multiple respiratory viruses: 45.1% in the GENDRES cohort, and 29.9% in the UK cohort, which is again in the upper end of the reported range (17 - 41%) [7,96,97].

In 12 cases there was discordance between a negative PCR and a positive diagnostic pretest. This could be due to the different time of sampling, and/or it might be due to false positives, which are known to occur more frequently in rapid tests. We also found five negative samples (2.5%) for both PCR and pretest tested pathogens. These differences might be explained by the time and mode of collection of the samples. In some patients, the initial conventional viral test was performed on hospital admission samples, whilst

PCR was performed using samples obtained after the patients were transferred to PICU and recruited for the study.

Some viruses were mainly present as co-infecting agents (hRV, IV, hBoV, AdV and hMPV) and rarely found as single pathogens. As previously reported [7,98,99], RSV was the most frequent pathogen in both cohorts, especially in younger children. The second virus most frequently detected by PCR was hRV. The clinical significance of a positive hRV PCR assay has been questioned, given that hRV has been detected in asymptomatic children even two weeks after the clinical symptoms had disappeared [100,101]. However, hRV has been identified as single pathogen in some ARIs in children [79]. In our study, hRV was found in one third of samples and as single pathogen in approximately 10% of the cases.

In the GENDRES cohort, infection by both RSV and hRV was the most common viral co-infection detected, but in the UK cohort the most common viral co-infections were RSV + hBoV and IV + hBoV. These differences most likely reflect the fact that UK patients were recruited during the 2009 pandemic influenza season, but they may also reflect local differences in epidemiology and recruitment (including a higher proportion of PICU cases in the UK cohort).

Bocavirus is a recently discovered virus that may cause ARIs, particularly in children, with the highest frequency found in hospitalized infants. Our results indicate that hBoV is commonly detected in respiratory samples of young children with LT-ARI, in agreement with previous reports [62,102]. In our study, hBoV was the third most frequently identified virus in the GENDRES

cohort, after RSV and hRV, and the fourth in the UK cohort. Our detection rates in both the GENDRES and UK cohorts (23.5% and 19.6%, respectively) are higher than those in other published series, which have reported variable prevalence ranges of 1.5–19%. Methodological factors may explain these differences: our cohorts included only hospitalized children, whereas other studies included inpatients and outpatients [62,103]. hBoV was rarely found as a single infecting agent: in most cases (87.5%) it was found together with other respiratory viruses, as previously observed [60,102,104]. RSV, hRV, AdV and hMPV viruses were the most frequently observed co-pathogens, as observed by other authors [105-107].

In young children, hMPV is an important cause of bronchiolitis, accounting for 5 – 15% of all cases [10,59,108]. In our study we found 27 (13.2%) hMPV-positive samples in the GENDRES cohort. Of these, 66.7% of hMPV were detected as a co-infection with another respiratory virus, and 33.3% were found as a mono-infection. In the UK cohort four (4.1%) samples were hMPV-positive, including two with co-infection. Co-infection with hMPV has been proposed to increase disease severity in some studies [8,109,110], but not in others [111]. Dual infection with RSV is reportedly common, reflecting the overlapping seasonal distributions. One study reported that 70% of children with severe RSV bronchiolitis were co-infected with hMPV, suggesting that the disease caused by RSV may be augmented by a concurrent hMPV infection [110]. However, population-based and case control studies of hospitalized children have found that hMPV and RSV co-infections are uncommon [108,109]. In our study, the low proportion of mono-infected patients suggests that hMPV rarely produces clinically significant

infection by itself, but co-infection of hMPV with RSV was also uncommon (only two cases).

Bezerra et al. [112] have observed that AdV is frequently detected as part of a co-infection, in contrast with the findings of Huang et al. [113]. AdV was reported to be responsible for 5 - 10% of ARI in children [11]. Our detection rate ranged between 9.3% (UK cohort) and 19.1% (GENDRES cohort) with a median age of 21.8 months.

Our study detected a broad range of common respiratory pathogens but it was not exhaustive, and indeed it may have missed as yet undescribed respiratory pathogens. The study considered only children admitted with LT-ARI, and did not include milder or asymptomatic infections. Several studies have shown that viruses can be found in children with no ARIs [93,114], and further research is needed to understand the respiratory viral carriage and infection. Although quantification of the virus load by PCR is possible, respiratory samples are heterogeneous, and different extractions of the same patient can lead to diverse results depending on chance variation in the amount of virus present in the aliquots extracted. Whilst the robustness of our findings is supported by the broad similarity between the two independent cohorts analyzed in the present study, its applicability to other populations is likely to be influenced by local epidemiological and host genetic factors.

5.2 Severity of the illness

Our study revealed that even though multiple viral detection is frequent in hospitalized children with LT-ARI, this association is not related to either disease severity or to any other clinical features studied. PICU admission, disease severity according to different scales, need for respiratory support, and length of hospital stay followed a similar pattern in viral mono- versus co-infected children. Contrariwise, bacterial superinfection increased the severity of the disease course, while pneumococcal vaccination played a protective role.

The detection of multiple coincident viruses in clinical settings is becoming more common since the introduction of molecular based multiplex tests, but the clinical significance of these findings remains unclear and seems to have no impact in disease severity [115]. Both an increase in disease severity in relation to dual infections [93,97,116,117] and the absence of this association [7,74,94-96,99,105,111,118,119] have been reported. Richard et al. [116] found that co-infected children were almost three times more likely to be admitted to the PICU than those with single viral infections. Compared to our study Richard et al. developed a retrospective and monocentric study in which they only considered dual infections, infants and bronchiolitis.

There is contradictory evidence linking disease severity with specific respiratory viruses. A shorter hospital stay has been reported in children with rhinovirus bronchiolitis than with RSV [120]. Rhinovirus and RSV co-infection is reported to increase the risk of severe disease [116] or the bronchiolitis relapse [121,122]. Other studies did not find significant differences in severity

between co-infection and single infection [96,118,123,124]. In our study we did not find increased severity of illness in children with RSV-rhinovirus dual infection. In our series, only RSV as mono-infection increased oxygen requirements, and rhinovirus as a co-infecting pathogen increased the Wood-Downes score in the Spanish cohort, but these isolated findings arising from the multivariate analysis could not be replicated in the UK cohort.

Several studies have reported increased severity with bocavirus (hBoV) co-infections [9,105-107]; this was not the case in our series (also in agreement with Pen et al. [95]). hBoV was commonly detected in our patients, with no impact in the severity of the illness. As hBoV was detected in alongside other respiratory viruses with an established pathogenic potential, it is possible that hBoV detection reflects asymptomatic persistence or prolonged viral shedding [60].

Bacterial superinfection was the only factor consistently linked to greater severity. Studies of the pandemic influenza indicate that respiratory viruses predispose to bacterial complication and interaction between viruses and bacteria in respiratory infections has been extensively reported in the literature [78], but the underlying mechanisms between viral and bacterial synergism are complex and remain unclear [125]. Common respiratory viral infections, such as influenza or respiratory syncytial virus have been linked to seasonal increases in *Streptococcus pneumoniae* disease [126]. The relationship between bacterial and viral infection is clouded by the low sensitivity of bacterial detection in sterile-site samples by traditional culture methods, and the reliance on non-specific clinical data for the for diagnosis of bacterial co-infection, including inflammatory markers, radiological findings

and / or appropriate cultures, resulting in 30% of the cases in the GENDRES cohort and 55% in the UK cohort. Suspected or confirmed bacterial infection increased most measures of severity in both cohorts (PICU admission, respiratory support requirement, GENVIP score, hospital stay length and respiratory distress).

Interestingly, pneumococcal vaccination was revealed as an independent protective factor of disease severity in our patients. Pneumococcal vaccine reduced the severity of viral LT-ARIs through a reduction in oxygen requirement, invasive and non-invasive ventilation, admission to PICU, respiratory distress, and GENVIP score. A reduced incidence of viral alveolar pneumonia has been previously reported after pneumococcal vaccination [126,127], although there was no demonstrable reduction in the number of confirmed pneumococcal infections. This is likely to reflect the limited sensitivity of culture-proven pneumococcal disease in pneumonia.

One of the limitations of the present study is that our samples were not tested for viral load by quantitative PCR and the viral load of certain viruses – like RSV- has been associated with the co-infection status and the severity [128]. Also, the study did not consider milder or asymptomatic children. In addition, bacterial super-infection rate in our series might be overestimated as diagnosis was accepted as true even without microbiological confirmation, just based on referring physicians' criteria.

Several studies had shown that viruses can be found in children with no respiratory infections [93,129], and further research is needed to understand the natural history of respiratory viral carriage and infection. However, our

findings were consistent in both independent cohorts very different between them, so this makes the outcomes more robust.

5.3 Seasonal and geographical area distribution

Similarly to other studies [99,130], our findings show a higher prevalence of hMPV infections in the late winter months and spring. It is usually detected from January to March in the northern hemisphere and peak of the hMPV seasonal cases is observed between March and April following the RSV and influenza infection seasons although it was also reported that the hMPV infection season overlaps with that of the RSV infection season[58]. García-García [99] found a similar seasonal pattern of hMPV and RSV between the years 2000 - 2006, therefore this seasonal distribution seems unlikely to change from one year to another. As in our study, in an observational study developed by Gil-Prieto in Spain during the years 1997 - 2011, an important seasonality was observed in hospitalizations due to RSV, with 76% of the patients occurring between November and March. hBoV was found in a similar monthly distribution than in Calvo et al. [131] and VonLinstow et al [132], although the latter was a study in non-hospitalized Danish infants under 1 year old.

Parainfluenzavirus is a common cause of respiratory illness and their seasonal epidemiology depends on the type; hPIV 1-2 has been reported to occur biennially usually during fall and early winter, hPIV 3 is endemic throughout the year but with peaks in April-May and hPIV 4 more irregularly and seldom [133].

5.4 Bacteremia

One out of every ten previously healthy children hospitalized due to RSV had bacteremia. These patients experienced a more severe disease and half of them had received empirical antibiotic therapy.

The prevalence of bacteremia in children with RSV infection reported in the literature is low, ranging between 0.6 and 1.1% [65-67,134-136]. Our study found rates of concurrent bacteremia ten times higher (10.6%). In the studies cited, only conventional cultures were performed, whereas molecular methods were applied in our series. RSV has been linked to seasonal increases in *S. pneumoniae* disease [126], as well as to other viruses such as influenza, but the underlying mechanisms between viral and bacterial synergism are complex and remain unclear. Immunization programs with conjugate vaccines against invasive *H. influenzae* serotype b and *Streptococcus pneumoniae* have changed the frequency of bacteremia in febrile infants [126,134]. In our series, only 14.3% of the PCR positive patients had been vaccinated against pneumococcal disease, as compared to PCR negative ones, with 52.5% having received the vaccine.

The diagnosis of bacterial superinfection is most often made on clinical grounds, and not always confirmed microbiologically. Antibiotics should not be administered to RSV-infected children unless complications such as secondary bacterial illness occur [137,138]. In our series, blood cultures were not carried out systematically, but only when a bacterial superinfection was suspected by the referring physician. Blood culture is considered the gold standard for bacteria detection, but has a low sensitivity and some bacteria

are difficult to culture. Blood PCR –much more sensitive– was performed in all the recruited patients but the clinical relevance of its positive result is not clear. Detecting circulating DNA in the blood by PCR has some limitations and it is essential to extrapolate always the results obtained to the clinical grounds to see if both PCR results and the clinical phenotype are consistent [139]. The quantitative PCR and the cycle threshold value are inversely correlated with the bacteria load and could be an indicator to avoid false positive results. In our cohort the cycle threshold was <20, but as we only have seven positive samples we could not establish maximum cutoff levels.

In our series, the physician suspected bacterial superinfection in half of the cases, a proportion similar to that in Thibeault et al.'s study [140]. Even though a blood culture was obtained for the majority of our patients (72.7%), only 3.8% (*n* = 1) yielded a positive result. Furthermore, we failed to find any correlation between PCR-confirmed bacteremia and clinical suspicion of bacterial superinfection (Cohen's kappa coefficient = 0.15). Therefore, in agreement with other studies [136,141], we found that children with uncomplicated bacterial RSV infection are often overtreated with empirical antibiotics.

Empirical antibiotic treatment is often prescribed in practice to children hospitalized due to a confirmed viral ARI based on fever presence or persistence. Between 23 and 31% of cases of bronchiolitis are associated with fever [142], and in our series the fever rate (37 and 50%) was in the range previously described of 45 to 65% for children hospitalized with RSV [143]. However, the risk of bacteremia is low in febrile children with

bronchiolitis [66,68,142], as corroborated in our series, where fever was not a predictor for bacteremia (*P*-value = 0.733).

Randolph et al. [144] studied the risk of bacterial infection in children infected with RSV admitted to PICU, and using blood culture they found a rate of bacteremia of only 0.6%. In our study, the rate of bacteremia found by PCR in children who required PICU admission is higher (25.0%), probably due to the higher sensitivity of molecular techniques. Bacteremic patients had a more severe course according to PICU admission rates, respiratory support necessity, clinical scales and length of hospitalization. Bloomfield et al. [136] suggest that empirical antibiotics should be considered for any child admitted to PICU with a RSV infection and requiring ventilator. In or series only half the patients who were admitted to PICU and required ventilatory support had a bacteremia revealed by PCR, meaning that antibiotic prescription might be superfluous in up to 50% of the cases.

6 CONCLUSIONS

1. The use of molecular techniques -namely multiplex nested PCR- in the diagnostic approach of children admitted to hospital with ARI significantly increases the detection yield of viruses in children.

2. The multiplex nested PCR used in this study provides a sensitive and specific approach to diagnose the most common and important viruses involved in respiratory infections.

3. RSV is the pathogen most frequently found in children hospitalized with acute respiratory infection, being present from one third to one half of the cases.

4. Co-infection with multiple viruses is very frequent in children hospitalizaed with acute respiratory infection, being found in 30 to 45% of the cases.

5. Co-infection with multiple viruses increases with age up to 48 months of age, being most frequent in the 12-24 months age group.

6. The virus co-infection pattern most frequently observed is RSV-Rhinovirus and RSV-bocavirus/bocavirus-influenza.

7. The pattern of viral co-infection did not correlate with any marker of disease severity.

8. The presence of more than one virus in children hospitalized with acute respiratory infection is very frequent although the clinical significance of this finding remains unclear.

9. The presence of more than one virus in hospitalized children with ARI is very frequent but it does not seem to have a major clinical impact in terms of severity.

10. Bacterial superinfection was associated with increased severity according to PICU admission rate, clinical score, need of respiratory support and length of hospital stay.

11. Pneumococcal vaccination was found to be a protective factor in terms of severity according to degree of respiratory distress, PICU admission rate, clinical score, need of respiratory support and oxygen necessity.

12. In previously healthy children with a positive RSV respiratory illness, a bacteria is found in one of each ten cases.

13. Concurrent bacteremia is not frequent in infants and children hospitalized with RSV respiratory infection, even in the presence of fever and despite the use of molecular techniques for the diagnosis, and thus, antibiotics are usually overused in the setting of RSV infection.

14. RSV infected children with bacteremia had more severe disease than those without bacteremia, according to PICU admission rate, need of respiratory support, Wood-Downes score, GENVIP scale and length of hospitalization.

15. Bacteremia may actually occur in children with RSV infection and we have not found a reliable predictive clinical pattern, although our sample size is limited to draw definite conclusions in this regard.

16. Further studies assessing co-infection in children with mild illness and healthy control groups are needed in order to better understand its clinical relevance.

17. Future studies are needed to investigate whether particular viruses, or combinations of virus, influence the risk of bacterial co-infection.

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7 FUTURE CHALLENGES

The findings of this thesis could contribute to the ongoing discussion of the importance of diagnostic ability to reliably detect multiple concurrent pathogens in a single patient. The multiplex PCR in nasopharyngeal samples and in blood, searching in this one for bacterial infection, provides useful information on the etiology of the respiratory infections. The use of this method also provides important information to better understand the epidemiology of respiratory infections as well as for infection control. One advantage of the use of the multiplex PCR method is that it may reduce antibiotic prescription rates at medical attendance in children with ARI, in an inpatient or even in an outpatient setting.

It is important to keep in mind that genome detection methods (as PCR) detect only the specific gene sequence of the microbial agent that the primers are designed to bind to. If the agent has mutated in such a way that it affects the gene sequence targeted, no amplification will occur and the analysis will be a false negative. However, in order to avoid this, the primers of all agents are targeted at a conserved region of the genome. It can also occur that a virus or a bacterium is not detected because it is not included in the PCR panel but may be present in the patient sample. This may happen especially in new or not discovered virus.

Consequently with the still open questions of this study, new lines of research have been developed:

For further studies, it would be interesting to undertake a large study including control samples. A large prospective study, investigating the prevalence of pathogens in samples from children with no respiratory symptoms would be very useful, and may put some of our results into context in terms of the likelihood of the particular pathogens being causative in each case.

Another area of interest would be to perform the same panel of 19 viruses to mild symptomatic children, and compare the PCR results, in the same epidemic period, with those who are admitted to hospital.

We also have developed genetic studies in which we aim to identify the genes that determine susceptibility and severity in respiratory infections of childhood. We will use RSV bronchiolitis as the prototypic model to develop an integrated staged approach to identify the genetic basis of both susceptibility to infection and severity of disease in those affected, as it is the

most prevalent ARI in children, and then apply this model to the other major respiratory infections of childhood. In this research line, extreme phenotypes of RSV infection are going to be studied. With this propose, we are going to carry out whole exome sequencing (WES) that permits analysis of the DNA sequence for all protein-coding areas of the human genome. By comparison of patients' DNA sequence to the reference genome, we can identify the DNA changes (variants) present in that individual that may have functional consequences.

Another research line is to study the transcriptome of RSV. Next generation sequencing (NGS) is capable of sequencing, in parallel and massively, millions of cDNA fragments in a single sequencing process rapidly and relatively inexpensively. Massive sequencing techniques of total RNA (RNA-Seq) offer the opportunity to obtain global information of transcriptomic status of a specific tissue, or even a single cell, not only providing information about gene expression levels but also allowing the identification of alternative splicing events, unknown transcripts, processes of gene fusion or identification of mutations simultaneously.

Finally, we would like to study in future studies drugs that could modify the respiratory illness and vaccines to prevent them. In this regard, we are involved in clinical trials with new RSV drugs and vaccines. In the other hand, we are investigating the potential protective role of vitamin D in respiratory infections. It is known that vitamin D is known to play a major role in calcium metabolism and bone health, stimulating intestinal absorption of calcium and phosphorus, and regulating serum calcium levels in order to maintain an adequate mineralization of the bones. But in recent years, aside from this

main role, observational studies suggest that low levels of vitamin D may contribute to an increased risk of many different diseases, including ARI. As the relation between levels of vitamin D and severity of the respiratory episode is not yet clear, our aim is to prospectively assess the influence of vitamin D levels in infants admitted to hospital due to an ARI and its possible relationship with the severity of involvement

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9 APPENDIX MATERIAL

Appendix Figure 1. GENDRES informed consent used for this study.

Appendix Figure 2. GENDRES case reported form used for the study.

Appendix Table 1. Demographic characteristics, family and patient medical history, clinical course and principal virus in children with ARI and disease severity, considering respiratory support and oxygen requirement the characteristics that described the severity of the illness of the main cohort. A binary logistic model was used. Data are presented as OR (95% confidence interval) and the level of statistical significance was set at 0.05. Two multiple test correction were considered: Bonferroni correction and FDR

Appendix Table 2. Variables analyzed in children with ARI and disease severity, considering the clinical scales the characteristics that described the severity of the illness of the main cohort. A linear model for continuous variables was used and the level of statistical significance was set at 0.05. Two multiple test correction were considered: Bonferroni correction and FDR.

Appendix Table 3. Demographic characteristics, family and patient medical history, clinical course and main virus in children with ARI and disease severity in GENDRES cohort. A binary logistic model was used for the binary variable (PICU admission) and a negative binomial regression model for counted data (hospital stay length). Data are presented as OR (confidence interval 95%) and the level of statistical significance was set at 0.05. Two multiple test correction were considered: Bonferroni correction and FDR.

Appendix Table 4. Children's with ARI characteristics and moderate and severe respiratory distress. A logistic multinomial model was used and mild status was fixed as category of reference. Level of statistical significance was set at 0.05.

Appendix Table 5. Comparison of virus and disease severity of the main cohort considering the virus as single pathogen or as co-infection in the sample. Different statistical models were considered to study the bivariate association between the variables depending on the dependent variable. A binary logistic model was used for the binary variables oxygen needed and respiratory support needed, and a negative binomial regression model for counted data (hospital stay length). Data are presented as OR (confidence interval 95%) and the level of statistical significance was set at 0.05

Appendix Table 6. Comparison of virus and disease severity of the replication cohort (UK cohort) considering the virus as single pathogen or as co-infection in the sample. Different statistical models were considered to study the bivariate association between the variables depending on the dependent variable. A binary logistic model was used for the binary variables oxygen needed and respiratory support needed, and a negative binomial regression model for counted data (hospital stay length). Data are presented as OR (confidence interval 95%) and the level of statistical significance was set at 0.05.

Appendix Table 7. Demographic characteristics, clinical course and main virus in children with ARI and disease severity, considering respiratory support and oxygen requirement the characteristics that described the severity of the illness of the UK-cohort are presented. A binary logistic model was used. Data are presented as OR (95% confidence interval) and the level of statistical significance was set at 0.05.

Appendix Table 8. Variables analyzed in the UK-cohort children and disease severity according to hospital stay length and PICU admission are shown. A binary logistic model was used for the binary variable (PICU admission) and a negative binomial regression model for counted data (hospital stay length). Data are presented as OR (confidence interval 95%) and the level of statistical significance was set at 0.05.

Appendix Figure 1

Proyecto-Gen-D-Res: Evaluación de la influencia del componente genético y de los niveles de vitamina D en la susceptibilidad individual y pronóstico de la infección por virus influenza H1N1 y otros virus respiratorios.

Consentimiento Informado

Si ha comprendido la información que se le ha proporcionado, ha resuelto cualquier duda que pudiese tener y decide que su hijo/a colabore con este estudio de investigación en los términos indicados en la hoja de información que se le entrega junto con este consentimiento, por favor, lea y firme a continuación.

Al firmar este documento, acepta que su hijo/a participe en este estudio de investigación y otorga permiso para que se utilice la información de su hijo/a de acuerdo a la legislación de protección de datos vigente, sin renunciar a ninguno de los derechos legales que le corresponde a su hijo.

1. Confirmo que he leído y entendido la hoja de información para padres/tutor legal (versión 2.0, del 31 de octubre de 2014) para el estudio Gen-D-Res y que se me ha dado una copia del presente documento para guardar. He tenido la oportunidad de preguntar mis dudas y estas han sido respondidas satisfactoriamente. ☐
2. Entiendo que la participación de mi hijo/a es voluntaria y que soy libre de retirar mi consentimiento en cualquier momento sin necesidad de dar ninguna explicación y sin que la atención de mi hijo/a o sus derechos se vean afectados en modo alguno. ☐
3. Comprendo que los datos personales y clínicos recogidos con motivo del estudio en relación con mi hijo/a serán exclusivamente manejados por personal responsable del estudio y preparado para hacerlo, garantizando la protección de estos datos de acuerdo a la ley vigente (Ley 15/1999). Doy mi permiso para que estas personas, y en las condiciones señaladas, puedan acceder al historial clínico de mi hijo/a. ☐
4 Acepto que las muestras obtenidas de mi hijo/a de sangre, saliva y moco se utilicen en este proyecto de acuerdo a lo descrito en la hoja de información al paciente ☐
5. Acepto que las muestras obtenidas de mi hijo/a en este estudio puedan ser utilizadas en otros estudios futuros siempre que hayan sido aprobados por un comité ético y se garanticen al menos las mismas condiciones que en el presente estudio. ☐
6. Acepto que mi hijo/a participe en este proyecto de investigación. ☐

_____
Nombre del sujeto participante

_____	_____	_____
Nombre del padre/tutor	Firma	Fecha
_____	_____	_____
Nombre de la madre/tutora	Firma	Fecha

Si solo un progenitor firma este documento, por favor, complete la siguiente casilla:

☐ Confirmo con la presente que el otro progenitor no se opone a la participación de nuestro hijo/a en el estudio.
☐ El firmante es el único tutor legal.

_____	_____	_____
Nombre del facultativo	Firma	Fecha

Appendix Figure 2

PROYECTO GEN-D-RES

Cuestionario de datos GENDRES

Pegatina paciente

Nº centro		Nº paciente		Código paciente web	
Score		Si		No	

Datos de filiación general y genética

Nº historia				**Sexo**		Hombre	Mujer
Fecha de nacimiento				**Edad (años/meses)**			
Etnia		Europeo occidental		Gitana		Americano Sur	
		Africano-Subsahariano		Norteafricano			
Provincia nacimiento paciente							
Provincia nacimiento padre				**Provincia nacimiento madre**			
Atención médica recibida		Atención		Ingreso	**Fecha de la atención / ingreso**		

Antecedentes familiares

Asma		Si		No
Problemas respiratorios		Si		No Especificar
Recibió madre durante embarazo suplem de vit. D		Si		No

Antecedentes personales

Algún episodio previo		Si		No	(bronquiolitis, hiperreact. bronquial, dificul. resp. baja)
Otra enferm. pulmonar crónica		Si		No	Especificar
Prematuro		Si		No	Especificar
Otros		Si		No	Especificar
Recibe suplemento de vit D		Si		No	Especificar dosis/duración
Otros ingresos de interés		Si		No	Especificar
Otros tratamientos de interés		Si		No	Especificar
Asma		Si		No	
Diabetes		Si		No	
Obesidad		Si		No	

Datos clínicos

Motivo de atención/ingreso								
Afectación respiratoria		Ninguna		Leve		Moderada		Grave
Score Wood-Downes	(0-10)							
Necesitó oxígeno		No		Si				
Necesitó soporte respiratorio		No		Ventilación no invasiva		Ventilación mecánica		

Datos diagnósticos

Diagnóstico		Bronquiolitis		Neumonía		Broncoespasmo		Infección vías altas
Otros diagnósticos		Reagudización asma		OMA		HRB		Otros. Especificar
Agente etiológico		VRS		Influenza H1N1		Influenza Otros. Especificar:		
		Otro agente. Especificar				No identificado		No pruebas microbiologicas
Sobreinfección bacteriana		Si		No	Especificar:			

Datos evolutivos

Vacunado		Gripe estacional		Antineumocócica	Especificar:		7		10	13
Tratamiento antivírico		Si		No	Especificar					
Tratamiento antibiótico		Si		No	Especificar					
Ingreso hospitalario		Si		No	Duración del ingreso (días)					
Evolución		Alta		Sin secuelas		Con secuelas. Especificar				
		Éxitus	Especificar				Cita CAP		Atención en urgencias	

Otros datos relevantes o de interés

Variable	Respiratory support (n = 203)				Oxygen needed (n = 203)			
	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P* - value	OR (95% CI)	*P*- value	Multiple OR (95% CI)	*P* -value
Demographic characteristics								
Sex (female proportion)	0.470 (0.191, 1.155)	0.100	0.463 (0.156, 1.216)	0.136	0.766 (0.416, 1.410)	0.392	0.709 (0.370, 1.361)	0.300
Age								
13 - 24 months					1.217 (0.498, 2.972)	0.667	1.421 (0.557, 3.829)	0.471
25 - 48 months					0.649 (0.243, 1.731)	0.388	3.532 (1.257, 11.237)	0.022
> 48 months					0.983 (0.322, 3.005)	0.976	1.756 (0.556, 6.269)	0.354
Family history								
Asthma	1.181 (0.539, 2.586)	0.678			1.500 (0.806, 2.793)	0.201		
Respiratory conditions	1.790 (0.695, 4.610)	0.228			1.158 (0.502, 2.671)	0.731		
Patient medical history								
Premature birth	2.159 (0.641, 7.269)	0.214			0.527 (0.186, 1.492)	0.228		
Pulmonary conditions	2.489 (0.460, 13.481)	0.290			0.325 (0.071, 1.499)	0.325		
Asthma	1.177 (0.372, 3.721)	0.782			2.458 (0.804, 7.518)	0.115		
Pneumococcal vaccine	**0.443 (0.199, 0.987)**	**0.046**	**0.324 (0.124, 0.790)**	**0.016**	**0.374 (0.199, 0.704)**	**0.002**♭#	**0.328 (0.163, 0.639)**	**0.001**
Clinical data								
Bacterial superinfection	**6.368 (2.724, 14.886)**	**<0.001**♭#	**7.484 (3.113, 19.254)**	**<0.001**	0.713 (0.367,1.383)	0.316		
Co-infection	1.859 (0.796, 4.346)	0.152			1.119 (0.597, 2.096)	0.726		
Virus								
RSV	1.630 (0.732, 3.629)	0.231			1.831 (1.003, 3.340)	0.049	2.040 (1.054, 3.998)	0.035
Rhinovirus	1.981 (0.902, 4.351)	0.809			0.979 (0.520, 1.840)	0.947		
Bocavirus	0.848 (0.302, 2.382)	0.755			1.123 (0.553, 2.280)	0.749		
Adenovirus	0.980 (0.392, 2448)	0.965			1.325 (0.600, 3.927)	0.486		

Appendix Table 1

Appendix Table 2

Variable	GENVIP score (n = 136)				Wood Downes score (n = 192)			
	Coefficient (95% CI)	*P*-value	Multiple coefficient (95% CI)	*P*-value	Coefficient (95% CI)	*P*-value	Multiple coefficient (95% CI)	*P*-value
Demographic characteristics								
Sex (female proportion)	-0.749 (-2.006, 0.508)	0.241	-0.469 (-1.710, 0.774)	0.457	-0.158 (-0.783, 0.467)	0.619	-0.080 (-0.713, 0.554)	0.804
Age								
13 - 24 months	-1.223 (- 2.990, 0.545)	0.174	-1.067 (-2.903, 0.769)	0.252	-0.454 (-1.380, 0.472)	0.335	-0.429 (-1.375, 0.497)	0.356
25 - 48 months	-1.744 (-4.087, 0.600)	0.144	0.032 (-2.410, 2.474)	0.980	-0.707 (-1.618, 0.203)	0.127	-0.694 (-1.613, 0.224)	0.138
> 48 months	1.023 (-1.946, 3.994)	0.497	3.274 (-0.148, 6.696)	0.061	-0.287 (-1.391, 0.816)	0.608	-0.283 (-1.390, 0.825)	0.615
Family history								
Asthma	-0.686 (-0.589, 1.960)	0.289			0.410 (-0.196, 1.017)	0.184		
Respiratory conditions	1.583 (0.068, 3.099)	0.041	1.168 (-0.391, 2.726)	0.141	0.002 (-0.807, 0.811)	0.995		
Patient medical history								
Premature birth	1.131 (-1.337, 3.599)	0.366			0.147 (-1.052, 1.347)	0.809		
Pulmonary conditions	-1.008 (-4.259, 2.244)	0.541			-0.471 (-2.069, 1.127)	0.561		
Asthma	-0.213 (-2.165, 1.739)	0.829			0.298 (-0.612, 1.207)	0.519		
Pneumococcal vaccine	**-1.510 (-2.714, -0.305)**	**0.014**	**-1.499 (-2.768, -0.231)**	**0.021**	-0.568 (-1.164, 0.029)	0.062		
Clinical data								
Bacterial superinfection	**2.124 (0.864, 3.385)**	**0.001**[‡]	**1.988 (0.737, 3.238)**	**0.002**	0.678 (-0.010, 1.366)	0.053		
Co-infection	-0.095 (-1.360, 1.171)	0.882			0.155 (-0.472, 0.783)	0.626		
Virus								
RSV	0.624 (-0.618, 1.865)	0.322			-0.006 (-0.610, 0.597)	0.984		
Rhinovirus	0.129 (-1.178, 1.426)	0.845			0.355 (-0.287, 0.997)	0.277		
Bocavirus	-0.881 (-2.331, 0.569)	0.232			-0.432 (-1.152, 0.287)	0.237		
Adenovirus	-0.573 (-2.231, 1.085)	0.495			0.235 (-0.535, 1.005)	0.548		

Variable	Hospital stay length (*n* = 180)				PICU admission (*n* = 131)			
	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P*-value
Demographic characteristics								
Sex (female proportion)	0.954 (0.809, 1.125)	0.578	1.010 (0.864, 1.181)	0.901	0.777 (0.342, 1.766)	0.546	0.901 (0.343, 2.266)	0.827
Age								
13 - 24 months	0.863 (0.675, 1.101)	0.236	0.944 (0.737, 1.206)	0.643	0.645 (0.196, 2.118)	0.470	0.858 (0.170, 3.417)	0.836
25 - 48 months	0.650 (0.502, 0.839)	<0.001	0.690 (0.515, 0.918)	0.011	0.323 (0.038, 2.735)	0.300	0.938 (0.046, 6.839)	0.956
> 48 months	1.030 (0774, 1.374)	0.838	1.152 (0.833, 1.589)	0.393	2.258 (0.304, 16.770)	0.426	2.529 (0.280, 23.037)	0.378
Family history								
Asthma	0.869 (0.737,1.024)	0.094			1.688 (0.773, 3689)	0.189		
Respiratory conditions	1.083 (0.869, 1.350)	0.480			0.941 (0.357, 2.477)	0.902		
Patient medical history								
Premature birth	1.340 (0.995, 1.815)	0.556			1.161 (0.333, 4.047)	0.814		
Pulmonary conditions	1.170 (0.787, 1.752)	0.440			1.648 (0.264, 10.281)	0.593		
Asthma	0.875 (0.679, 1.128)	0.303			1.053 (0.258, 4.307)	0.943		
Pneumococcal vaccine	0.849 (0.725, 0.994)	0.042	0.874 (0.747, 1.022)	0.091	**0.368 (0.169, 0.804)**	**0.012**	**0.301 (0.116, 0.735)**	**0.011**
Clinical data								
Bacterial superinfection	**1.480 (1.261, 1.737)**	**<0.001**♭#	**1.468 (1.257, 1.715)**	**<0.001**	**2.851 (1.300, 6.252)**	**0.009**	**3.342 (1.438, 8.093)**	**0.006**
Co-infection	0.914 (0.775, 1.078)	0.287			0.847 (0.381, 1.885)	0.684		
Virus								
RSV	1.095 (0.933, 1.286)	0.266			1.133 (0.520, 2.468)	0.754		
Rhinovirus	1.071 (0.905, 1.268)	0.425			1.514 (0.689, 3.326)	0.302		
Bocavirus	0.769 (0.634, 0.931)	0.007	0.904 (0.741, 1.101)	0.316	0.643 (0.237, 1.744)	0.386		
Adenovirus	0.930 (0.757, 1.143)	0.490			0.855 (0.285, 2.566)	0.780		

Appendix Table 3

Appendix Table 4

Variable	Respiratory distress			
	Moderate		Severe	
	Coefficient (95% CI)	*P*-value	Coefficient (95% CI)	*P*-value
Demographic characteristics				
Sex (female proportion)	1.060 (0.557, 2.018)	0.860	0.693 (0.248, 1.933)	0.483
Family history				
Asthma	2.274 (0.467, 3.473)	0.636	1.262 (0.289, 5.517	0.757
Respiratory conditions	1.000 (0.419, 2.385)	1.000	1.462 (0.434, 4.923)	0.540
Patient medical history				
Premature birth	0.666 (0.201, 2.205)	0.666	2.063 (0.500, 8.514)	0.317
Pulmonary conditions	0.237 (0.042, 1.334)	0.102	0.598 (0.063, 5.642)	0.598
Asthma	1.274 (0.467, 3.473)	0.636	1.262 (0.289, 5.517)	0.757
Pneumococcal vaccine	1.212 (0.644, 2.280)	0.552	2.917 (1.078, 7.889)	0.035
Clinical data				
Bacterial superinfection	0.877 (0.420, 1.831)	0.727	4.356 (1.564, 12.128)	0.005
Co-infection	0.915 (0.477, 1.756)	0.790	1.615 (0.570, 4.578)	0.367
Virus				
RSV	0.858 (0.459, 1.604)	0.632	1.406 (0.532, 3.718)	0.492
Rhinovirus	1.251 (0.634, 2.469)	0.519	1.765 (0.657, 4.739)	0.260
Bocavirus	1.036 (0.498, 2.152)	0.925	0.643 (0.188, 2.199)	0.481
Adenovirus	1.404 (0.606, 3.248)	0.429	1.111 (0.307, 4.024)	0.873

Risk Factor	Hospital stay length (n = 163)		Oxygen needed (n = 186)		Respiratory support (n = 186)		Wood Downes score (n = 177)		GENVIP score (n = 125)		PICU admission (n = 119)	
	OR (95% CI)	*P* - value	OR (95% CI)	*P* - value	OR (95% CI)	*P* – value	Coefficient (95% CI)	*P* - value	Coefficient (95% CI)	*P* - value	OR (95% CI)	*P* - value
Mono-infected												
RSV	1.092 (0.868, 1.374)	0.450	3.154 (1.302, 7.966)	0.012	3.556 (0.832, 24.487)	0.122	0.571 (-0.284,1.425)	0.188	1.384 (-0.307, 3.075)	0.107	1.121 (0.362, 3.728)	0.846
Rhinovirus	0.785 (0.562, 1.094)	0.154	0.327 (0.109, 0.962)	0.042	1.167 (0.165, 5.257)	0.854	-0.823 (-1.932, 0.285)	0.144	-1.134 (-4.095, 1.826)	0.447	1.410 (0.330, 5.410)	0.623
Bocavirus	0.999 (0.646, 1.548)	0.997	0.435 (0.076, 2.482)	0.327			-0.187 (-2.270, 1.897)	0.859	-2.610 (-5.840, 0.619)	0.111		
Adenovirus	1.211 (0.733, 2.019)	0.456					0.859 (-1.217, 2.935)	0.413	1.581 (-4.841, 8.002)	0.624		
Co-infected												
RSV	1.150 (0.892, 1.481)	0.281	0.938 (0.361, 2.364)	0.892	1.550 (0.509, 5.328)	0.456	-0.222 (-1.217, 2.935)	0.646	0.243 (-1.829, 2.316)	0.815	1.426 (0.402, 5.887)	0.597
Rhinovirus	1.443 (1.135, 1.836)	0.003	1.642 (0.658, 4.158)	0.288	2.921 (0.923, 11.199)	0.085	1.289 (0.387, 2.192)	0.006	0.920 (-1.157, 2.997)	0.379	2.169 (0.104, 1.398)	0.146
Bocavirus	0.697 (0.551, 0.883)	0.003	1.243 (0.499, 3.168)	0.642	0.889 (0.290, 2.632)	0.832	-0.654 (-1.590, 0.282)	0.168	-0.551 (-2.583, 1.481)	0.589	1.174 (0.352, 3.910)	0.794
Adenovirus	0.938 (0.730, 1.204)	0.613	1.000 (0.396, 2.604)	1.000	0.682 (0.198, 22.080)	0.515	0.290 (-0.677, 1.257)	0.553	-0.848 (-2.947, 1.252)	0.422	1.426 (0.383, 5.305)	0.597

Appendix Table 5

Appendix Table 6

Variable	PICU admission (n = 97)		Respiratory support (n = 94)		Oxygen needed (n = 95)		Hospital stay length (n = 94)	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Mono-infected								
RSV	1.938 (0.658, 5.877)	0.233	1.385 (0.449, 4.264)	0.568	3.200 (1.000, 11.614)	0.059	0.789 (0.430, 1.478)	0.449
Rhinovirus	2.132 (0.536, 9.330)	0.287	2.000 (0.487, 8.270)	0.327	7.200 (1.203, 138.501)	0.072	0.573 (0.264, 1.375)	0.181
Bocavirus	0.389 (0.019, 3.265)	0.427	0.561 (0.027, 4.745)	0.628	0.594 (0.067, 5.279)	0.617	0.822 (0.290, 3.122)	0.739
Adenovirus	2.609 (0.236, 58.189)	0.445	1.789 (0.068, 47.003)	0.687	0.606 (0.023, 15.898)	0.728	2.224 (0.750, 10.046)	0.210
Co-infected								
RSV	0.804 (0.174, 3.574)	0.774	0.444 (0.085, 2.093)	0.313	1.400 (0.310, 6.604)	0.662	0.667 (0.327, 1.382)	0.267
Rhinovirus	0.635 (0.136, 2.803)	0.551	0.833 (0.173, 3.916)	0.816	1.400 (0.310, 6.604)	0.662	1.324 (0.649, 2.724)	0.440
Bocavirus	0.700 (0.153, 3.123)	0.638	1.200 (0.255, 5.791)	0.816	0.389 (0.076, 1.779)	0.234	0.523 (0.260, 1.035)	0.064
Adenovirus	0.218 (0.010, 1.640)	0.194	0.220 (0.011, 1.683)	0.199	0.286 (0.034, 1.804)	0.198	0.697 (0.301, 1.788)	0.423

Variable	Oxygen needed (n = 95)				Respiratory support (n = 94)			
	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P*-value
Sex (female proportion)	0.734 (0.321, 1.661)	0.458	0.860 (0.338, 2.190)	0.749	1.197 (0.521, 2.772)	0.671	1.268 (0.478, 3.403)	0.633
Age								
13 - 24 months	0.179 (0.047, 0.606)	0.008	0.187 (0.045, 0.692)	0.015	0.238 (0.048, 0.887)	0.046	0.327 (0.059, 1.433)	0.158
25 - 48 months	0.720 (0.2145 2.522)	0.596	0.714 (0.187, 2.816)	0.623	0.396 (0.109, 1.286)	0.136	0.593 (0.142, 2.283)	0.454
> 48 months	0.360 (0.120, 1.045)	0.063	0.455 (0.129, 1.547)	0.211	0.507 (0.169, 1.443)	0.210	0.251 (0.055, 0.967)	0.055
Pneumoccocal vaccine	0.543 (0.215,1.320)	0.184			0.372 (0.147, 0.913)	0.032	0.267 (0.070, 0.901)	0.040
Bacterial superinfection	4.151 (1.778, 10.112)	0.001	3.842 (1.541, 10.058)	0.005	3.432 (1.434, 8.699)	0.007	3.218 (1.230, 8.898)	0.020
Co-infection	0.824 (0.326, 2.100)	0.681			1.203 (0.462, 3.088)	0.701		
Virus								
RSV	2.500 (1.047,6.297)	0.044	2.295 (0.780, 7.204)	0.138	0.925 (0.385, 2.181)	0.859		
Rhinovirus	2.535 (0.935, 7.701)	0.079			1.331 (0.503, 3.459)	0.557		
Bocavirus	0.587 (0.210, 1.620)	0.302			1.031 (0.345, 2.925)	0.954		
Influenza	0.462 (0.174, 1.190)	0.112			0.692 (0.239, 1.858)	0.476		

Appendix Table 7

Appendix Table 8

Variable	PICU admission (n=97)				Hospital stay length (n=94)			
	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	Multiple OR (95% CI)	*P*-value
Sex (female proportion)	1.150 (0.515, 2.579)	0.733	1.396 (0.499,4.014)	0.526	1.228 (0.784, 1.921)	0.368	1.481 (0.952, 2.311)	0.073
Age								
13 - 24 months	0.120 (0.024, 0.442)	0.003	0.114 (0.018, 0.533)	0.020	0.524 (0.280, 1.011)	0.047	0.526 (0.284, 1.000)	0.046
25 - 48 months	0.233 (0.063, 0.765)	0.021	0.239 (0.051, 0.982)	0.055	1.367 (0.753, 2.576)	0.315	1.409 (0.790, 2.597)	0.254
> 48 months	0.400 (0.137, 1.121)	0.085	0.127 (0.022, 0.551)	0.010	0.935 (0.540, 1.645)	0.813	0.837 (0.487,1.455)	0.519
Pneumoccocal vaccine	0.324 (0.129, 0.786)	0.014	**0.208 (0.046, 0.776)**	**0.027**	0.755 (0.461, 1.216)	0.255		
Bacterial superinfection	4.571 (1.947, 11.350)	0.001	**5.864 (2.122, 18.063)**	**0.001**	1.663 (1.068, 2.583)	0.024	**1.865 (1.198, 2.902)**	**0.005**
Co-infection	0.875 (0.348, 2.162)	0.774			1.086 (0.672, 1.789)	0.741		
Virus								
RSV	1.308 (0.567, 3.023)	0.528			0.783 (0.493, 1.265)	0.308		
Rhinovirus	1.084 (0.424, 2.743)	0.864			0.979 (0.586, 1.699)	0.937		
Bocavirus	0.613 (0.210, 1.668)	0.348			0.743 (0.436, 1.320)	0.290		
Influenza	0.594 (0.216, 1.541)	0.294			1.623 (0.994, 2746)	0.061		

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PUBLICATIONS

Below is a summary of the publications related to this project.

1. ***Viral co-infections in pediatric patients hospitalized with lower tract acute respiratory infections.*** Cebey-Lopez M, Herberg J, Pardo-Seco J, Gómez-Carballa A, Martinón-Torres N, Salas A, Martinón-Sánchez JM, Gormley S, Sumner E, Fink C, Martinón-Torres F. PLoS One. 2015 Sep 2;10(9):e0136526.

2. ***Bacteremia in hospitalized children with respiratory syncytial virus infection***. Cebey-Lopez M, Pardo-Seco J, Rivero-Calle I, Gómez-Carballa A, Martinón-Torres N, Justicia A, Salas A, Martinón-Sánchez JM, Pinnock E, Fink C, Martinón-Torres F, and GENDRES network (www.gendres.org). PlosOne (2015). Submitted.

3. ***Impact of viral co-infections in children hospitalized with acute respiratory infection.*** Cebey-Lopez M, Herberg J, Pardo-Seco J, Gómez-Carballa A, Martinón-Torres N, Salas A, Martinón-Sánchez JM, Justicia A, Rivero I, Sumner E, Fink C, Martinón-Torres F, and GENDRES network (www.gendres.org). Pediatric Research. (2015). Submitted.

I would now like to mention some other activities undertaken during the elaboration of the study:

- ***Vitamin D role in hospitalized children with lower tract acute respiratory infections.*** Cebey-Lopez M, Pardo-Seco J, Gómez-Carballa A, Martinón-Torres N, Rivero-Calle I, Justicia A, Redondo L, Martinón-Sánchez JM, Martinez-Padilla MC, Giménez-Sánchez F, Salas A, Martinón-Torres F, GENDRES network (www.gendres.org). Journal of Pediatric Gastroenterology & Nutrition. (2015).

- ***Impact of Rotavirus Vaccination on Childhood Hospitalization for Seizures***. Pardo-Seco J, Cebey-López M, Martinón-Torres N, Salas A, Gómez-Rial J, Rodriguez-Tenreiro C, Martinón-Sánchez JM, Martinón-Torres F. Pediatr Infect Dis J. 2015 Jul;34(7):769-73.

- ***Comparison of the Antidepressive Effects of Trans-Resveratrol and 5-Methoxy-7H-Dibenzo[de,h]Quinolin-7-One***. Cebey-LopezM, Fontenla JA, Uriarte E, Santana L, Sobarzo-Sánchez E; Current Topics in Medicinal Chemistry, 2014, 14, 234-238. A. 3.453.

- ***Indian signatures in the westernmost edge of the European Romani diaspora: new insight from mitogenomes.*** Gómez-Carballa A, Pardo-Seco J, Fachal L, Vega A, Cebey M, Martinón-Torres N, Martinón-Torres F, Salas A. PLoS One. 2013 Oct 15;8(10):e75397.

- ***A reverse evidence of rotavirus vaccines impact.*** Martinón-Torres F, Aramburo A, Martinón-Torres N, Cebey M, Seoane-Pillado MT, Redondo-Collazo L, Martinón-Sánchez J M.; Human Vaccines & Immunotherapeutics 2013, 9:7, 1–3. A. 2.131.

• Meeting communications:

National congress:

-Sociedad Española de Infectología Pediátrica - SEIP (Santiago de Compostela 6-8 Marzo 2014)

- **Co-infecciones virales en niños hospitalizados por infecciones respiratorias agudas.** M. Cebey López, J. Pardo Seco, A. Gómez Carballa, J. Herberg, E. Sumner, E. Pinnock, F. Giménez Sánchez, M. Martínez Padilla, F. Martinón-Torres, Red Gendres (www.gendres.org). Oral communication.

- **Influencia de la vitamina d en la severidad de las infecciones respiratorias virales (Proyecto Gen-D-Res).** M. Cebey López, J. Pardo Seco, A. Gómez Carballa, N. Martinón Torres, C. Rodríguez-Tenreiro, L. Redondo Collazo, A. Justicia Grande, F. Martinón-Torres, Red Gendres (www.gendres.org). Oral communication.

- **Red para la investigación de las enfermedades respiratorias pediátricas en España: Proyecto Gen-D-Res.** M. Cebey López, I. Villanueva González, A. Gómez Carballa, E. Bernaola Iturbe, T. González López, F. Giménez Sánchez, C. Calvo Rey, P. Alonso Quintela, C. Calvo Monge, M.D.C. Martínez Padilla, M. Baca Cots, D. Moreno Pérez, S. Beatriz Reyes, F. Martinón Torres Red Gendres (www.gendres.org). Poster

-62 Congreso de la Asociación Española de Pediatría - AEP (Madrid 5-7 Junio 2014)

- **La influencia de la vitamina D en las infecciones respiratorias virales (Proyecto Gen-D-Res).** Miriam Cebey López, Jacobo Pardo Seco, Alberto Gómez Carballa, Carmen Curros Novo, Federico Martinón-Torres, Red Gendres (www.gendres.org). Oral communication

- **Co-Infecciones Virales En Pacientes Pediátricos Hospitalizados Por Infecciones Respiratorias Agudas.** Miriam Cebey López, Jacobo Pardo Seco, Alberto Gómez Carballa, Francisco Giménez Sánchez, María Del Carmen Martínez Padilla, Federico Martinón-Torres, Red Gendres (www.gendres.org). Poster with defense.

- **Red para la investigación de las enfermedades respiratorias pediátricas en España: Proyecto Gen-D-Res.** Miriam Cebey López, Alberto Gómez Carballa, Jacobo Pardo Seco, Natalia Fernández Pedrós, Lucía Vilanova Trillo, Federico Martinón-Torres, Grupo Gen-D-Res (www.gendres.org). Poster with defense.

-Reunión de primavera de la SCCALP (León 10-11 April 2015)

- **Co-Infecciones virales en pacientes pediátricos hospitalizados por infecciones respiratorias agudas**; Cebey López M, Alonso Quintela P, Martínez Saenz de Jubera J, Giménez Sánchez F, Martínez Padilla MC, Martinón-Torres F, Red Gendres (www.gendres.org). Oral communication.

- **La influencia de la vitamina D en las infecciones respiratorias virales (Proyecto Gen-D-Res);** Alonso Quintela P, Cebey López M, Naranjo Vivas D, Giménez Sánchez F, Martínez Padilla MC, Martinón-Torres F, Red Gendres (www.gendres.org) ***(Best oral comunication award)***

-63 Congreso de la Asociación Española de Pediatría - AEP (Bilbao 11-13 June 2015)

- **Bacteremia en lactantes hospitalizados con infecciones por virus respiratorio sincitial;** M Cebey López, Irene Rivero Calle, A. Gómez Carballa, A. Justicia Grande, L. Vilanova Trillo, F. Martinón-Torres, Red GENDRES. ***(Special mention award for best posters)***

International meetings:

- 32nd Meeting of the European Society for Paediatric Infectious Diseases - ESPID (Dublin, Ireland 6-10 May 2014)

- **Viral co-infections in hospitalized children with acute respiratory infections.** M. Cebey, J. Pardo-Seco, A. Gómez-Carballa, N. Martinón-Torres, M. Montero-Martín, L. Pías, C. Rodríguez-Tenreiro, L. Vilanova, J. Herberg, E. Sumner, C. Fink, F. Giménez-Sánchez, M.C. Martínez-Padilla, C. Curros, F. Martinón-Torres, GENDRES network - www.gendres.org

- **The influence of vitamin d on the severity of viral respiratory infections (GENDRES Project).** M. Cebey, J. Pardo-Seco, A. Gómez-Carballa, N. Martinón-Torres, C. Rodriguez-Tenreiro, L. Redondo-Collazo, A. Justicia-Grande, F. Martinón-Torres, GENDRES network

- 33rd Meeting of the European Society for Paediatric Infectious Diseases - ESPID (Leipzig, Germany 12-16 May 2015)

- **Bacteremia in hospitalized children with respiratory syncytial virus infection;** Miriam Cebey, Jacobo Pardo-Seco, Alberto Gómez-Carballa, Nazareth Martinón-Torres, José María Martinón-Sánchez, Antonio Justicia, Irene Rivero, Elli Pinnock, Antonio Salas, Colin Fink, Federico Martinón-Torres, GENDRES network (www.gendres.org). Poster