UNIVERSIDADE FEDERAL DA BAHIA

Faculdade de Medicina da Bahia

Programa de Pós-graduação em Ciências da Saúde

Largo do Terreiro de Jesus, s/n. Centro Histórico

40.026-010 Salvador, Bahia, Brasil.

Tel.: 55 71 3283.5582 | Fax: 55 71 3283.5567

www.possaude.ufba.br | pos.saude@ufba.br

Tese de Doutorado

Avaliação da carga de patógenos e da resposta imune em crianças portadoras de infecção respiratória aguda.

Kiyoshi Ferreira Fukutani

Salvador

2016

Modelo de ficha catalográfica fornecido pelo Sistema Universitário de Bibliotecas da UFBA para ser confeccionada pelo autor

Ferreira Fukutani, Kiyoshi
Avaliação da carga de patógenos e da resposta imune em crianças portadoras de infecção respiratória aguda. / Kiyoshi Ferreira Fukutani. -- Salvador, 2016.
91 f.

Orientadora: Camila Indiani de Oliveira.
Tese (Doutorado - Programa de Pós-graduação em Ciências da Saúde) -- Universidade Federal da Bahia, Faculdade de Medicina da Bahia, 2016.

1. . I. Indiani de Oliveira, Camila. II. Título.

UNIVERSIDADE FEDERAL DA BAHIA

Faculdade de Medicina da Bahia

Programa de Pós-graduação em Ciências da Saúde

Largo do Terreiro de Jesus, s/n. Centro Histórico

40.026-010 Salvador, Bahia, Brasil.

Tel.: 55 71 3283.5582 | Fax: 55 71 3283.5567

www.possaude.ufba.br | pos.saude@ufba.br

Tese de Doutorado

Avaliação da carga de patógenos e da resposta imune em crianças portadoras de infecção respiratória aguda.

Kiyoshi Ferreira Fukutani

Professor orientador: **Camila Indiani de Oliveira**

Tese apresentada ao Colegiado do PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE, da Faculdade de Medicina da Bahia da Universidade Federal da Bahia, como pré-requisito obrigatório para o exame de qualificação.

Salvador

2016

<u>Tese de Doutorado</u>*: Avaliação da carga de patógenos e da resposta imune em crianças portadoras de infecção respiratória aguda*, de **KiyoshiFerreira Fukutani**.

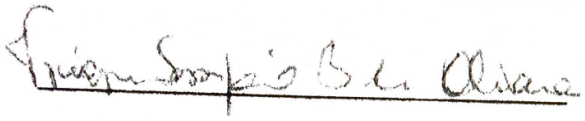
Professor orientador(a): **Camila Indiani de Oliveira**

<u>COMISSÃO AVALIADORA</u>:

Bruno Andrade

Pesquisador em saúde pública

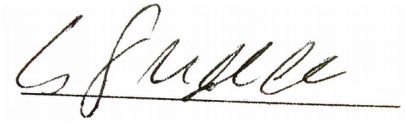
CPqGM-FIOCRUZ



Viviane B**o**aventura

Pesquisador em saúde pública

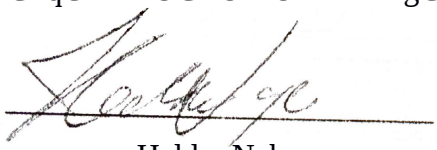
CPqGM-FIOCRUZ-UFBA



Lucas Carvalho

Pesquisador em saúde pública

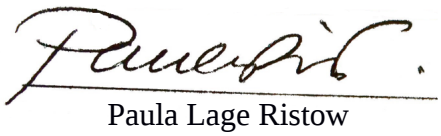
CPqGM-FIOCRUZ-UFBA-PPgCS



Helder Nakaya

Professor/Pesquisador

Universidade São Paulo



Paula Lage Ristow

Professora/Pesquisadora

CPqGM-FIOCRUZ-UFBA

"Aprendi. Não tanto quanto quis, mas vi que, conhecendo o universo ao meu redor, aprendo a me conhecer melhor, e, assim, escutarei o tempo, que ensinará a tomar a decisão certa em cada momento. E partirei em busca de muitos ideais. Mas sei que hoje se encontram meu passado, futuro e presente. Hoje sinto em mim a emoção da despedida. Hoje é um ponto de chegada, e, ao mesmo tempo, ponto de partida. Se em horas de encontros pode haver tantos desencontros, que a hora da separação seja, tão somente, a hora de um verdadeiro, profundo e coletivo encontro. De tudo ficarão três coisas: a certeza de estar sempre começando, a certeza de que é preciso continuar e a certeza de ser interrompido antes de terminar. Fazer da queda um passo de dança, do medo uma escada, do sonho uma ponte e da procura um encontro. Um simples encontro!"

Fernando Sabino

Dedicatória

Dedico esse trabalho as pessoas importantes nesse período da minha vida: Camila Indiani de Oliveira, sem ela estaria largado no meio do caminho; meus pais Yoshikazu Fukutani e Lizia Ferreira Fukutani, o motivo da minha forca e aos meus irmãos Yoshio Ferreira Fukutani e Kazuo Ferreira Fukutani. No final, a vida me ensinou de forma dura o valor que vocês têm, perto não notava, longe sinto o vazio.

EQUIPE

- Aluno: Kiyoshi Ferreira Fukutani. e-mail: ferreirafk@gmail.com;
- Professor(a)orientador(a):Dr(a).Camila Indiani de Oliveira. email:camila@bahia.fiocruz.br;
- Colaboradores: Dr. Johan Van Weyenbergh, Universidade de Leuven, KULEUVEN; Dra. Cristiana Nascimento de Carvalho, UFBA; MsC. Maiana Bouzas, UFBA e MsC. Juliana Oliveira, UFBA.

INSTITUIÇÕES PARTICIPANTES

UNIVERSIDADE FEDERAL DA BAHIA

- Faculdade de Medicina da Bahia (FMB)
- Curso de Pós Graduação em Ciências da Saúde (PPgCS)

UNIVERSIDADE DE LEUVEN

- REGA Instituto (REGA)
- Laboratório de Virologia

FUNDAÇÃO OSWALDO CRUZ

- Centro de Pesquisa Gonçalo Moniz

FONTES DE FINANCIAMENTO

1. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)
2. Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB)
3. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPE*S*)
4. Programa Ciências sem Fronteiras (CsF-CNPq)

AGRADECIMENTOS

As pessoas imprescindíveis para esse trabalho:
CPqGM, FIOCRUZ: Dra Aldina Barral, colegas do LIP, LIMI, demais laboratórios e alunos das gerações antigas.
UFBA: Dra. Cristiana Nascimento Carvalho, Dra. Maiara Bouzas, Dra. Juliana Oliveira e PPgCS.
KULEUVEN: Dr. Johan van Weyenbergh, Dr. Ricardo Khouri, Dra. Annemieke Van Damme, REGA e amigos de Leuven.
Banca avaliadora: Dr. Bruno Andrade, Dra. Viviane Boaventura, Dr. Lucas Carvalho, Dr. Helder Nakaya e Dra. Paula Carvalhal Ristow
Fontes financiadores: Ciências sem fronteiras, CNPq, FAPESB e CAPES.

SUMÁRIO

LISTA DE FIGURAS

I. RESUMO

Introdução: Infecções respiratórias agudas (IRA) apresentam uma elevada morbidade e representam um problema de saúde pública. **Objetivos:** Detectar , via a presença de RNA, os agentes virais e bacterianos presentes em aspirado nasofaríngeo (ANF) de crianças com idades entre 6-23 meses, portadoras de IRA e detectar o perfil de resposta imune associados. **Metodologia:** ANF foram submetidos à extração de RNA e esse foi utilizado em ensaios de nCounter empregando sondas desenhadas para a detecção viral e bacteriana e empregando sondas padrão para a detecção da resposta imune. **Resultados:** na coorte de 60 ANFs obtidos de crianças com IRA, detectamos transcritos de Parainfluenza (1-3), Vírus Sincicial Respiratório (A e B) (21%), Metapneumovirus humanos (5%), Bocavírus, Coronavírus e Vírus Influenza A (3%), Rinovírus (2%), *Staphylococcus aureus* (77%), *Haemophilusinfluenzae* (69%), *Streptococcuspneumoniae* (26%), *Moraxellacatarrhalis* (8%), *Mycoplasmapneumoniae* (3%) e *Chlamydophilapneumoniae* (2%). Dentre os 60 pacientes, 28 apresentaram infecção bacteriana única, 22 pacientes apresentaram a presença de bactérias e vírus, e cinco pacientes apresentaram infecção viral apenas. Em cinco pacientes, a detecção dos transcritos ficou abaixo do ponto de corte e esses foram considerados negativos. Também observamos uma modulação diferencial de genes imunes em ANFs; o número de genes modulados foi reduzido por redução de dimensões. Encontramos 30 genes diferencialmente expressos. Para avaliar as associações entre todas as variáveis, utilizamos a rede Bayesiana e observamos uma associação entre marcadores da resposta imune com carga microbiana. **Conclusão:** O nCounter é uma técnica sensível para identificar o transcriptoma de ANF, tendo como alvo tanto a resposta imune quanto os patógenos. A análise integrada dos dados

revelou um subconjunto de genes relacionados à resposta que interagem diretamente com a carga microbiana e com os sintomas clínicos.

II. OBJETIVOS

II.1 Objetivo Geral: Identificar, por método transcriptômico, os agentes etiológicos e os marcadores imunes associados em crianças com infecção respiratória aguda, atendidas no Centro Pediátrico Hosannahde Oliveira, Salvador – BA.

II.2 Objetivos Específicos

1.Detectar transcritos microbianos presentes em aspirado nasofaríngeo, coletado de crianças com infecção respiratória aguda.

2.Identificar o perfil de resposta imune, por meio da assinatura gênica presente no aspirado nasofaríngeo, de crianças com infecção respiratória aguda.

3. Correlacionar a presença de patógenos com as resposta imune, por meio de modelos matemáticos.

III. INTRODUÇÃO

As infecções respiratórias agudas (IRAs) constituem a maior causa de morbidade e a quarta causa de morte no mundo (Stein & Marostica, 2007). A morbidade gera impactos econômicos tais como o aumento na utilização dos serviços de saúde, como o aumento nos gastos familiares com assistência médica e aumento nos gastos com medicamentos (Lambert et al., 2008). Segundo a Organização Pan-americana de Saúde (OPAS), ocorrem anualmente cerca de 500 a 900 milhões de casos de IRA (Maria et al., n.d.). Desses casos pelo menos 75% ocorrem nos países em desenvolvimento (Mahony, 2008) e a sua incidência é dez vezes maior nos países em desenvolvimento em comparação aos países desenvolvidos (Stein and Marostica 2007). No Brasil, na década de 80, as IRAs ocupou o primeiro lugar na causa morte por doenças infecciosas ultrapassando as doenças diarréicas; e em 2010 46% dos leitos do Sistema Único de Saúde (SUS) foram ocupado por pacientes com IRA (Silva, et al., 2012). No estado da Bahia, as doenças respiratórias são responsáveis por pelo menos 35,7% das hospitalizações (DATASUS, 2011) e os agentes etiológicos não são comumente identificados (Mahony, 2008).

A IRA e descrita como infecções que acometem as vias respiratórias superiores e não acometem o trato respiratório inferior (Tregoning & Schwarze 2010). Estruturalmente o trato respiratório superior é constituído pelas narinas, cavidade nasal, boca, faringe e laringe. A parte mais externa do trato respiratório superior é colonizada por micróbios comensais e potencialmente patogênicos (Brealey & Chappell, 2015). Os sinais mais comuns associadas a IRA, são: i) a rinofaringite aguda que acomete vias aéreas superiores, comum em crianças menores de cinco anos e é causada exclusivamente por agentes virais.; ii) a sinusite aguda, definida como

infecção bacteriana dos seios paranasais; iii) a faringo amigdalite aguda estreptocócica, infecção aguda da orofaringe, que acomete crianças após os cinco anos de vida, mas pode ocorrer, não raramente, em menores de três anos, iv) a faringite viral aguda, uma inflamação da porção subglótica da laringe (Pitrez & Pitrez, 2003).

O risco de infecção está correlacionado com a idade (sendo as faixas etárias acima de 65 e menores de 5 anos as mais susceptíveis à infecção), o que torna a população idosa e pediátrica com maiores riscos de infecções (Ren et al. 2009). Sazonalmente a maioria dos casos de IRA ocorrem durante os meses de inverno com a circulação de partículas microbianas em ambientes fechados. A transmissão dos agentes causadores da IRA ocorre por contato com os aerossol, gota, ou contato direto com secreções infectadas (Hartzell et al., 2003). As medidas preventivas para se evitar a IRA são medidas simples, como cuidados básicos de higiene (Rabie and Curtis 2006), cuidado com as secreções provenientes de pessoas com sintomas respiratórios, evitar o contato com pessoas vulneráveis (Bulla & Hitze 1978). Apesar da vacina da gripe e da vacina pneumocócica serem normalmente utilizadas com sucesso em grupos de alto risco (Girard et al., 2005).

Dentre os micróbios patogênicos, os mais comumente relacionados com a IRA são: *Staphylococcus pneumoniae*, *Haemophilus influenzae*, *Moraxela catarrhalis*, vírus sincicial respiratório humano (RSV A e B), vírus parainfluenza (PIV) dos tipos 1 a 3, adenovírus (AV), vírus influenza (IVA e IVB) e rinovírus (RV) (Costa et al., 2006; Uitti et al., 2015). A ocorrência dos patógenos no Brasil, especificamente no estado de São Paulo, aponta uma maior prevalência do RV (37,7%), seguido pelo RSV (20,7%) e pelo IVB (17,7%) (Bonfim et al. 2011). No estado da Bahia, em pacientes com pneumonia adquirida na comunidade, identificou-se uma

predominância do RV (21%), seguido pelo PIV (17%) e RSV (15%) (Nascimento-Carvalho et al., 2008).

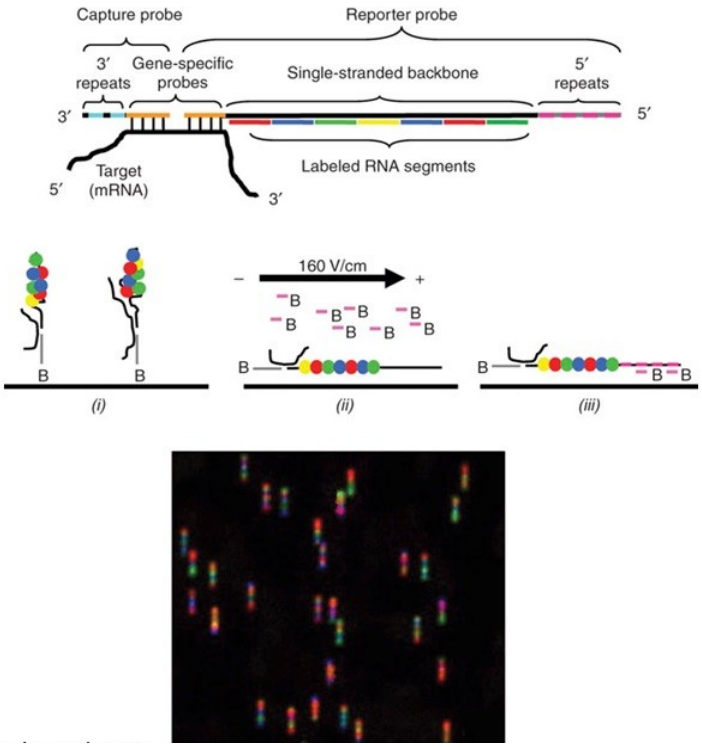
A avaliação clínica dos pacientes com IRA, tanto para o tipo viral como para o tipo bacteriano,é dificultada por apresentar características semelhantes e pela co-existência de ambos tipos de agentes etiológicos na microbiota do individuo saudável (Ellis et al., 2007; Oved et al., 2015; Smith et al., 2012). Os sinais clínicos comumente encontrados são: febre, rinite, congestão nasal, tosse, rouquidão, conjuntivite, dor de garganta, dor no peito, chiado, coriza, espirros, edema e cefaléia (Bulla & Hitze 1978), sendo a coriza uma causa comum e recorrente nas consultas médicas (Fendrick et al., 2003). Estudos recentes sobre o microbioma de pacientes com infecção respiratória descrevem a existência de uma cinética para o aparecimento dos sintomas: assim que uma população microbiana patogênica se estabelece de forma oportunista sobre uma população simbiótica, surgem os sintomas (Brealey et al., 2015; Teo et al., 2015; Thorburn, 2006).

Os vírus respiratórios e as bactérias podem ser detectados por cultura, pela busca de anticorpos e por métodos de detecção baseados em ácidos nucléicos. Dentre as técnicas baseadas na detecção dos ácidos nucléicos, a reação em cadeia da polimerase (PCR) é a técnica mais utilizada (Yang &Rothman 2004). Ele se baseia na amplificação de uma região conhecida de DNA ou de cDNA utilizando iniciadores específicos. Essa técnica tem elevada sensibilidade, é rápida, possui baixo custo e apresenta vários ensaios padronizados. Porém para cada organismo é necessário fazer uma reação diferente, o que impossibilita a identificação de múltiplos patogénos de uma só vez.

Com a dificuldade de se identificar os agentes etiológicos com base nos sintomas, houve um aumento de interesse no desenvolvimento dos testes diagnósticos

para a detecção dos patógenos respiratórios (Caliendo et al., 2011, Falsey et al., 2013). Com a introdução das técnicas moleculares de alto rendimento (*high throughput*) (Chaussabel & Banchereau, 2010) se tornou possível detectar múltiplas moléculas (Barczak et al. 2012; Malkov et al. 2009; Payton et al. 2009). O nCounter (NanoString Technologies) é uma técnica molecular de contagem digital de RNA mensageiro. A técnica utiliza duas sondas, uma que se ancora ao material genético e outra que está ligada a um código de barras único, o qual identifica o alvo por hibridação específica. Após a hibridação do código de barras, a presença e a freqüência dos alvos são reveladas pela contagem dos códigos hibridizados. Cada reação permite a identificação de vários códigos de barras de uma só vez, em uma única reação, não sendo necessária a síntese da molécula complementar ao RNA (cDNA) (Geiss et al. 2008) (Figura 1). A grande vantagem do nCounter em comparação às demais técnicas é a sua capacidade de medir transcritos pouco abundantes, ou seja, moléculas de RNA presentes em baixa concentrações, fator que ocorre com freqüência nas amostras clínicas (Payton et al. 2009).

Figura 1 – Diagrama metodológico do nCounter (Nanostring) (Geiss et al., 2008)



As técnicas de alto rendimento são utilizadas para a investigação da expressão de um grande número de genes ao mesmo tempo (Chaussabel et al. 2010). Umas das técnicas mais comum é o transcriptoma, que avalia a quantidade de transcritos de RNAm, nas amostras, tais como transcritos: virais, bacterianos, protistas e fungicos (Barczak et al. 2012), podendo ser ampliado para quantificar também genes da resposta imune do hospedeiro. Estudos recentes mostraram a utilidade desse tipo de abordagem, como o trabalho de Mejias e colaboradores (2013), que identificaram por transcriptômica, biomarcadores capazes predizer a piora nos sinais respiratório em crianças com infecção viral por RSV. Heinonen e colaboradores (2015), que diferenciaram a assinatura imune de pacientes sintomáticos versus assintomáticos, infectados com RV. O trabalho de Andres-Terre e colaboradores (2015) identificaram um padrão imunológico específico para as infecções respiratórias abrindo o leque de pesquisa para a busca de biomarcador ou vacina. Por último, Oved e colaboradores (2015) encontraram moléculas capazes de distinguir a infecção viral e bacteriana em pacientes com infecções respiratórias agudas.

O que torna as abordagens de alto rendimento uma excelente maneira de se identificar os agentes etiológicos, bem como na identificação de biomarcadores associados à resposta imunes. Nesse contexto o presente trabalho teve como objetivo identificar os patógenos presentes em uma coorte de crianças com infecção respiratória aguda e, ao mesmo tempo, medir as assinaturas imunes associadas no aspirado nasofaríngeo.

IV. RESULTADOS GERAIS.

IV.I MANUSCRITO I.

Pathogen transcriptional profile in nasopharyngeal aspirates of children with Acute Respiratory Tract Infection.**Journal of Clinical Virology.**

Journal of Clinical Virology 69 (2015) 190–196

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Pathogen transcriptional profile in nasopharyngeal aspirates of children with acute respiratory tract infection

Kiyoshi F. Fukutani[a,b], Cristiana M. Nascimento-Carvalho[b,c], Winke Van der Gucht[d], Elke Wollants[d], Ricardo Khouri[a,b], Tim Dierckx[d], Marc Van Ranst[d], Lieselot Houspie[d], Maiara L. Bouzas[b], Juliana R. Oliveira[b], Aldina Barral[a,b,e,f], Johan Van Weyenbergh[d], Camila I. de Oliveira[a,b,f,*]

[a] Centro de Pesquisas Gonçalo Moniz (CPqGM)-FIOCRUZ, Salvador, BA, Brazil
[b] Post-Graduation Program in Health Sciences, Federal University of Bahia School of Medicine, Salvador, Brazil
[c] Department of Pediatrics, School of Medicine, Federal University of Bahia, Salvador, BA, Brazil
[d] Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium
[e] Department of Pathology, School of Medicine, Federal University of Bahia, Salvador, BA, Brazil
[f] Instituto de Investigação em Imunologia, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 30 May 2015
Accepted 8 June 2015

Keywords:
ARI
nCounter
Diagnostics
RSV

ABSTRACT

Background: Acute respiratory tract infections (ARI) present a significant morbidity and pose a global health burden. Patients are frequently treated with antibiotics although ARI are most commonly caused by virus, strengthening the need for improved diagnostic methods.
Objectives: Detect viral and bacterial RNA in nasopharyngeal aspirates (NPA) from children aged 6–23 months with ARI using nCounter.
Study design: A custom-designed nCounter probeset containing viral and bacterial targets was tested in NPA of ARI patients.
Results: Initially, spiked control viral RNAs were detectable in ≥6.25 ng input RNA, indicating absence of inhibitors in NPA. nCounter applied to a larger NPA sample ($n = 61$) enabled the multiplex detection of different pathogens: RNA viruses Parainfluenza virus (PIV 1–3) and RSV A-B in 21%, *Human metapneumovirus* (hMPV) in 5%, *Bocavirus* (BoV), CoV, Influenza virus (IV) A in 3% and, Rhinovirus (RV) in 2% of samples, respectively. RSV A-B was confirmed by Real Time PCR (86.2–96.9% agreement). DNA virus (AV) was detected at RNA level, reflecting viral replication, in 10% of samples. Bacterial transcripts from *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* were detected in 77, 69, 26, 8, 3 and 2% of samples, respectively.
Conclusion: nCounter is robust and sensitive for the simultaneous detection of viral (both RNA and DNA) and bacterial transcripts in NPA with low RNA input (<10 ng). This medium-throughput technique will increase our understanding of ARI pathogenesis and may provide an evidence-based approach for the targeted and rational use of antibiotics in pediatric ARI.

1. Background

Acute respiratory tract infection (ARI) accounts for both high mortality and morbidity in children <5 years old [1]. In developing countries, 75% of all acute morbidities were assigned as ARI but identification of the etiological agent in these cases is not common [2]. Upper respiratory tract infections are most commonly caused by virus and only 10% of cases are attributed to bacteria [3]; nonetheless, most patients are treated with antibiotics [1].

Respiratory viruses can be diagnosed by culture, antibody-based and nucleic acid–based detection methods. Polymerase chain reaction (PCR) is the most widespread technique for pathogen identification [4]; however, simultaneous detection of different pathogens by multiplex PCR demands reaction standardization. To this end, nCounter (NanoString Technologies), a digital mRNA expression profiling method, can overcome these drawbacks: it identifies genetic material using two probes, one anchored to the genetic material and the other anchored to a singular bar code that identifies the target by specific hybridization. Upon bar code

* Corresponding author at: LIP–CPqGM–FIOCRUZ, Rua Waldemar Falcão, 121, Candeal – Salvador – BA 40296710, Brazil. Fax: +55 71 31762279.
E-mail address: camila@bahia.fiocruz.br (C.I. de Oliveira).

http://dx.doi.org/10.1016/j.jcv.2015.06.005
1386-6532/

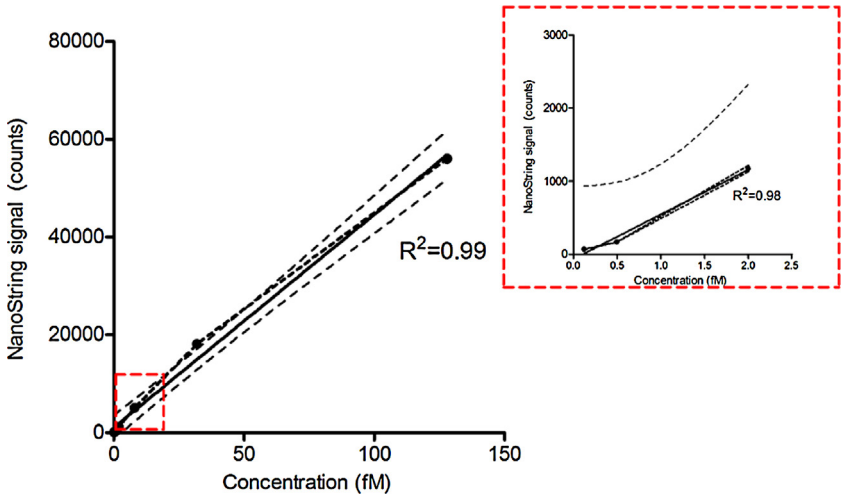


Fig. 1. Quantification of the positive control RNAs by nCounter.
Number of counts for exogenous positive control RNAs (background-subtracted) plotted against the different concentrations tested (0.125–128 fM). The correlation coefficients (R^2 values) of a linear fit to the mean ($n = 61$) are indicated for the entire concentration range tested and, in the insert, for the concentrations <0.25 fM.

hybridization, presence and frequency of the target is revealed and multiple barcodes in the same reaction allow multiplex analysis [5].

2. Objectives

We designed a codeset for quantification of viral (RV, RSV A and B, IV A and B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV, CoV) and bacterial (*Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Mycoplasma pneumoniae* and *Chlamydia pneumoniae*) transcripts in one multiplex reaction employing nasopharingeal aspirates (NPA) from children presenting ARI.

3. Study design

3.1. Clinical samples

Our study complied with the guidelines of the Declaration of Helsinki and was approved by the institutional review board of the Universidade Federal da Bahia (UFBA) (#067/2009). Written informed consent was obtained from all legal guardians. This was a prospective cohort study involving a sample of children aged 6–23 months, seen at UFBA's Emergency Unit between 2009 and 2011 [6]. Community-dwelling patients fulfilling inclusion criteria (fever, cough, sneeze or nasal obstruction for a period up to seven days and without previous episode of wheezing) were enrolled and NPA were collected. Control samples (nasopharyngeal wash) were obtained from individuals ($n = 7$) undergoing routine outpatient visits. After collection, samples were place in Nuclisens lysis buffer (Biomerieux) and frozen at −70 °C.

3.2. RNA extraction and nCounter analysis

Viral and bacterial detection in NPA was performed using nCounter transcriptomic analysis (NanoString Technologies), allowing us to simultaneously assay for viral and bacterial targets. Total RNA (10–50 ng), obtained from a subset of randomly selected NPAs, was extracted using Qiagenís RNEasy, following manufacturerís instructions, and was subsequently hybridized against probes targeting RV, RSVA-B, IV A-B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV and CoV (229A and OC43), *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus*, *C. pneumoniae* and *M. pneumoniae*(Supplementary Table 1), synthesized by NanoString Technologies. Hybridization was also performed with control viral and bacterial RNA pools [kindly donated by K. Bruyninckx, UZ Leuven], in three different concentrations (6.25, 12.5 and 25 ng for viral RNA and 1.5, 3 and 6 ng for bacterial RNA).

3.3. Real-Time PCR

RSV A and B were independently quantified by Real-Time PCR, as described [7].

3.4. Data analysis

Raw data were pre-processed using both nSolver 2.0 software (Nanostring Technologies) and the NanoStringNorm R package [8]. Preprocessing sequentially corrects for three factors: technical variation, background and sample content. First, using a set of exogenous positive control RNAs present in each sample (see also Fig. 1), technical variation is accounted for by adjusting the counts in each sample to the geometric mean of counts for positive control counts in all samples. Subsequently, a background correction is performed by subtracting the maximum count value of the negative control probes in a sample from each probe output within the sample. Transcripts counts that are negative after background correction are set to 1. Each sample is then normalized for RNA content by adjusting the counts to the geometric mean of 15 housekeeping genes (Supplementary Table 2). Finally, the data were log 2 transformed. Full details of each step can be found in the supplementary information of Waggott et al. [8]. Network data were designed by association matrix and the graph file was created using Gephi. Nodes were aligned by Force Atlas algorithm and the centrality was calculated by the betweenness. Statistical analyses were performed using Graphpad Prism version 5.0.

4. Results

4.1. Pathogen detection in NPAs by nCounter transcriptomics

The digital nCounter profiling technology captures and counts specific nucleic acid molecules in a complex mixture [5]. Briefly, unique pairs of capture and detection probes are designed against each gene of interest. The capture probe consists of a sequence complimentary to a target pathogen mRNA and a short common

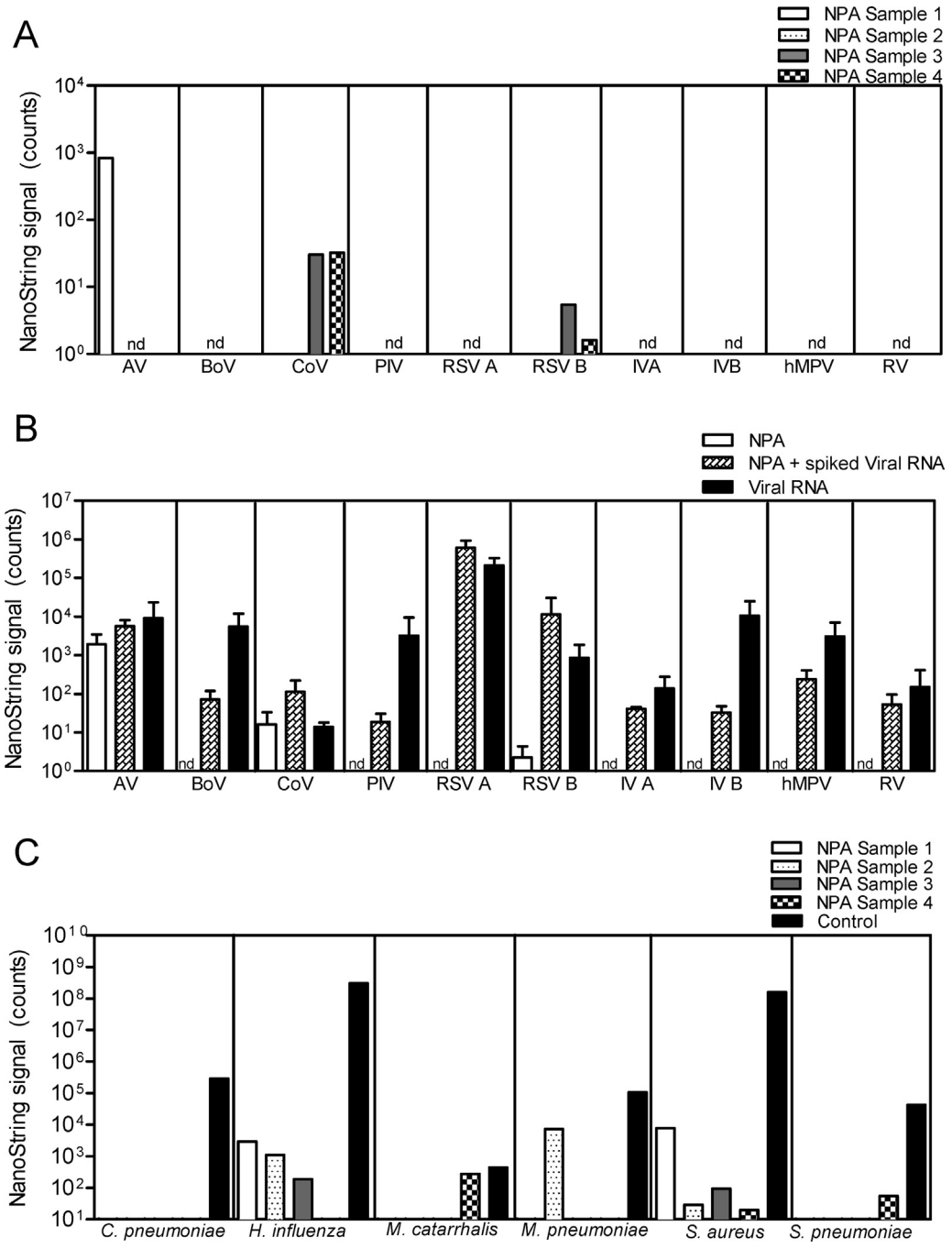


Fig. 2. nCounter profile of pathogen transcripts in nasopharyingeal aspirates (NPA) from patients with ARI.
NPA ($n = 4$) was submitted to RNA extraction and, subsequently, to nCounter analysis for the detection of (A) Adenovirus (AV), *Bocavirus* (BoV), *Coronavirus* (CoV), Parainfluenza virus (PIV), Respiratory Syncytial virus A and B (RSV A and B), Influenza virus A and B (IV A and B), *Human metapneumovirus* (hMPV) and Rhinovirus (RV). Data are shown as the number of counts (transcripts), for each target. (B) Pooled NPA RNA ($n = 4$) were hybridized alone (NPA) or in the presence of control viral RNA (NPA + spiked viral RNA). Black bars represent hybridization with control viral RNA alone. Data are shown as the mean number of counts (transcripts), for each target ± SD. (C) Detection of *Chlamydophila pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Mycoplasma pneumoniae, Staphylococcus aureus* and *Streptococcus pneumoniae* in each individual NPA sample. Black bars reflect hybridization with control bacterial RNA (pooled). Data are shown as the number of counts (transcripts), for each target.

sequence coupled to an affinity tag (biotin). The reporter probe also contains the complementary sequence but is coupled to a color-coded tag, providing the detection signal. The color code in each tag is unique, allowing the accurate discrimination of the different targets, in a complex mixture. Capture and reporter probes are mixed with total RNA in a single hybridization reaction, resulting in the formation of tripartite structures (target bound to the specific reporter and capture probes). Unhybridized probes are washed away and remaining complexes are exposed to streptavidin. Targets bound to the specific probes are immobilized on a solid surface that is scanned; the unique reporter probe then identifies each target and the level of expression is measured by the digital readout of transcript counts. The reaction occurs in the absence of nucleic acid amplification and/or enzymatic reactions. To estimate target concentration, nCounter uses a series of positive controls covering a range of known concentrations (0.1–100 fM, comparable to 0.2–200 copies per cell). Based on the counts obtained for these known concentrations, the concentration of mRNA transcripts can

be estimated. This estimate is done by plotting the counts obtained for the positive controls versus the concentrations tested and by fitting a regression line through the average (Fig. 1). The regression line allows the quantification of mRNA transcript concentration. The correlation between the counts for positive controls and the concentration was 0.99 and 0.98 for the lower concentration range.

Next, we assessed the potential of nCounter as a diagnostic technique for ARI. In pilot experiments, we used RNA obtained from NPA of four patients and the set of probes custom-designed for detection of viral [RV, RSVA-B, IV A-B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV and CoV (229A and OC43)] and bacterial (*S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus, C. pneumoniae,* and *M. pneumoniae*) targets (Supplementary Table 1). We detected the presence of AV, CoV and RSVB (Fig. 2A), whereas BoV, PIV, RSVA, IV A-B, hMPV and RV were detected in none of the four samples initially tested. Detection was also positive in NPA spiked with known viral RNA (6.25–12.5–25 ng), indicating absence of inhibitors in RNA isolated from NPA (Fig. 2B). In addition, use of tRNA (an RNA carrier) to ARI samples did not improve viral detection (Supplementary Fig. 1), confirming that even NPA containing low input RNA (<10 ng) is suitable. Bacterial RNA (*H. influenzae, M. catarrhalis, M. pneumoniae, S. aureus,* and *S. pneumoniae*) was also detected in both patientsí and control NPA (Fig. 2C).

For comparison purposes, we performed RSV A and B detection by Real-Time PCR. Agreement between nCounter and Real-Time PCR was 86.2% for RSVA (kappa = 0.231) and 96.9% for RSVB (kappa = 0.82) (Table 1). RNA counts detected by nCounter were significantly correlated to the number of copies detected by Real-Time PCR for both RSVA ($r = 0.25$, Spearman; $p = 0.049$) and RSVB ($r = 0.83$; Spearman $p = 0.001$).

Table 1
RSVA and RSVB agreement between Real Time PCR and nCounter results in NPA samples from ARI patients.

nCounter	Real-Time PCR					
	RSVA			RSVB		
	Positive	Negative	Total	Positive	Negative	Total
Positive, *n*	2	4	6	5	0	5
Negative, *n*	5	54	59	2	58	60
Total	7	58	65	7	58	65

4.2. Pathogen network in NPA samples of children with ARI

We then expanded nCounter analysis to a set of 61 randomly selected NPA and included seven healthy control samples (Fig. 3). Using this extended group, we detected RV (2% of samples), BoV (3%), CoV (229E and OC43, 3%), IV A (3%), hMPV (5%), PIV (genotypes 1 and 3, 21%) and RSV A and B (21%). In addition, detection of DNA virus AV (types 2 and 5) was confirmed at the RNA level in 10% of samples, indicating ongoing (intracellular) viral replication. Transcripts for *C. pneumoniae, M. catarrhalis*, *M. pneumoniae* and *S. pneumoniae* were detected in 2, 3, 8 and 26% of samples, respectively. *H. influenzae* and *S. aureus* transcripts were ubiquitous (69 and 77% of samples, respectively) and were also detected in healthy controls (28 and 100% of samples, respectively). Using this data set, we observed bacterial presence in the majority of children (55.1%), whereas in 43.5% of children, both viruses and bacteria were present (Fig. 4A). A low percentage of children (1.5%) presented viruses only. Upon observation of the number of transcripts per type of pathogen (Fig. 4B), AV 2 and RSB (A and B) showed

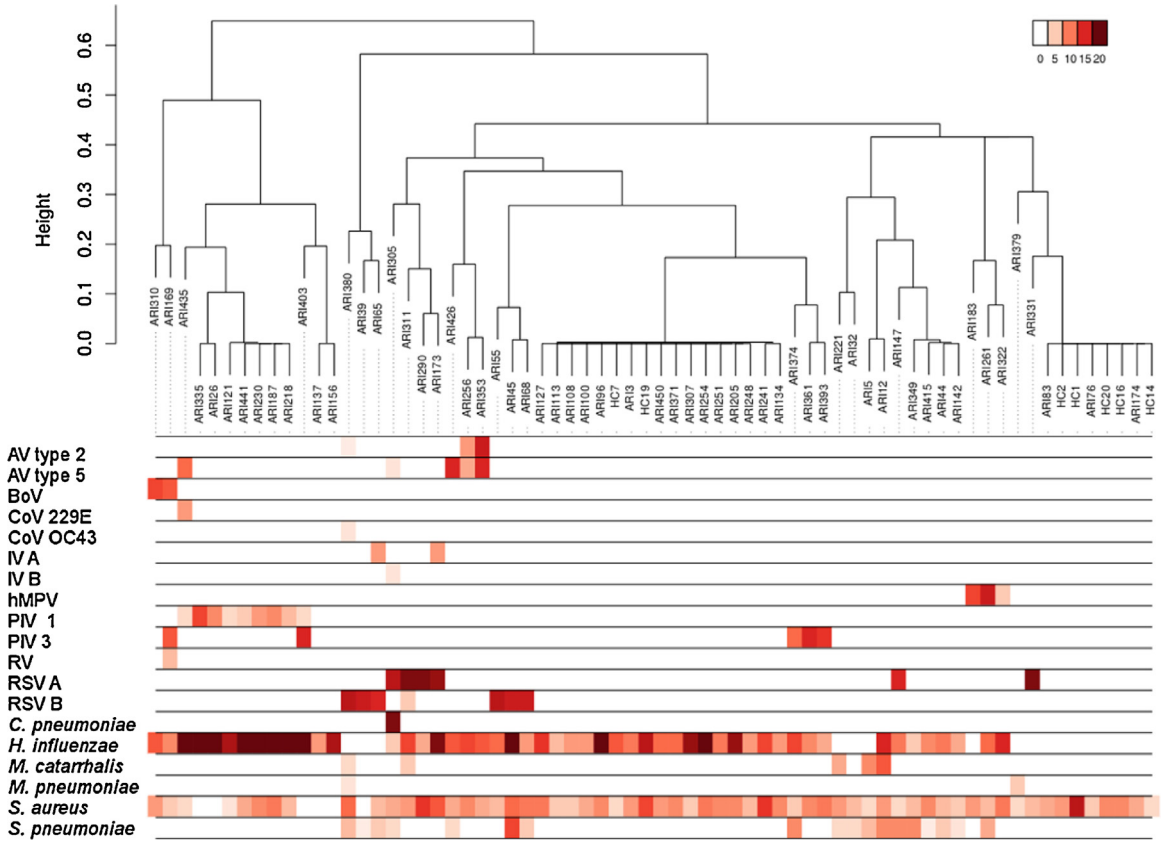


Fig. 3. Heat map dendrogram of pathogen detection by nCounter in children with ARI.
RNA from ARI patients ($n = 61$) and from healthy controls ($n = 7$) were hybridized against probes for adenovirus (AV), *Bocavirus* (BoV), *Coronavirus* (CoV), Influenza virus (IV), *Human metapneumovirus* (hMPV), parainfluenza virus (PIV), Rhinovirus (RV), Respiratory Syncytial Virus (RSV), *C. pneumoniae, H. influenzae, M. catarrhalis, M. pneumoniae, S. aureus* and *S. pneumoniae.* Samples were clustered by complete linkage of spearman correlation and color intensity indicates probe counts in each sample. The processed data were log2-transformed.

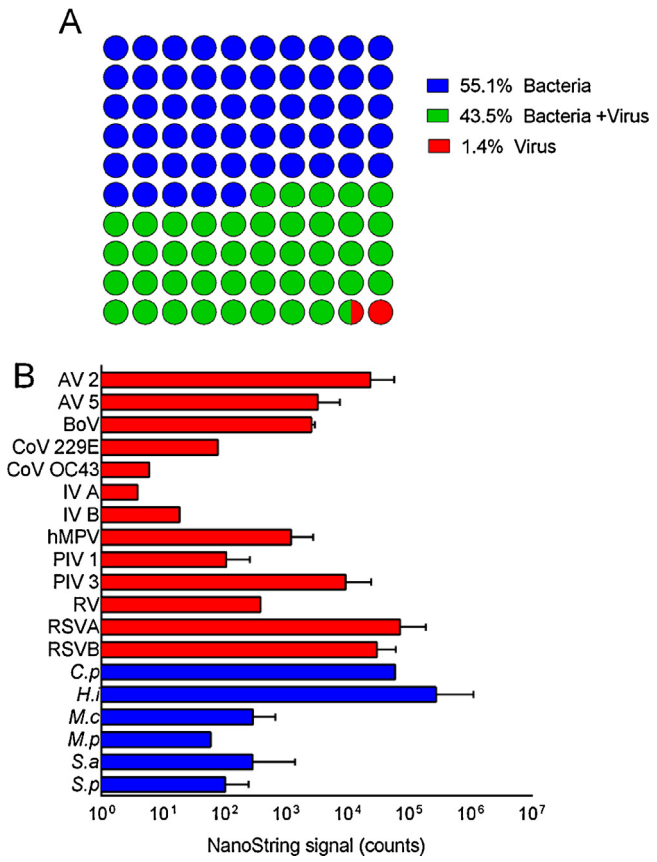


Fig. 4. Microbe counts (transcripts) in NPA of children with ARI, determined by nCounter.
(A) Chart showing the percentages of samples ($n=61$) presenting counts (transcripts) for bacteria (blue), viruses (red) or both viruses and bacteria (green). (B) Bar plot representing the mean number of counts (transcripts) detected for each pathogen in NPA samples ($n=61$). Adenovirus (AV), *Bocavirus* (BoV), *Coronavirus* (CoV), Influenza virus (IV), *Human metapneumovirus* (hMPV), parainfluenza virus (PIV), Rhinovirus (RV), Respiratory Syncytial Virus (RSV), *C.p*, *C. pneumoniae*; *Hi*, *H. influenzae*, *M.c*, *M. catarrhalis*; *M.p*, *M. pneumoniae*; *S.a*, *S. aureus* and *S.p*, *S. pneumoniae*.

the highest mean transcript numbers (>10.000), comparing the 61 samples. The mean number of transcripts detected for AV 5, BoV and PIV 3 was <10.000. CoV (229E and OC43), IV (A and B), hMPV, PIV 1 and RV presented the lowest mean number of transcripts ($\leq$1000). For bacteria, the mean transcript number of *C. pneumoniae*, across the 61 NPA samples, was comparable to that detected for AV and RSV ($\sim$10.000). For the other bacterial targets (*H. influenzae*, *M. catarrhalis*, *M. pneumoniae*, *S. aureus* and *S. pneumoniae*), the mean number of transcripts was $\leq$1000.

Network analysis also revealed the complex interactions that occur between individual viruses and bacteria detected in NPA (Fig. 5). For example, the strongest links were observed for bacteria: *H. influenzae*/*S. aureus* and, secondly, *H. influenzae*/*S. aureus* or *H. influenzae*/*S. pneumoniae*. Comparing virus and bacteria, PIV1/*H. influenzae* showed a strong interaction as did RSV (A and B)/*S. aureus*. Of note, the interactions of CoV 229E were different from those observed for CoV OC43.

5. Discussion

Our results demonstrate that nCounter is a robust medium-throughput technique for detection of both viral and bacterial RNA transcripts in ARI patients. The sensitivity and reproducibility of nCounter has already been compared with Openarray® [9], Affymetrix microarray [10] and TaqMan PCR [5]. In the latter, nCounter was equally reproducible and sensitive regarding detection of differentially modulated genes; however, nCounter detected low abundant transcripts, not detected by microarray. nCounter was also successfully used for detection of pathogen RNA transcripts in clinical samples [11], and an important advantage is transcript detection without the need for purification or amplification, yielding a rapid indicator of pathogen replication.

Transcriptomic profiling by nCounter allowed simultaneous detection of both viral and bacterial transcripts in ARI patients. We detected the presence of AV, BoV, CoV, IV, hMPV, PIV, RV and RSVA-B, which are associated with respiratory tract infections [1]. To our knowledge, this is the first description of detection of a pathogenic DNA virus (Adenovirus type 2 and 5) at the RNA level in clinical samples. In contrast to a standard DNA PCR, which quantifies DNA in (mostly extracellular) viral particles, nCounter enables demonstration of ongoing (intracellular) viral replication, which we hypothesize to be more relevant to ARI pathogenesis. We also, simultaneously detected transcripts for *C. pneumoniae*, *M. catarrhalis* and *S. pneumoniae*, which are commonly isolated from the respiratory tract [12], as is *H. influenzae*. We observed a good agreement between Real-Time PCR and nCounter for RSV (A and B) detection, reinforcing the possibility of using the latter as an alternative method for pathogen detection. Of note, RSVA strains present a high variability in the target used for detection (G protein domain) [13], also in Brazil [6], which could explain the weaker correlation observed for nCounter and Real-Time PCR, as compared to RSVB.

The healthy upper respiratory tract is colonized by commensals and potential pathogens kept in check by the immune system. There is evidence that viral respiratory infections can stimulate an increase in bacterial load and this association can lead to secondary complications [14]. Preterm infants with RSV and bacterial co-infection were hospitalized for longer periods and more frequently admitted to the intensive care unit, compared to children infected with RSV alone [15]. In our samples, the majority of ARI patients presented *H. influenzae* and *S. aureus*, which, together with *S. pneumoniae* and *M. catarrhalis* are the usual opportunistic pathogens co-detected with RSV [16–18]. Indeed, transcripts for all these agents were detected in our NPA samples from children with ARI. Network analysis showed strong links between PIV 1 and *H. influenzae* and between RSV B and *S. pneumoniae*, for example. Both *S. pneumoniae* [19] and RSV activate the inflammasome [20]; *S. pneumoniae* triggers NF-kB activation leading to an inflammatory response whereas, RSV infection results in a strong interferon response. The possible overlap between the signaling pathways triggered by each pathogen may synergize and amplify the inflammatory signal during co-infection. This exacerbated immune response could accentuate damage in situ and increase morbidity. Given this scenario, knowing the effective pathogen load, as shown here by nCounter, can contribute towards understanding the contribution of each pathogen to disease severity. As a proof-of-principle, our group has already used nCounter to simultaneously quantify both human and retroviral transcriptomes; thus, revealing intact IFN signaling in HTLV-1 infection [21].

We conclude that nCounter can be used as an alternative medium-throughput method to identify, in a single reaction, the presence of multiple pathogens in NPA from ARI patients. Given that respiratory infections are one of the most common reasons for visits to physicians, improved diagnostic methods such as nCounter can contribute to better patient management. Moreover, a transcriptomic approach involving pathogen detection, and as discussed above, immune response transcripts will enable the identification of biomarkers in ARI clinical samples, even those presenting low RNA yield (10–50 ng) such as NPA. This approach, combined with long-term clinical follow-up, will increase our understanding of ARI pathogenesis, disease progression (wheez-

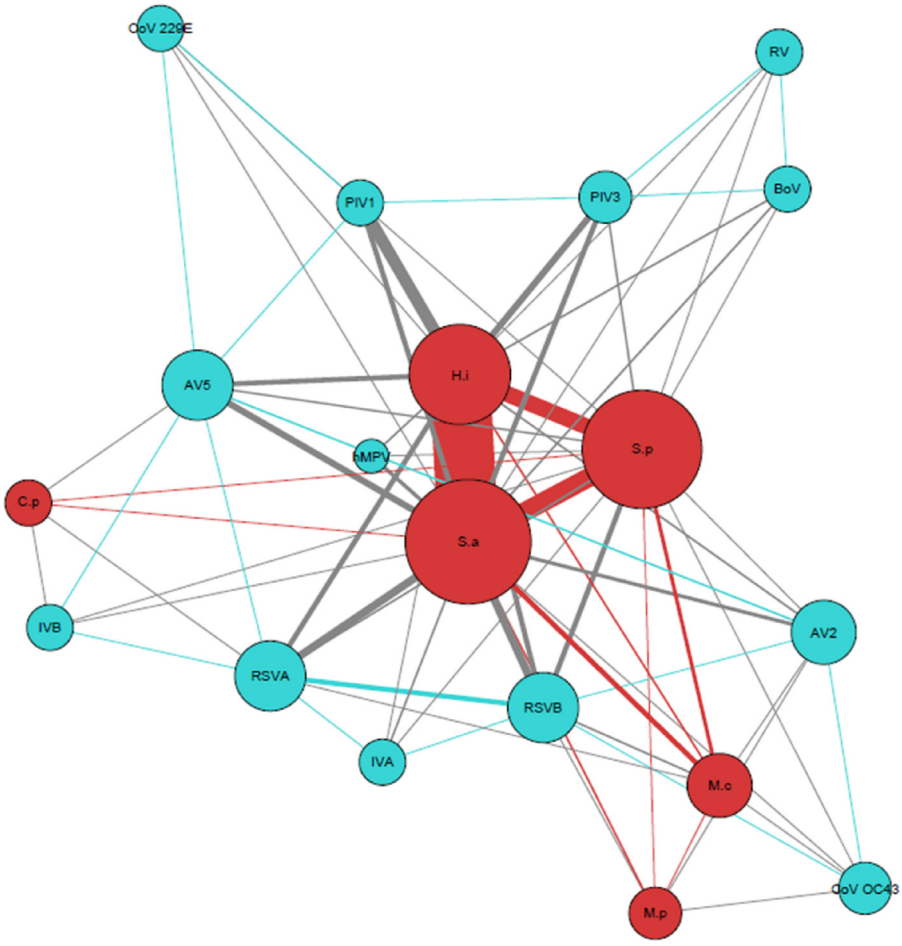


Fig. 5. Network distribution of viral and bacterial agents in NPA samples of children with ARI.
Each system component [Virus; RV, RSVA-B, IV A-B, PIV (types 1 and 3), AV (types 2 and 5), hMPV, BoV and CoV (229A and OC43) and bacteria (*S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus, C. pneumoniae and M. pneumoniae*) is indicated by a blue or red node, respectively] and is a partner of the network. Each line is a link between two nodes and its color represents interactions with similar category circles (blue or red) or with a different category (grey). The width of each line is a measure of the strength of the link between two nodes. The circles are represented according to the ForceAtlas2 scheme.

ing, asthma) and, more importantly, provide an evidence-based approach for the targeted and rational use of antibiotics in pediatric ARI.

Competing interests

Authors declare they have no conflicts of interest

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB; grant PNX0019/2009). KFF was supported by fellowships from Science without Borders (Conselho Nacional de Desenvolvimento Científico e Tecnológico; CNPQ) and FAPESB. RK was supported by a fellowship from CNPq. AB, CN-C and CIO are senior investigators from CNPq.

Ethical approval

Our study complied with the guidelines of the Declaration of Helsinki and was approved by the institutional review board of the Universidade Federal da Bahia (UFBA) (#067/2009). Written informed consent was obtained from all legal guardians.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2015.06.005

References

[1] A.T. Pavia, Viral infections of the lower respiratory tract: old viruses, new viruses, and the role of diagnosis, Clin. Infect Dis. 52 (Suppl. 4) (2011) S284–9.
[2] J.B. Mahony, Detection of respiratory viruses by molecular methods, Clin. Microbiol. Rev. 21 (2008) 716–747.
[3] T. Fahey, N. Stocks, T. Thomas, Systematic review of the treatment of upper respiratory tract infection, Arch. Dis. Child 79 (1998) 225–230.
[4] S. Yang, R.E. Rothman, PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings, Lancet Infect. Dis. 4 (2004) 337–348.
[5] G.K. Geiss, R.E. Bumgarner, B. Birditt, T. Dahl, N. Dowidar, D.L. Dunaway, et al., Direct multiplexed measurement of gene expression with color-coded probe pairs, Nat. Biotechnol. 26 (2008) 317–325.
[6] M.L. Bouzas, M.R. Cardoso, D. Sole, A. Barral, C.M. Nascimento-Carvalho, Acute Respiratory I, et al., Validating report of first episode of wheezing with pediatrician-detected wheezing among children, Pediatr. Allergy Immunol. 25 (2014) 198–200.
[7] L. Houspie, P. Lemey, E. Keyaerts, E. Reijmen, V. Vergote, A. Vankeerberghen, et al., Circulation of HRSV in Belgium: from multiple genotype circulation to prolonged circulation of predominant genotypes, PLoS One 8 (2013) e60416.
[8] D. Waggott, K. Chu, S. Yin, B.G. Wouters, F.F. Liu, P.C. Boutros, NanoStringNormn: an extensible R package for the pre-processing of NanoString mRNA and miRNA data, Bioinformatics 28 (2012) 1546–1548.

[9] S.D. Prokopec, J.D. Watson, D.M. Waggott, A.B. Smith, A.H. Wu, A.B. Okey, et al., Systematic evaluation of medium-throughput mRNA abundance platforms, RNA 19 (2013) 51–62.
[10] J.E. Payton, N.R. Grieselhuber, L.W. Chang, M. Murakami, G.K. Geiss, D.C. Link, et al., High throughput digital quantification of mRNA abundance in primary human acute myeloid leukemia samples, J. Clin. Invest. 119 (2009) 1714–1726.
[11] A.K. Barczak, J.E. Gomez, B.B. Kaufmann, E.R. Hinson, L. Cosimi, M.L. Borowsky, et al., RNA signatures allow rapid identification of pathogens and antibiotic susceptibilities, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 6217–6222.
[12] J. Robinson, Colonization and infection of the respiratory tract: what do we know, Paediatr. Child Health 9 (2004) 21–24.
[13] C. Silva Rde, M.A. Siqueira, E.M. Netto, J.S. Bastos, C.M. Nascimento-Carvalho, A.L. Vilas-Boas, et al., Epidemiological aspects of influenza A related to climatic conditions during and after a pandemic period in the city of Salvador, Northeastern Brazil, Mem Inst. Oswaldo Cruz. 109 (2014) 229–235.
[14] M.M. Pettigrew, J.F. Gent, R.B. Pyles, A.L. Miller, J. Nokso-Koivisto, T. Chonmaitree, Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection, J. Clin. Microbiol. 49 (2011) 3750–3755.
[15] B. Resch, W. Gusenleitner, W.D. Mueller, Risk of concurrent bacterial infection in preterm infants hospitalized due to respiratory syncytial virus infection, Acta Paediatr. 96 (2007) 495–498.
[16] H. Hishiki, N. Ishiwada, C. Fukasawa, K. Abe, T. Hoshino, J. Aizawa, et al., Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients, J. Infect. Chemother. 17 (2011) 87–90.
[17] L. Duttweiler, D. Nadal, B. Frey, Pulmonary and systemic bacterial co-infections in severe RSV bronchiolitis, Arch. Dis. Child 89 (2004) 1155–1157.
[18] K. Thorburn, S. Harigopal, V. Reddy, N. Taylor, H.K. van Saene, High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis, Thorax 61 (2006) 611–615.
[19] J. Segovia, A. Sabbah, V. Mgbemena, S.Y. Tsai, T.H. Chang, M.T. Berton, et al., TLR2/MyD88/NF-kappaB pathway, reactive oxygen species, potassium efflux activates NLRP3/ASC inflammasome during respiratory syncytial virus infection, PLoS One 7 (2012) e29695.
[20] R. Fang, K. Tsuchiya, I. Kawamura, Y. Shen, H. Hara, S. Sakai, et al., Critical roles of ASC inflammasomes in caspase-1 activation and host innate resistance to *Streptococcus pneumoniae* infection, J. Immunol. 187 (2011) 4890–4899.
[21] B. Moens, C. Pannecouque, G. Lopez, M. Talledo, E. Gotuzzo, R. Khouri, et al., Simultaneous RNA quantification of human and retroviral genomes reveals intact interferon signaling in HTLV-1-infected CD4+ T cell lines, Virol. J. 9 (2012) 171.

IV.I MANUSCRITO II.

Immune signatures of nasopharyngeal aspirates associated with microbial presence in children with Acute Respiratory Infection.

Title page

Immune signatures of nasopharyngeal aspirates associated with microbial presence in children with Acute Respiratory Infection.

Kiyoshi F.Fukutani[a,b], Cristiana M. Nascimento-Carvalho[b,c], Maiara L. Bouzas[b], Juliana R. Oliveira[b], AldinaBarral[a,b], Tim Dierckx[d], Ricardo Khouri[a,b], HelderI. Nakaya[e], Bruno B. Andrade[a,f], Johan Van Weyenbergh[d], Camila I. de Oliveira[a,b]

[a]InstitutoGonçaloMoniz-FIOCRUZ, Salvador, BA, Brazil

[b]Post-graduation Program in Health Sciences, Federal University of Bahia School of Medicine, Salvador,BA, Brazil

[c]Department of Pediatrics, School of Medicine, Federal University of Bahia, Salvador, BA, Brazil

[d]Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium

[e]School of Pharmaceutical Sciences, University of São Paulo, São Paulo,Brazil

[f]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, FundaçãoJosé Silveira, Salvador, Brazil

Keywords: network analysis, bayesian analysis, ARI immune response, nCounter

Running title: Biomarkers of ARI

Correspondence and requests for reprints should be addressed to Camila I. de Oliveira, InstitutoGonçaloMoniz-FIOCRUZ, Salvador, BA, Brazil, RuaWaldemarFalcão, 121, Candeal - Salvador/BA CEP: 40296-710, E-mail: camila@bahia.fiocruz.br

Summary of the article's main point: We investigated the microbial load and immune response in ARI, using nasopharyingeal aspirates and a transcriptional approach. We identified three immune markers associated with bacterial load and one marker associated with viral load, none of which clustered with clinical symptoms.

ABSTRACT

Background: Acute Respiratory Infection (ARI) is the most frequent cause for hospitalization in infants and young children. The microbial agents (viruses and bacteria) most frequently associated with ARI development are currently known, however thehost immune responses involved in this process, particularly at the nasopharyngeal interface, have not been thoroughly investigated.

Methods: The purpose of this study was to investigate the microbial and concomitant immune signature present in nasopharyngeal aspirates (NPAs) of children with ARI. To address these questions, NPAs (n = 58) from children <2yr with clinical diagnosis of ARI, enrolled over a period of 2 years, were examined using multiplexed nCounter technology, digitally quantifyinga total of 600 human, 14 viral and 5 bacterial probes.

Results: In this study sample, 27 (46.6%) NPAs presented transcripts for bacteria only, 21(36.6%) presented transcripts for bacteria and virus (co-detection) and 5 (8.6%) presented transcripts for virus only. In five (8.6%) NPAstranscript quantification scored below our established cut-off values and these were defined as indeterminate. Clinical presentation was not significantly different among children classified as viral,bacterial or co-detection. Further analyses identified 30 immune response genes that were commonly expressed in NPAs. Network analysis pinpointed four genes directly associated with microbial loadwhereas Bayesian network analysis did not show associations between microbial load and clinical symptoms.

Conclusion: By examining NPA samples from children with ARI, we simultaneously determined the microbial burden and the ongoing immune signatures, enabling the identification of biomarkers of the host response to ARI.

INTRODUCTION

Respiratory tract infections (RTI) cause great morbidity and mortality in children across the globe. The annual worldwide incidence of RTI was estimated in 156 million cases, 43% of which were infants or young children [1]. In 2013, Pneumonia was among the top three infectious diseases leading to child mortality [2]. Acute respiratory infection (ARI) is mainly associated with viral infections and is confined to the upper respiratory tract, causing cough and hoarseness [3]. However, infection may expand to the lower respiratory tract causing wheezing and respiratory distress. ARI caused by the association between viruses and bacteria can lead to secondary complications such as otitis media [4],whereas viral-alone infection may cause recurrent wheezing, a risk factor for subsequent asthma development[5]. This complex scenario is aggravated by the fact that it is not possible to differentiate between viral and bacterial ARI based on clinical signs or radiology. Therefore, precisedetection of the microbial agents could optimize patient care by reducing the use of unwanted antibiotics.

Transcriptome analyses provide a comprehensive and efficient way to elucidate the host's immune response to infectious disease [6, 7] and vaccines [8]. Recent investigationshave used blood transcriptomic analysis to identify immune signatures in community-acquired pneumonia [9], respiratory syncytial virus infection [10] and rhinovirus infection [11]. Additionally, such analyses arerobust enough to distinguish bacterial from viral respiratory infection, suggesting that host gene expression can improve diagnosis[12]. We have previously reported the use of nCounter technology to identify viral and bacterial

agents in nasopharyngeal aspirates (NPAs) from children with ARI, enrolled at a reference Pediatric Emergency Unit in Brazil [13]. In the present study, weanalyzed the microbial (viral and bacterial load) in these same NPAs, through RNA detection, and studied the accompanying immune-related signature. Using dimensional reduction analyses, we identified 30 genes that were commonly expressed in NPAs classified according to the type of microbe found. Network analysis further pinpointed four genes directly associated with viral and bacterial load. Bayesian analysis showed that clinical symptoms are dissociated from expression of these four markers.

METHODS

Study Design and Patients

We performed a cross-sectional study involving NPAs obtained from children with confirmedARI. A total of 576patientswere enrolled, at the Pediatric Emergency Unit of the Federal University of Bahia (UFBA, Salvador, Brazil) between September 2009 and October 2013.Inclusion criteria were presence of clinical symptoms of ARI such as fever, cough, sneeze or nasal obstruction for a period up to seven days, age 6–23 months and negative history of previous wheezing episodes. Written informed consent was obtained from all legal guardians. The study complied with the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of the Universidade Federal da Bahia (067/2009).

RNA extraction and gene expression analysis

60 NPAs, randomized from the larger cohort (n = 576), were used for RNA extraction,performed using RNEasy (Qiagen), according tomanufacturer'sinstructions and RNA was quantified using Qubit RNA HS assay (Invitrogen). Total RNA (50 ng) was subsequently hybridized against probes custom designed for microbial detection [Adenovirus (AV) 2 and 5, human Bocavirus (hBoV), Coronavirus (CoV) 229E and OC43, Respiratory Syncytial Virus (RSV) A and B, Influenza virus (IV) A and B, Parainfluenzavirus (PIV) 1, 2 and 3, Rhinovirus (RV), human Metapneumovirus(hMPV), *Chlamydiapneumoniae, Haemophilusinfluenzae, Moraxella catarrhallis, Mycoplasma pneumoniae and Stretococcuspneumoniae*], as previously described [13]. RNA was also hybridized against probes in the immunology custom set V2 (http://www.nanostring.com/products/gene_expression_panels), all synthesized and designed by NanoStringTechnologies®. The nCounter (NanoString®) reactions were performedat NucleomicsCore Facility (VIB, Leuven-Belgium), as previously described [14].

Data Analyses

Raw data were pre-processed using both nSolver 2.0 software (Nanostring Technologies®) and the NanoStringNorm R package [15], as described [13]. All data were log10 transformed. To determine microbial load, we established the cut off values for each NPA tested as the mean number of digital counts detected in eight exogenous internal negative controls (spiked in each samples) plus three standard deviations. Samples presenting microbial counts above the cut-off were considered positive (n = 53) and were classified as viral, bacterial or co-detection (viral and bacterial). Samples presenting microbial counts below

the cutoff were considered indeterminate (n=5) (Supplementary Table1).Outliers, regarding the expression of immune transcripts, were identified using ROUT [16] and two samples (ARI197 and ARI198) were removed from further analyses. Immune response transcripts were selected by multiplet-test. Analyses were performed to identify differentially expressed genes in each defined group (bacterial, viral, co-detection and indeterminate), considering $p<0.05$ and only genes differentially modulated in ≥3 comparisons were considered, normalized by Z-score and visualized in a heat map, obtained in JMP (SAS, v.11). The individual gene expression perturbation was measured by molecular distance to health. The distance of each sample (viral, bacterial or co-detectionfrom the indeterminate (control baseline) was calculated as described [17, 18]. Gene Ontology enrichment test was performed using Enrichr[19]. The correlation network displaying significant interactions ($p<0.001$, Pearson) among immune response-related genes and microbial load was constructed using GraphPad (Prism, V.5) and the modular community analysis was obtained using Gephi modulatory statistical package. Network stability was examined using atlas force 2 algorithm. Strength connection was assessed with a non-parametric bootstrap (100× replicates) and arcs with bootstrap over 60% were considered.Bayesian network learning [20] was used to describe and visualize conditional dependencies between the multiple variables. Dependencies were represented qualitatively by a directed acyclic graph where each node corresponds to a variable and a direct arc between nodes represents a direct influence. Robustness of the arcs was scored by a non-parametric bootstrap test (100 × replicates) and only arcs with more than 20% support were depicted.

RESULTS

NPAswere submitted to transcriptomicanalysis targeting both microbial transcripts and immune-related genes. Microbial transcripts were detected in most samples (53/58, 92.4%), which were then classified as bacterial, viral or co-detection (Figure 1). Five NPAs were classified as indeterminate since transcript counts were below the established cut off(Figure 1A). Five NPAs (5/58, 8.6%) had exclusivelyviral transcripts, including transcripts for RSV A and B (2/5, 40%), hMPV and IVA (1/5, 20%). The largest group of NPAs had exclusively bacterial transcripts (27/58, 46.6%), and among these *H. influenzae* was most prevalent (89.2%)followed by *S. pneumoniae* (14.2%), *M. catarrhalis* (7.1%) and *M. pneumoniae* (3.5%) (Figure 1B). Co-detection (21/58, 36.2%) was determined based on the presence of both viral and bacterial transcripts: *H. influenzae* (90.9%) and *S. pneumoniae* (27.2%) were ubiquitous whereas PIV3 (22.7%), AV5 and RSVA (18.1%) were the most common viruses co-detected with bacteria. In co-infected samples, PIV1, RSVB, AV2, BoV, CoV, hMPV and RVwere present in less than 15% of NPAs.The presence of different viruses(n=1), multiple bacteria (n=3) and multiple viruses plus bacteria (n=9) were also observed (Supplemental Table 2).No significant differences regarding age, gender or clinical symptoms were found when comparing NPAsclassified as bacterial, viral, co-detection or indeterminate were found (Table 1). The most common symptoms at the time of admission were cough (presented by 81-100% of children), sneeze (76-100%) and fever (78-100%).

After identifying the NPA microbial signatures, we next searched for immune-related gene signatures. Comparison of NPAs classified as bacterial, viral, co-detection and indeterminate yielded lists of differentially expressed genes (Figure 2A) that include a broad range of innate and adaptive immune-related

genes. For instance, compared to indeterminate samples, viral NPAsshowed overall lower expression of 33 genes, including*CD19*, *CCR5* and *IL-7* (Figure 2A). On the other hand, 15 and 7 genes were differentially expressed in NPAs classified as co-detection or bacterial, respectively, compared to indeterminate NPAs. To identify a global molecular signature of ARI, we searched for genes that were differentially expressed (DEGs) in at least three comparisons out of 6 total comparisons (Figure 2B). This dimensional reduction analysis yielded a list of 30 genes (Figure 2C) that characterizes the NPA transcriptional signature ofARIandincluded: cytokines and chemokines/chemokine receptors(*IL6*, *IFNL3*, *IL19*, *TNFSF12*, *CCL8* and *CCR6*), markers of innate response (*MRC1*, *C4BPA*, *CD6*,*CR2* and *DEFB4A*), and molecules related to B cell responses (*AICDA*, *CXCL13*, *CD19*, *MS4A1*, *CD22*, *CD5*, *CD79A* and the BAFF receptor *TNFRSF13C*), among others.

Examinationof the overall expression pattern of the 30-gene set, according to the NPA classification, showeda stronger upregulation in NPAs classified as co-detecion and general downregulation in NPAs classified as viral infection (Figure 3A). Functional enrichment analysis using Gene Ontology (GO) terms indicated the “humoral immune response” as the top enriched term (Figure 3B). This analysis suggests that theNPA immune response is marked by activation of B cell responses, in line with the differential expresses of genes such as *AICDA*, *CD19*, *CD79*, as described earlier.

To examine the degree of transcriptional perturbation in the signatures observed for NPAs classified as bacterial, viral and co-detection, we calculated the

molecular distance to health (MDTH)[17, 18], using as baseline NPAs classified as indeterminate.Using the 30 DEGs(Figure 2),the degree of perturbation (MDTH) was significantly higher in NPAs classified as bacterial, viral and co-infected (Figure 4A), compared to indeterminatesamples.However, MDTH did not differ comparing the type of microbe (viral, bacterial or codetected) present in NPAs. Examination of the degree of perturbation per individual NPAalso did not indicate an associationbetween MDTH vs. viral presence or co-detection (Figure 4B); however, we observed a borderline correlation between MDTH and bacterial presence (r = 0.37, p = 0.053). Similar results were obtained when we compared perturbation in gene expression considering all immune transcriptsdetected (data not shown).

Given the weak association between global gene perturbation and microbial load, we next examined whether single or combined genes could bettercorrelate with microbial load. To answer this question, we performed a correlation network analysis including the 30 DEGs in NPAs and bacterial or viral load as additional parameters (Figure 5). We thus identified a largergroupof immune -related genes (containing 24 genes) further subdividied into smaller subgroups. One of these subgroups directly correlated to thegroup containing bacterial load and genes related to innate response (MRC1 and C4BPA) and B cell response (AICDA3). Another of these subgroups directly correlated to another and distinct group containing viral load and IFNL3, an anti-viral cytokine, indicating that these are biomarkers of ARI associated with bacterial and viral presence, respectively. A fourth independent module grouped genes related to antigen presentation (HLADQ).

In univariateanalysis (Table 1), we did not find significant differences in the presence of clinical symptoms among NPAs classified as viral and/or bacterial or classified as indeterminate.Given the network association among four single genes (C4BPA, MRC1, AICDA and IFNL3) with bacterial and viral load, respectively (Figure 5), weemployed a Bayesian network (BN) analysis compiling these markers and clinical information (Table 1) to identify direct and indirect relationships. The BN approach confirmed the connection of viral load and IFNL3 (Figure 6) and showed the connection of viral and bacterial load and the latter with AICDA and MRC1, again corroboration network findings (Figure 5). Considering the clinical symptoms, hoarseness was connected to sneeze, cough and nasal occlusion and with CD19, a marker of B cell response. Nasal occlusion was also connected to CD79A, a molecule involved in expression and function of the B- cell receptor. The BN also revealed that clinical symptoms were dissociated from microbial load and from the immune markers associated with it (AICDA, MRC1 and IFNL3).

DISCUSSION

Despite the global impact of ARI, understanding of the immune response that occurs at the nasopharyngeal interface is still incomplete. Herein, we characterized the immune response at the NPA interface in children with ARI and probed for associations with microbial load and clinical symptoms.

Regarding microbial presence, few samples (<10%) presented viral transcripts only and RSV (A and B) was the most prevalent virus, followed by hMPV and IV.

These results are in agreement with a report showing the presence of these viruses in children attending clinics and hospitals in Northeastern Brazil [21]. In terms of bacteria, 46,6% NPAs presented transcripts for, mostly, *H. influenzae* followed by *S. pneumoniae and M. catarrhalis,* all of which are part of the nasopharynxmicrobiome[22]. When new bacterial or viral acquisition occurs, a potential disturbance in the microenvironment can lead to invasion, dissemination and complications.Following experimental introduction, established populations of *H. influenzae* and *S. pneumoniae*inihibited invasion of new *S. aureus* population [23]. Recurrent otitis media has been associated in children presenting higher detection rates of RV, PIV, AV and RSV in the nasopharynx[24]. RV infection stimulated *S. pneumoniae* adhesion to airway epithelial cells [25] whereas RSV and PIV2 increased the expression of receptors for pathogenic bacteria [26], these being two possible mechanistic explanations for complicated disease in simultaneous bacterial/viral infection. Although we detected certain NPAs presenting multiple microbial transcripts (for example, PIV, RSV and IV were co-detected with *H. influenzae*), clinical symptoms presented by such children did not differ significantly from those harboring a single infection, at least at the time of admission. Regarding viral ARI, increased disease severity in the case of multiple co-detectionhas not been clinically observed (reviewed in[27]). However, most of the works reviewed thereinemployedDNA detection which may reflect a range of conditions, differently from our strategy that employed mRNA detection, thus mirroring active viral replication.

Following the classification of NPAs according to the microbial load, we sought to determine the immune signature. Initially, we compared the profile in NPAs

classified as viral, bacterial and/or co-detection, resulting in a set of 30 genes that are differentially modulated in at least three of these groups. Examination of the profiles showed overexpression of these 30 genes in NPAs classified as co-detectionand a general suppressionin NPAs classified as viral; humoral immune response was the top GO enrichment category. MDTH analysis revealed that a significant degree of transcriptomic perturbation was observed in all NPA groups, independent of the classification (viral, bacterial or co-detection) and irrespective of the microbial load. This somewhat surprising dissociation between microbial load and gene expression profile, in terms of the immune response, led us to use network analysis to better understand the relationship between these parameters. Indeed, of the 30 differentially expressed genes, IFNL3 was the only marker associated with viral load,suggesting a bystander effect of the remaining 24 differentially expressed genes. Type III IFN such as IFLN3 (IL28B) induces a variety of ISGS, interfering with viral replication [28]. IFNL3is expressed in response to RSV [29]and IV [30], both detected in the NPAs, and IFNLs seem to be the major source of IFN in airway epithelial cells [31].With regards to the bacterial module, three genes were associated with bacterial load: AICDA, MRC1 and C4BPA . C4BPA (C4b-binding protein)inhibits classical pathway complement activation and subsequent opsonization followed by lysis. C4BPA is bound by several bacterial pathogens, including *H. influenzae[32]* and *M.catarrhalis[33]*, enhancing bacterial adhesion. The mannose receptor MRC1 recognizes bacterial polyssacharideand is involved in the uptake of *S. pneumoniae[34]*. Finally, AICDA is a DNA editing protein that plays an essential role in somatic hypermutation, class switch recombination and Ig gene conversion. Collectively, these data indicate that bacterial presence

in NPAs is sensed by MRC1 and expression of C4BPA may be involved in pathogenesis and triggering of a humoral immune response. Viral presence, on the other hand, triggers and anti-viral response, characterized by expression of IFNL3.

The Bayesian network reinforced certain findings of the correlation network: a connection between bacterial load and the immune markers AICDA and MRC1and between viral load and IFNL3. Importantly, it highlightedthe direct association between bacterial load and viral load, reinforcing that the interplay between viral and microbial loadcontributes to the dynamics of the nasopharynxmicrobiome. Indeed, simultaneous viral and bacterial presence can disturb an existing equilibrium leading tocomplications such as otitis media, for example[4]. Although we did not probe for secondary complications herein, this is an existing possibility since 36.2% NPAs harbored both viral and bacterial transcripts.The Bayesian network also showed that symptoms such as hoarseness, sneeze, cough and nasal occlusion were dissociated from either bacterial or viral load, reinforcing the need to identify more specific biomarkers of viral and/or bacterial infection in ARI [11, 35]. Although RSV and RV have been implicated in wheezing and viral presence is a significant predictor of asthma development in children [36], we did not find an association between viral load and wheezing herein, possibly due to the limited number of NPAs classified as viral.We also observed a direct association between hoarseness and CD19 and between nasal occlusion and CD79A, a protein associated with activation of the B cell receptor, indicating a link between these two clinical symptoms and humoral immune response.

In conclusion, this study addressed the immune response in NPAs from children with ARI. Upon classification of the NPAs as viral, bacterial or co-detection, we identified a group of 30 genes that are commonly modulated in ARI. Further network analyses allowed us identify four genes directly associated with microbial load, none of which were connected to the common symptoms of ARI such as cough, sneeze and nasal occlusion. Approaches such as the one employed here can help elucidate factors contributing to ARI in pediatric patients, leading to identification of candidate molecules for further investigation.

Funding

Thisworkwassupportedby Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB; grantPNX0019/2009to AB). KFF was supported by a fellowship from Science without Borders. TD was supported by the Institute for the Promotion of Innovation through Science and Technology Flanders (IWT-Vlaanderen), project 141614CMNC, AB and CIO are senior investigators from ConselhoNacional de PesquisaCientífica (CNPq).

Potential conflicts of interest. All authors: No reported conflicts.

Acknowledgments

The authors are in debt to the pediatricians and nurses of the Professor Hosannah de Oliveira Pediatric Center, FederalUniversity of Bahia, Salvador, Brazil, for their cooperation in recruiting the patients.

References

1. Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H. Epidemiology and etiology of childhood pneumonia. Bull World Health Organ **2008**; 86(5): 408-16.
2. Liu L, Oza S, Hogan D, et al. Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet **2015**; 385(9966): 430-40.
3. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev **2010**; 23(1): 74-98.
4. Ruohola A, Pettigrew MM, Lindholm L, et al. Bacterial and viral interactions within the nasopharynx contribute to the risk of acute otitis media. J Infect **2013**; 66(3): 247-54.
5. Inoue Y, Shimojo N. Epidemiology of virus-induced wheezing/asthma in children. Front Microbiol **2013**; 4: 391.
6. Chaussabel D, Pascual V, Banchereau J. Assessing the human immune system through blood transcriptomics. BMC Biol **2010**; 8: 84.
7. Nakaya HI, Gardner J, Poo YS, Major L, Pulendran B, Suhrbier A. Gene profiling of Chikungunya virus arthritis in a mouse model reveals significant overlap with rheumatoid arthritis. Arthritis Rheum **2012**; 64(11): 3553-63.
8. Nakaya HI, Clutterbuck E, Kazmin D, et al. Systems biology of immunity to MF59-adjuvanted versus nonadjuvanted trivalent seasonal influenza

vaccines in early childhood. Proc Natl Acad Sci U S A **2016**; 113(7): 1853-8.

9. Parnell GP, McLean AS, Booth DR, et al. A distinct influenza infection signature in the blood transcriptome of patients with severe community-acquired pneumonia. Crit Care **2012**; 16(4): R157.
10. Mejias A, Dimo B, Suarez NM, et al. Whole blood gene expression profiles to assess pathogenesis and disease severity in infants with respiratory syncytial virus infection. PLoS Med **2013**; 10(11): e1001549.
11. Heinonen S, Jartti T, Garcia C, et al. Rhinovirus Detection in Symptomatic and Asymptomatic Children: Value of Host Transcriptome Analysis. Am J Respir Crit Care Med **2015**.
12. Suarez NM, Bunsow E, Falsey AR, Walsh EE, Mejias A, Ramilo O. Superiority of transcriptional profiling over procalcitonin for distinguishing bacterial from viral lower respiratory tract infections in hospitalized adults. J Infect Dis **2015**; 212(2): 213-22.
13. Fukutani KF, Nascimento-Carvalho CM, Van der Gucht W, et al. Pathogen transcriptional profile in nasopharyngeal aspirates of children with acute respiratory tract infection. J Clin Virol **2015**; 69: 190-6.
14. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol **2008**; 26(3): 317-25.
15. Waggott D, Chu K, Yin S, Wouters BG, Liu FF, Boutros PC. NanoStringNorm: an extensible R package for the pre-processing of NanoString mRNA and miRNA data. Bioinformatics **2012**; 28(11): 1546-8.

16. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinformatics **2006**; 7: 123.

17. Pankla R, Buddhisa S, Berry M, et al. Genomic transcriptional profiling identifies a candidate blood biomarker signature for the diagnosis of septicemic melioidosis. Genome Biol **2009**; 10(11): R127.

18. Berry MP, Graham CM, McNab FW, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature **2010**; 466(7309): 973-7.

19. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics **2013**; 14: 128.

20. Kouri V, Khouri R, Aleman Y, et al. CRF19_cpx is an Evolutionary fit HIV-1 Variant Strongly Associated With Rapid Progression to AIDS in Cuba. EBioMedicine **2015**; 2(3): 244-54.

21. Cuevas LE, Nasser AM, Dove W, Gurgel RQ, Greensill J, Hart CA. Human metapneumovirus and respiratory syncytial virus, Brazil. Emerg Infect Dis **2003**; 9(12): 1626-8.

22. Bogaert D, Keijser B, Huse S, et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS One **2011**; 6(2): e17035.

23. Margolis E, Yates A, Levin BR. The ecology of nasal colonization of Streptococcus pneumoniae, Haemophilus influenzae and

Staphylococcus aureus: the role of competition and interactions with host's immune response. BMC Microbiol **2010**; 10: 59.

24. Wiertsema SP, Chidlow GR, Kirkham LA, et al. High detection rates of nucleic acids of a wide range of respiratory viruses in the nasopharynx and the middle ear of children with a history of recurrent acute otitis media. J Med Virol **2011**; 83(11): 2008-17.
25. Ishizuka S, Yamaya M, Suzuki T, et al. Effects of rhinovirus infection on the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells. J Infect Dis **2003**; 188(12): 1928-39.
26. Avadhanula V, Rodriguez CA, Devincenzo JP, et al. Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. J Virol **2006**; 80(4): 1629-36.
27. Nascimento-Carvalho CM, Ruuskanen O. Clinical significance of multiple respiratory virus detection. Pediatr Infect Dis J **2015**.
28. Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. Nat Rev Immunol **2008**; 8(7): 559-68.
29. Spann KM, Tran KC, Chi B, Rabin RL, Collins PL. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. J Virol **2004**; 78(8): 4363-9.
30. Durbin RK, Kotenko SV, Durbin JE. Interferon induction and function at the mucosal surface. Immunol Rev **2013**; 255(1): 25-39.
31. Khaitov MR, Laza-Stanca V, Edwards MR, et al. Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial

cells and peripheral blood mononuclear cells. Allergy **2009**; 64(3): 375-86.

32. Hallstrom T, Jarva H, Riesbeck K, Blom AM. Interaction with C4b-binding protein contributes to nontypeable Haemophilus influenzae serum resistance. J Immunol **2007**; 178(10): 6359-66.

33. Nordstrom T, Blom AM, Forsgren A, Riesbeck K. The emerging pathogen Moraxella catarrhalis interacts with complement inhibitor C4b binding protein through ubiquitous surface proteins A1 and A2. J Immunol **2004**; 173(7): 4598-606.

34. Zamze S, Martinez-Pomares L, Jones H, et al. Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. J Biol Chem **2002**; 277(44): 41613-23.

35. Hu X, Yu J, Crosby SD, Storch GA. Gene expression profiles in febrile children with defined viral and bacterial infection. Proc Natl Acad Sci U S A **2013**; 110(31): 12792-7.

36. Jackson DJ, Gangnon RE, Evans MD, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am J Respir Crit Care Med **2008**; 178(7): 667-72.

Table 1. Demographic and clinical characteristics of children with ARI .

Parameter	Bacteria	Co-detection	Virus	Indeterminate[a]	*p*
Count	27	21	5	5	
Age (months)	8.6	9.1	11.7	14.4	0.488
	[7.1-14.3]	[7.1-13.7]	[7.7-15.9]	[7.9-19.5]	
Gender	12F, 15M	15F,6M	3F, 2M	4F,1M	0.203
Cough	25 (93%)	17 (81%)	5 (100%)	4 (80%)	0.474
Sneeze	24 (89%)	16(76%)	5 (100%)	4 (80%)	0.475
Nasal occlusion	3 (11%)	3 (14%)	2 (40%)	1 (20%)	0.425
Fever	21 (78%)	18 (86%)	4 (80%)	5 (100%)	0.644
Hoarseness	7 (26%)	9(43%)	5 (100%)	5 (100%)	0.001
Otalgia	4 (15%)	0 (0%)	2 (40%)	2 (40%)	0.027
Wheezing	5 (18%)	2 (9%)	1 (20%)	0 (0%)	0.619

Median values [min-max range] or number (percent).

[a]NPAs that scored below the established cut off.

Statistics: for continuous variables, Kruskall-Wallis test was used; for discrete variables, Chi-square test was used.

Figure 1.

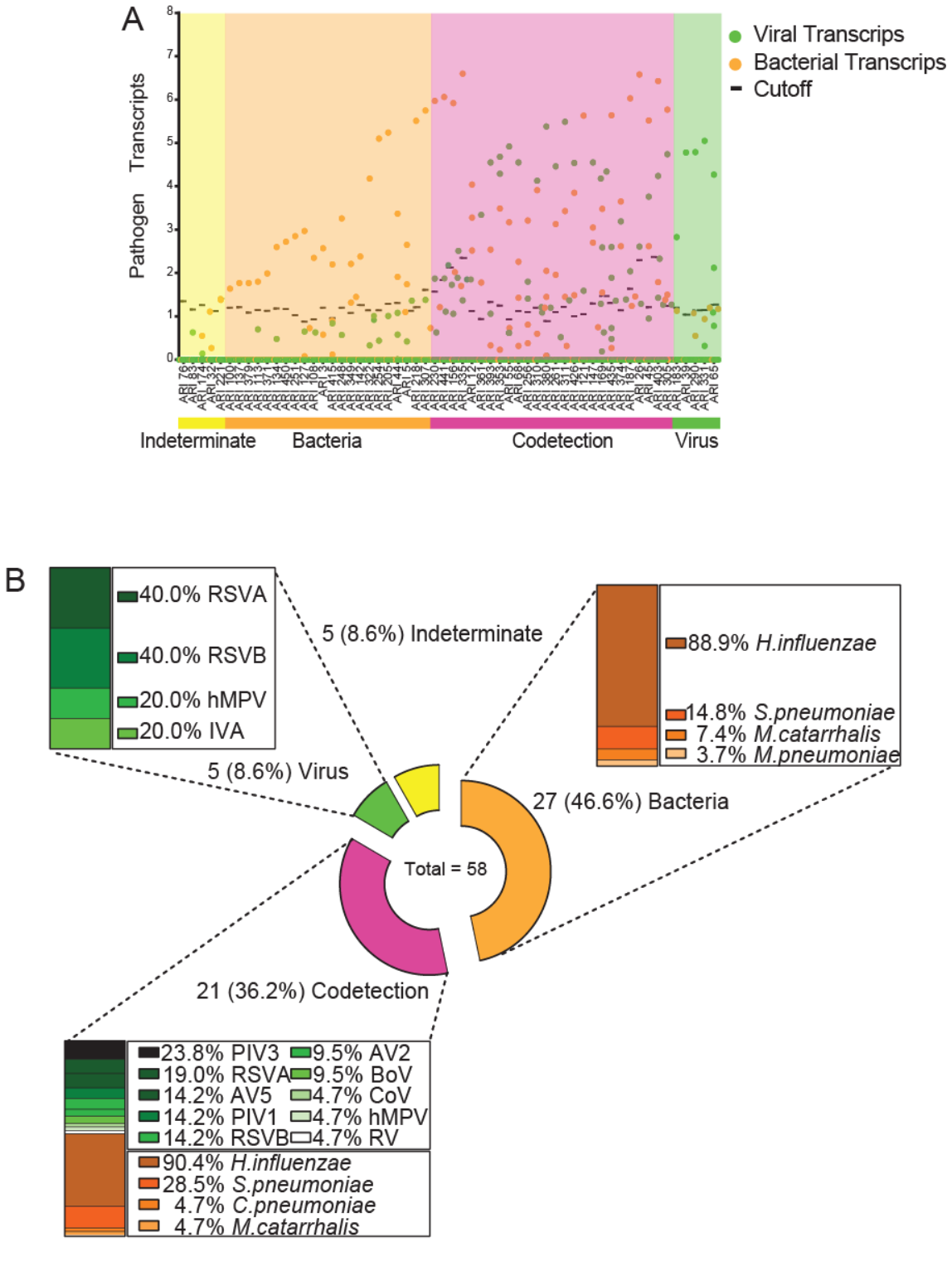

A
Pathogen Transcripts
Viral Transcrips
Bacterial Transcrips
Cutoff
Indeterminate
Bacteria
Codetection
Virus
B
40.0% RSVA
40.0% RSVB
20.0% hMPV
20.0% IVA
5 (8.6%) Virus
5 (8.6%) Indeterminate
88.9% H.influenzae
14.8% S.pneumoniae
7.4% M.catarrhalis
3.7% M.pneumoniae
27 (46.6%) Bacteria
Total = 58
21 (36.2%) Codetection
23.8% PIV3
19.0% RSVA
14.2% AV5
14.2% PIV1
14.2% RSVB
9.5% AV2
9.5% BoV
4.7% CoV
4.7% hMPV
4.7% RV
90.4% H.influenzae
28.5% S.pneumoniae
4.7% C.pneumoniae
4.7% M.catarrhalis

Figure 2.

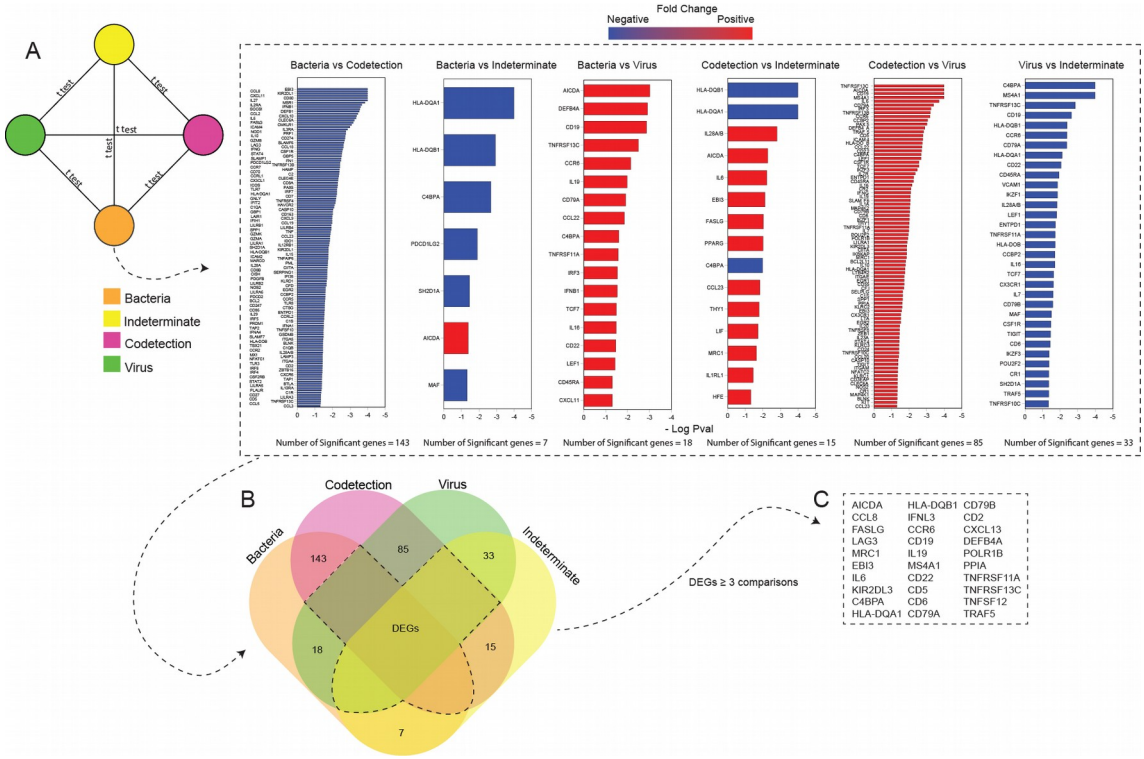

A
t test
Bacteria
Indeterminate
Codetection
Virus
Fold Change
Negative
Positive
Bacteria vs Codetection
Bacteria vs Indeterminate
Bacteria vs Virus
Codetection vs Indeterminate
Codetection vs Virus
Virus vs Indeterminate
- Log Pval
Number of Significant genes = 143
Number of Significant genes = 7
Number of Significant genes = 18
Number of Significant genes = 15
Number of Significant genes = 85
Number of Significant genes = 33
B
Codetection
Virus
Bacteria
Indeterminate
143
85
33
DEGs
18
15
7
DEGs ≥ 3 comparisons
C
AICDA HLA-DQB1 CD79B
CCL8 IFNL3 CD2
FASLG CCR6 CXCL13
LAG3 CD19 DEFB4A
MRC1 IL19 POLR1B
EBI3 MS4A1 PPIA
IL6 CD22 TNFRSF11A
KIR2DL3 CD5 TNFRSF13C
C4BPA CD6 TNFSF12
HLA-DQA1 CD79A TRAF5

Figure 3.

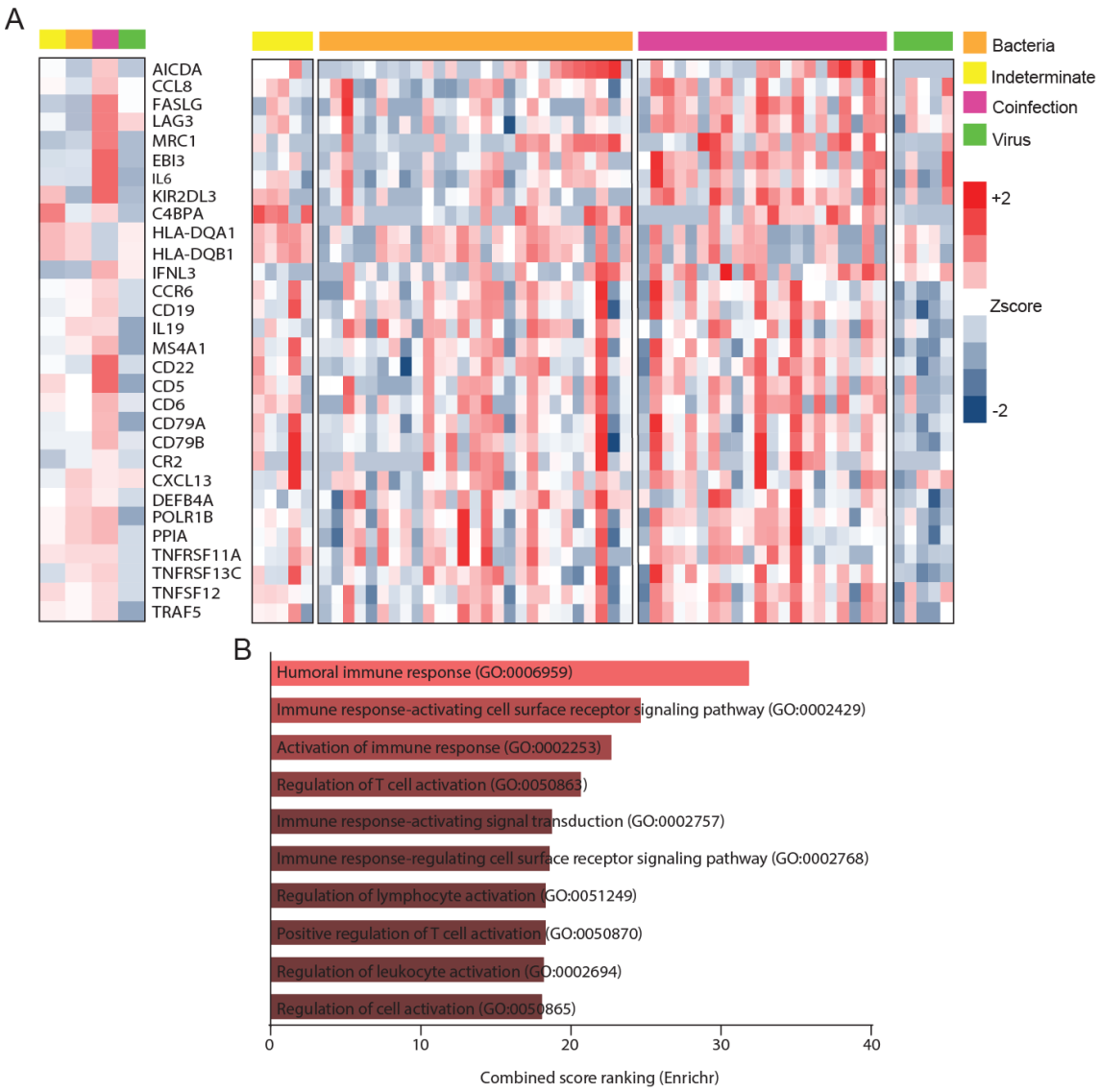

A
AICDA
CCL8
FASLG
LAG3
MRC1
EBI3
IL6
KIR2DL3
C4BPA
HLA-DQA1
HLA-DQB1
IFNL3
CCR6
CD19
IL19
MS4A1
CD22
CD5
CD6
CD79A
CD79B
CR2
CXCL13
DEFB4A
POLR1B
PPIA
TNFRSF11A
TNFRSF13C
TNFSF12
TRAF5
Bacteria
Indeterminate
Coinfection
Virus
+2
Zscore
-2
B
Humoral immune response (GO:0006959)
Immune response-activating cell surface receptor signaling pathway (GO:0002429)
Activation of immune response (GO:0002253)
Regulation of T cell activation (GO:0050863)
Immune response-activating signal transduction (GO:0002757)
Immune response-regulating cell surface receptor signaling pathway (GO:0002768)
Regulation of lymphocyte activation (GO:0051249)
Positive regulation of T cell activation (GO:0050870)
Regulation of leukocyte activation (GO:0002694)
Regulation of cell activation (GO:0050865)
0
10
20
30
40
Combined score ranking (Enrichr)

Figure 4.

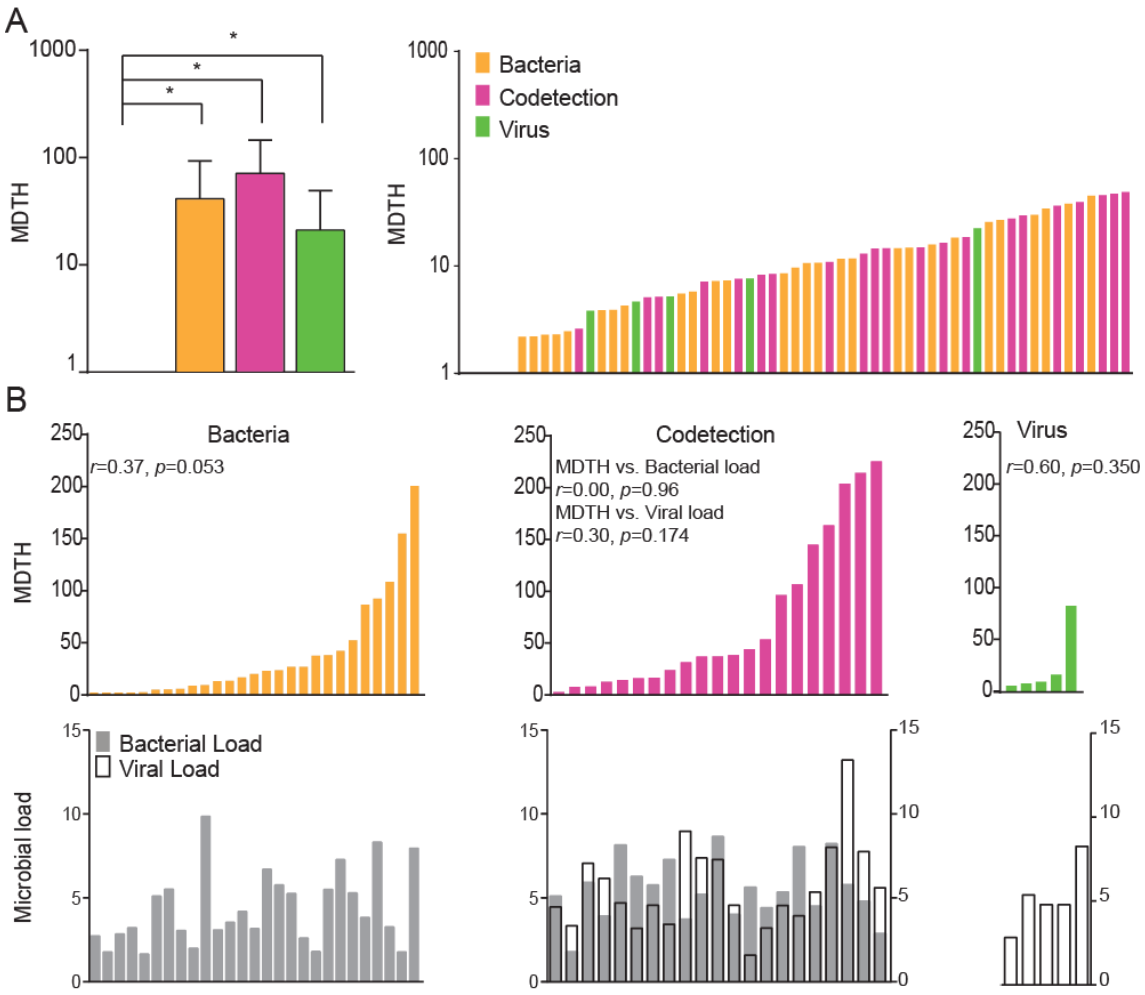

A
MDTH
1000
100
10
1
*
*
*
Bacteria
Codetection
Virus
B
Bacteria
r=0.37, p=0.053
Codetection
MDTH vs. Bacterial load
r=0.00, p=0.96
MDTH vs. Viral load
r=0.30, p=0.174
Virus
r=0.60, p=0.350
250
200
150
100
50
0
Bacterial Load
Viral Load
Microbial load
15
10
5
0

Figure 5.

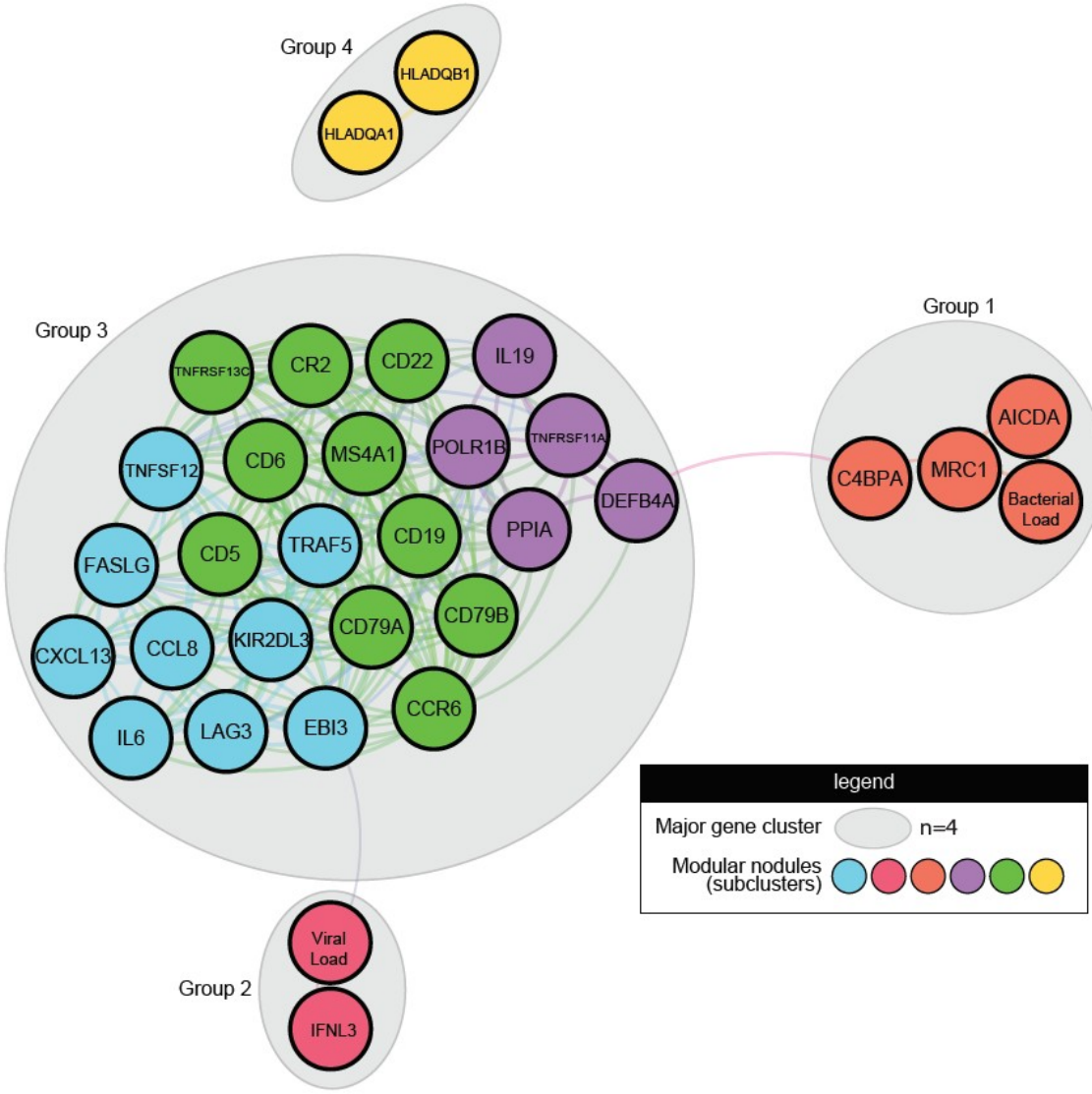
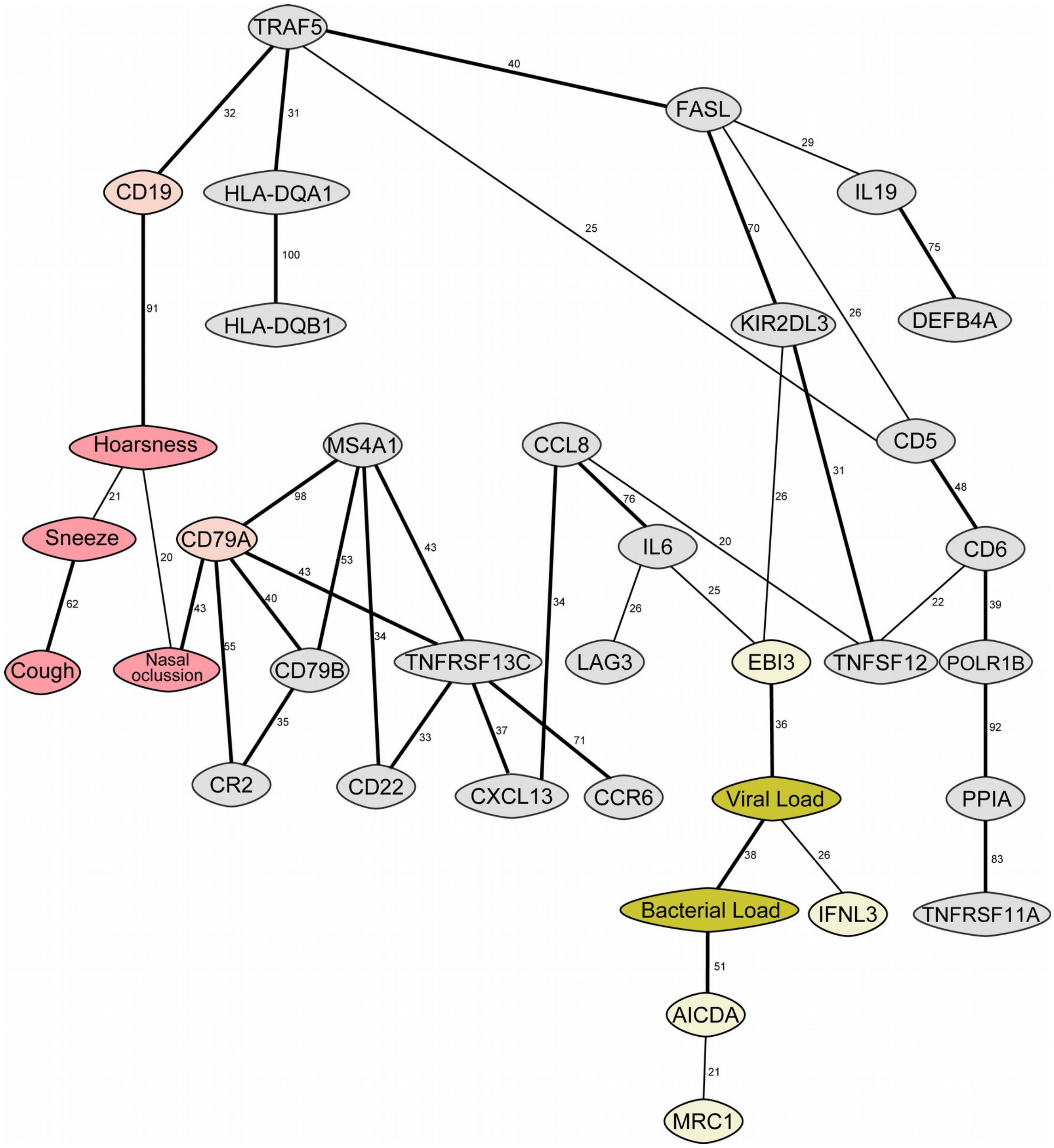

Group 4
HLADQB1
HLADQA1
Group 3
TNFRSF13C
CR2
CD22
IL19
TNFSF12
CD6
MS4A1
POLR1B
TNFRSF11A
DEFB4A
FASLG
CD5
TRAF5
CD19
PPIA
CXCL13
CCL8
KIR2DL3
CD79A
CD79B
IL6
LAG3
EBI3
CCR6
Group 1
AICDA
C4BPA
MRC1
Bacterial Load
Group 2
Viral Load
IFNL3
legend
Major gene cluster
n=4
Modular nodules (subclusters)

Figure 6.



TRAF5
FASL
CD19
HLA-DQA1
IL19
HLA-DQB1
KIR2DL3
DEFB4A
Hoarsness
MS4A1
CCL8
CD5
Sneeze
CD79A
IL6
CD6
Cough
Nasal oclussion
CD79B
TNFRSF13C
LAG3
EBI3
TNFSF12
POLR1B
CR2
CD22
CXCL13
CCR6
Viral Load
PPIA
Bacterial Load
IFNL3
TNFRSF11A
AICDA
MRC1

Supplemental Table 1. Microbial load in nasopharyngeal aspirates of children with ARI.

Samples	AV2	AV5	BoV	CoV 229E	CoV OC43	IVA	IVB	HMPV	PIV 1	PIV 2	PIV 3	RV	RSV A	RSV B	*C.p*	*H.i*	*M.c*	*M.p*	*S.p*	Viral Load	Bacterial Load	Microbial Load[a]	Negative control[b]	Cutof[c]	Classification[d]
ARI003	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2,57	0	0	0,58	0	3,15	3,15	0,34	1,66	Bacteria
ARI005	0	0	0	0,42	0	0	0	0	0	0	0	0	0	0	0	1,1	2,65	0	1,74	0,42	5,49	5,91	0,21	1,4	Bacteria
ARI012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4,04	3,28	0	2,52	0	9,84	9,84	0,31	1,6	Bacteria
ARI026	0	0	0	0	0	0	0	0	2,64	1,29	0	0	0	0	0	6,58	0	1,46	0	3,93	8,04	11,97	0,27	2,64	Bacteria and Virus
ARI032	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,27	0	0	1,39	0	1,66	1,66	0,4	1,72	Indeterminate
ARI039	0	0	0	0	0	0	0	0	0	0	0	0	4,78	0	0	0	0	0	1,13	4,78	1,13	5,91	0,18	1,23	Virus
ARI044	1,08	0	0	0	0	0	0,01	0	0	0	0	0,58	0	0	0	3,37	0	0	1,91	1,67	5,28	6,95	0,26	1,74	Bacteria
ARI045	0	0	0	0	0	0	0	0	0,95	0	0	0	3,76	0	0	5,52	0	0	2,62	4,71	8,14	12,85	0,32	1,74	Bacteria and Virus
ARI055	0,62	0	0	0	0	0	0	0	0	0	0	0,62	4,92	0	0	3,17	0,73	0	0	6,16	3,9	10,06	0,18	1,2	Bacteria and Virus
ARI065	1,09	0	0	0,78	0	2,12	0	0	0	0	0	0	4,27	0	0	1,17	0	0	1,53	8,26	2,7	10,96	0,36	1,73	Virus
ARI068	0	0	0	0	0	0	0	0	0	0	0	0	4,55	0	0	2,26	0,32	0	1,43	4,55	4,01	8,56	0,31	1,61	Bacteria and Virus
ARI076	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,46	2,01	Indeterminate
ARI083	0	0	0	0	0	0	0,63	0	0	0	0	0	0	0	0	0	0	0	0	0,63	0	0,63	0,39	1,73	Indeterminate
ARI100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1,64	0	0	0	0	1,64	1,64	0,26	1,55	Bacteria
ARI108	0	0	0	0	0	0	0	0	0	0	0	0,63	0	0	0	2,35	0	0	0,73	0,63	3,08	3,71	0,24	1,27	Bacteria
ARI113	0	0	0	0	0	0	0	0	0	0	0	0,7	0	0	0	1,8	0	0	0	0,7	1,8	2,5	0,32	1,59	Bacteria
ARI121	0	0	0	0	0	0	0	0	1,59	0	0	0	0	0	0	5,63	0	0	0	1,59	5,63	7,22	0,27	1,45	Bacteria and Virus

ARI127	0	0	0	0	0	0	0	0	0	0	0	0,65	0	0	0	2,97	0	0	0,08	0,65	3,05	3,7	0,26	1,18	Bacteria
ARI134	0	0	0	0	0	0	0	0	0	0	0	0,48	0	0	0	2,6	0	0	0	0,48	2,6	3,08	0,33	1,74	Bacteria
ARI137	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1,77	0	0	0	0	1,77	1,77	0,36	1,64	Bacteria
ARI142	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2,38	0	0	1,45	0	3,83	3,83	0,35	1,84	Bacteria
ARI147	0	0	0	0	0	0	0	0	0	0	0	0	0	4,55	0	3,05	0	0	2,7	4,55	5,75	10,3	0,35	1,88	Bacteria and Virus
ARI156	1,73	0	1,06	0	0	0	0	0	1,89	0	0	0	0	0	0	5,92	0	0	2,02	4,68	7,94	12,62	0,25	2,44	Bacteria
ARI169	0,19	0,63	4,18	1,29	0	0	0	0	0	0	4,34	2,59	0	0	0	3,48	0	0,75	1,55	13,22	5,78	19	0,29	1,84	Bacteria and Virus
ARI174	0	0	0	0,14	0	0	0	0	0	0	0	0	0	0	0	0,55	0	0	0	0,14	0,55	0,69	0,34	1,82	Indeterminate
ARI183	0	0	0	0	0	0	0	2,83	0	0	0	0	0	0	0	0	0	0	0	2,83	0	2,83	0,31	1,7	Virus
ARI187	0	0	1,39	0	0	0	0	0	2,04	0	0	0	0	0	0	6,03	0	1,24	0	3,43	7,27	10,7	0,26	1,9	Bacteria and Virus
ARI197	0	0	1,63	0	0	0	0	0	2,63	1,06	2,85	0	0	0	0	6,68	0	1,55	1,84	8,17	10,07	18,24	0,31	2,97	Bacteria[e]
ARI198	0	3,76	1,9	0	0	0	0	0	2,79	1,57	0	2,01	0	0	0	6,77	0	2,07	2,08	12,03	10,92	22,95	0,3	2,93	Bacteria and Virus[e]
ARI205	0	0	0	0	0	0	0	0	1,01	0	0	0	0	0	0	5,24	0	0	0	1,01	5,24	6,25	0,47	2,03	Bacteria
ARI218	0	0	0	0	0	0	0	0	1,36	0	0	0	0	0	0	5,51	0	0	0	1,36	5,51	6,87	0,32	1,7	Bacteria
ARI221	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1,63	0	1,38	0	3,01	3,01	0,28	1,87	Indeterminate
ARI230	0	0	0,63	0	0	0	0	0	1,87	0	0	0	0	0	0	5,97	0	0,73	0	2,5	6,7	9,2	0,4	2,17	Bacteria
ARI248	0	0	0	0	0	0	0	0	0	0,57	0	0	0	0	0	3,26	0	0	0	0,57	3,26	3,83	0,42	1,82	Bacteria
ARI251	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2,85	0	0	0	0	2,85	2,85	0,3	1,42	Bacteria
ARI254	0	0	0	0	0	0	0	0	0,44	0	0	0	0	0	0	5,1	0	0	0	0,44	5,1	5,54	0,39	1,63	Bacteria
ARI256	1,8	1,41	0	0	0	0	0	0	0	0	0	0	0	0	0	3,21	0	0,38	0,81	3,21	4,4	7,61	0,31	1,62	Bacteria and Virus
ARI261	0	0	0	0	0	0	0	4,46	0	0	0	0	0	0	0	3,13	0	0	1,96	4,46	5,09	9,55	0,29	1,56	Bacteria and Virus
ARI290	0	0	0	0	0	0	0	0	0	0	0	0	0	4,79	0	1,32	0,55	0	0	4,79	1,87	6,66	0,38	1,67	Virus

ARI305	0	1,27	0	0	0	0	1,27	0	0	0	0	0	0	4,74	5,77	1,5	0	0	1,38	7,28	8,65	15,93	0,33	1,75	Bacteria and Virus
ARI307	0	0	0	0	0	0	0	0	1,38	0	0	0	0	0	0	5,75	0	0	0	1,38	5,75	7,13	0,38	2,23	Bacteria
ARI310	1,21	0	4,13	0	0	0	0	0	0	0	0	0	0	0	0	3,91	0	0	0,6	5,34	4,51	9,85	0,38	1,72	Bacteria and Virus
ARI311	0	0	0	0	0,52	0	0	0	0	0	0	0	1,37	5,49	0	3,43	1,45	0,32	0	7,38	5,2	12,58	0,31	1,54	Bacteria and Virus
ARI322	0,33	0	0	0	0	0	0	1,32	0	0	0	0	0	0	0	4,18	0	0	0	1,65	4,18	5,83	0,3	1,63	Bacteria
ARI331	0	0	0	0	0	0	0	0	0	0	0	0,32	0	5,05	0	0,94	0	0	0	5,37	0,94	6,31	0,28	1,54	Virus
ARI335	0	0	1,85	0	0	0	0	0	2,51	1,37	0	0	0	0	0	6,6	0	1,7	0	5,73	8,3	14,03	0,28	2,7	Bacteria
ARI349	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1,32	0	0	2,21	0	3,53	3,53	0,38	1,64	Bacteria
ARI353	4,68	4,29	0	0	0	0	0	0	0	0	0	0	0	0	0	3,49	0	0	0,23	8,97	3,72	12,69	0,44	1,91	Bacteria and Virus
ARI361	0	0	0	0	0	0	0	0	0	0	3,34	0	0	0	0	1,78	0	0	0	3,34	1,78	5,12	0,29	1,26	Bacteria and Virus
ARI371	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1,99	0	0	0	0	1,99	1,99	0,25	1,5	Bacteria
ARI374	0	0	0	0	0	0	0	0	0	0	3,19	0	0	0	0	3,65	0	0	2,62	3,19	6,27	9,46	0,42	1,77	Bacteria and Virus
ARI379	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1,77	0	0	1,77	1,77	0,39	1,52	Bacteria
ARI380	1,09	0	0	0	1,2	0	0	0	0	0	0	0	5,38	0,09	0	0,09	1,45	1,2	2,05	7,76	4,79	12,55	0,29	1,27	Bacteria and Virus
ARI393	0	0	0	1,06	0	0	0	0	0	0	4,55	0	0	0	0	2,54	0	0,33	0	5,61	2,87	8,48	0,5	2,13	Bacteria and Virus
ARI403	0	0	1,43	0	0	0	0	0	2,33	0	4,24	0	0	0	0	6,43	0	1,79	0	8	8,22	16,22	0,28	2,73	Bacteria and Virus
ARI415	0	0	0	0	0	0	0	0	0	0	0	0,84	0	0	0,12	2,2	0	0	0,9	0,84	3,22	4,06	0,29	1,31	Bacteria
ARI426	0	4,54	0	0	0	0	0	0	0	0	0	0	0	0	0	3,85	0	0	1,49	4,54	5,34	9,88	0,24	1,33	Bacteria and Virus
ARI435	0,73	2,6	0,48	1,89	0	0	0	0	1,36	0	0	0	0	0	0	5,64	0	0,27	0	7,06	5,91	12,97	0,31	1,66	Bacteria and Virus
ARI441	0	0	1,11	0	0	0	0	0	1,85	0	0	0	0	0	0	6,06	0	1,21	0	2,96	7,27	10,23	0,31	2,26	Bacteria
ARI450	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2,72	0	0	0	0	2,72	2,72	0,42	1,81	Bacteria

Nasopharyngeal aspirates (NPAs, n=60) randomized from a largercohort of children (n= 576) with ARI were submitted gene expression analysis. Data (Log10-transformed) are reported as the number of transcripts detected for each target virus [Adenovirus (AV 2 and 5), Bocavirus (BoV), Coronavirus (CoV 229E and OC43), Parainfluenza virus (PIV 1, 2 and 3), Respiratory Syncytial virus (RSVA and B), Influenza virus (IV A and B), Human metapneumovirus (hMPV) and Rhinovirus (RV)] or bacteria (Chlamydia *penumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis, Mycoplasma pneumoniae* and *Streptococcus pneumoniae*).

[a]Number of microbial transcripts.

[b]Mean number of transcripts for internal negative controls.

[c]Mean of transcripts for internal negative controls plus three SD.

[d]Classification according to the presence of microbial transcript, considering cut off.

[e]Outliers removed (n = 2), after analysis by ROUT (see Methods for details).

Supplemental Table 2. Co-detection of microbial transcripts in children with ARI

Transcript	n (%)
MultipleVirus	**1 (1,8)**
IVA + RSVA	1 (1,8)
MultipleBacteria	**3 (5,5%)**
H.influenzae +*S.pneumoniae*	1 (1,8)
M.catarrhalis + *S.pneumoniae*	1 (1,8)
M.catarrhalis+ *M.pneumoniae* + *S.pneumoniae*	1 (1,8)
Virusandbacteria	**14 (25%)**
H.influenzae + PIV1	3 (5,5%)
H.influenzae + PIV3	3 (5,5%)
H.influenzae + RSVA	2 (3,6%)
H.influenzae + RSVB	2 (1,8)
H.influenzae + AV5	1 (1,8)
H.influenzae + AV2	1 (1,8)
H.influenzae + BoV	1 (1,8)
C.pneumoniae + RSVB	1 (1,8)
Multiplevirusandbacteria	**8 (15%)**
H.influenzae + *S.pneumoniae*+ RSVA	1 (1,8)
H.influenzae + BoV + PIV3 + RV	1 (1,8)
H.influenzae + *S.pneumoniae* + hMPV	1 (1,8)
H.influenzae + AV2 + AV5	1 (1,8)
H.influenzae + *S.pneumoniae* + PIV3	1 (1,8)
H.influenzae + *S.pneumoniae* + AV5	1 (1,8)
H.influenzae + AV5 + COV 229E	1 (1,8)
M.catarrhalis + *S.pneumoniae* + RSVA	1 (1,8)

Supplemental Data Set 1 - nCounter readings for pathogen probes, negative controls and immune-related genes, depicted per individual NPA.

Figure Legends

Figure 1. Microbial load and diversity in nasopharyngeal aspirates of children with ARI. The presence of microbial [Adenovirus (AV 2 and 5), Bocavirus (BoV), Coronavirus (CoV 229E and OC43), Parainfluenza virus (PIV 1, 2 and 3), Respiratory Syncytial virus (RSVA and B), Influenza virus (IV A and B), Human metapneumovirus (hMPV) and Rhinovirus (RV), *Haemophilusinfluenzae, Streptococcus pneumoniae, Mycoplasma pneumoniae, Moraxella catarrhalis*and *Chlamydia penumonia]* transcripts was determined by transcriptomic analysis of NPAs.(A) The number of pathogen transcripts (Log10) is depicted for each NPA and colors represent NPA classification (See Methods and Sup. Table 1 for details). (B) Number (percentage) of children presenting bacterial, viral or both transcripts (co-detected) as well as those classified as indeterminate.

Figure 2. Identification of immune genes differentially expressed in NPAs. (A) Differences in gene expression were identified through statistical comparison among NPAs classified according to the microbial load (bacterial, orange; indeterminate, yellow; co-detection, pink and viral, green)(non-parametric t test, $p<0.05$). (B) Venn diagram depicting the number of Differentially Expressed Genes (DEGs) identified according to the pairwise comparison performed. (C) List of 30 DEGs identified across ≥3 comparisons.

Figure 3. Differentially expressed genes in NPAs classified as viral, bacterialor co-detection. (A) DEGs (n=30, rows) in NPAs classified according to the microbial load (bacterial, orange; indeterminate, green; co-detection, pink

and viral, green) (non-parametric t test, $p<0.05$). Level of expression is shown per NPA group (left panel) or per individual NPA (right panel). (B) Gene ontology enrichment of the 30 DEGs.

Figure 4. Global transcription perturbation in NPAs classified as viralbacterial or co-detection. (A) Bar graph representing molecular distance to health (MDTH), calculated using the 30 DEGs, shown per group of NPA, left (ANOVA; *$p<0.05$) and shown individually for ach NPA, right. (B) Top graphs show MDTH per individual NPA, according to the type of microbial load (bacteria, co-detection or virus). Bottom graphs show total viral and microbial load for the same NPAs depicted at the top. Values (r and p) are from Spearman rank test.

Figure 5. Correlation network of immune markers and microbial load identified in NPAs. Lines display significant interactions ($p<0.001$, Pearson's test) among immune markers, viral and bacterial load. Network stability was examined using atlas force 2 algorithm. Markers are grouped according to the interaction profile, yielding major gene clusters (indicated in larger grey circles),subclusters are depicted in different colors, according to theintraconnectivity among markers. Connection strength was assessed by non-parametric bootstrap (100× replicates) and arcs with bootstrap over 60% are depicted.

Figure 6. Bayesian network depicting immune markers, clinical features and microbial load. The associations show direct influences (arcs) amongviral

and bacterial loadand immune-related genes (colored in yellow) and among clinical symptoms and immune-related genes (colored in red). Connection strength was assessed by non-parametric bootstrap (100× replicates) and arcs with bootstrap over 20% are depicted in bold.

V. DISCUSSÃO

Os nossos resultados foram subdivididos em dois capítulos, o primeiro mostra aplicação do nCounter na detecção dos patógenos respiratórios e o segundo mostra a associação da resposta imune dos pacientes com IRA com a carga microbiana.

O nCounter já teve sua sensibilidade e reprodutibilidade avaliadas em comparação com outras técnicas de alto rendimento como: Openarray® (Prokopec et al. 2013), Affymetrix (Payton et al. 2009) e PCR TaqMan (Geiss et al. 2008). O nCounter teve a capacidade de detectar transcritos pouco abundantes, não detectados por micro arranjo (Barczak et al. 2012). A partir dessa característica, decidimos aplicar esse conceito na detecção de RNA mensageiro de microorganismos nas amostras clínicas de crianças com IRA. O nCounter permitiu a detecção simultânea de transcritos virais e bacterianos). Em resumo, encontramos Rinovírus (RV) em 2% das amostras, Bocavírus (BoV) (3%), Coronavírus (CoV) (229E e OC43, 3%), Influenzavírus (IV) A (3%), metapneumovírus humano (hMPV) (5%), Adenovríus (AV) (10%), Parainfluenzavírus (PIV) (1 e 3, 21%), Vírus Sincicial Respiratório (RSV) (A e B (21%). Além disso, detectamos transcritos bactérianos, como: *H. influenzae* e *S. aureus* (69% e 77% das amostras, respectivamente). Apesar das diferenças sazonais de distribuição dos patógenos (Soediono 1989; Silva et al. 2014), todos esses patógenos são associados com a infecções do trato respiratório e são comumente isolados (Pavia, 2011, Robinson 2004).

A fim de compreender a relação entre os transcritos microbianos detectados, fizemos uma análise de redes utilizado o algoritmo ATLASFORCE2 (Jacomy et al. 2011), o qual leva em consideração a força de atração e repulsão que os nódulos possuem na configuração espacial da rede. Cada nódulo foi representado por um patógeno e cada arco representa a associação entre o patógenos. Assim, *H. influenzae*,

S. pneumoniae e *S. aureus* se localizaram centralmente na rede refletindo uma forte interação entre esses patógenos enquanto que outros como CoV OC43 e 229E apresentam menor interação, se localizando na parte mais externa da rede. A partir desse modelo, podemos interpretar que há maior prevalência da detecção conjunta do *H. influenzae*, *S. pneumoniae* e *S. aureus* e pouca co-ocorrência deles com CoV OC43 e 229E. Ruohola e colaboradores (2009), descreveram a preferência de alguns vírus em aparecer de forma conjunta, por exemplo as enterovíroses, foram comumente encontradas com a presença de AV.

Após a detecção dos patógenos por nCounter, prosseguimos com a detecção dos transcritos da resposta imune no hospedeiro. Para isso estratificamos as amostras em três grupos: presença de transcritos bacterianos apenas, virais apenas e bacterianos e virais. Nessa estratificação, encontramos que <10% dos pacientes apresentaram transcritos exclusivamente virais e os vírus mais prevalentes foram: RSV (A e B), seguido por hMPV e IV. Em relação às bactérias, 46,6% das amostras apresentaram transcritos unicamente bacterianos e com a maior prevalência de *H. influenza* seguido por *S. pneumoniae* e *M. catarrhalis*. Esses microrganismos, juntamente com *S. aureus* são colonizadores transientes comuns da nasofaringe em crianças saudáveis (Wiertsema et al. 2010; Uitti et al. 2015). As amostras nas quais houve co-detecção de transcritos virais e bacterianos representaram 36,6%, fato que tem se tornado comum, devido a utilização de técnicas moleculares modernas (Brealey et al. 2015).

Os sintomas clínicos apresentados pelas crianças que apresentaram transcritos mistos (virais + bacterianos) não diferiram significativamente daqueles observamos em crianças com transcritos unicamente virais ou bacterianos, pelo menos no momento de admissão. Já foi sugerido também que infecções virais por mais de um vírus não resulta em aumenta na gravidade da doença (Nascimento-Carvalho & Ruuskanen 2016). Na

nossa amostra, todos os pacientes apresentaram sintomas de IRA, o que poderia explicar a diversidade do microbioma da nasofaringe. Determinar a contribuição de cada micróbio para a gravidade da doença é complexo, especialmente na população pediátrica, porque há uma grande quantidade de diferentes vírus e de espécies bacterianas, as quais podem causar sintomas respiratórios semelhantes na nasofaringe (Brealey et al. 2015).

Além disso, a ocorrência de sintomas já foi correlacionada com a supressão de uma população simbiótica por uma população patogênica (Teo et al., 2015; Thorburn et al., 2006). Uitti e colaboradores (2015) encontraram uma associação entre febre e a presença dos vírus respiratórios e outra associação entre rinite, congestão nasal e tosse com a presença da *M. catarrhalis*. A rede Bayesianaque obtivemos associações entre a obstrução nasal e tosse. Essa associação pode ter sido evidenciada indiretamente pela presença de *M.catarrhalis* que apesar de não ter sido usada na estratificação das amostras, pode ter tido seu efeito conservado. Isso também pode ter ocorrido com a associação entre a febre e a infecção viral, pois a prevalência dos vírus respiratórios é descrita em associação com a população pediátrica, ou seja de baixa faixa etária (Brealey et al. 2015).

Após a classificação dos ANFs de acordo com a carga microbiana, foi comparado o perfil dos transcritos, para os pacientes com: vírus, bactérias e/ou co-detecção, resultando num conjunto de 30 genes diferencialmente modulados, em pelo menos três desses grupos. O perfis mostraram a sobre-expressão destes 30 genes na ANF classificada como, resposta imune humoral adquirida pelo enriquecimento do Gene Ontology. A perturbação molecular revelou que um grau significativo de disturbio no transcriptoma foi observada em todos os grupos, independente da classificação e independentemente da carga microbiana. Esta dissociação de certa forma surpreendente

entre a carga microbiana e perfil de expressão de genes, em termos de resposta imune, levou-nos a utilizar a análise de rede para melhor compreender a relação entre estes parâmetros. IFNL3 foi o único marcador associado com a carga viral, o que sugere um efeito espectador dos restantes 24 genes expressos diferencialmente expressos. O Interferon do Tipo III, tais como IFLN3 (IL28B) induz uma variedade de ISGs, que tem o papel de interferir com a replicação viral (Spann et al., 2004). IFNL3 e expressa em resposta a RSV (Spann et al., 2004) e IV (Durbin et al., 2013), ambos detectados nas ANFs, e o IFNL parece ser a fonte principal de IFN em células epiteliais das vias respiratórias (Khaitov et al. 2009). Com respeita ao módulo bacteriana, três genes foram associados com carga bacteriana: AICDA, MRC1 e C4BPA. C4BPA (proteína C4b vinculativo) inibe a ativação da via clássica do complemento e opsonização subsequente seguido de lise. C4BPA está vinculado a vários patógenos bacterianos, incluindo H. influenzae (Hallstrom et al., 2007) e M.catarrhalis (Nordstrom et al., 2004), melhorando a adesão bacteriana. O receptor de manose MRC1 reconhece polisacarideos bacterianos e está envolvido na absorção de S. pneumoniae (Nordstrom et al., 2004). Finalmente, o AICDA que é uma proteína de edição DNA que desempenha um papel essencial na mutação somática, recombinação e mudança de classe de Ig. Colectivamente, estes dados indicam que a presença de bactérias em ANFs é detectada por MRC1 e expressão de C4BPA pode estar envolvido na patogénese e desencadeamento de uma resposta imune humoral. presença viral, por outro lado, disparadores e resposta anti-viral, caracterizado por expressão do IFNL3.

A rede Bayesiana reforçada certos achados da rede de correlação: uma conexão entre a carga bacteriana e os marcadores imunológicos AICDA e MRC1 e entre carga viral e IFNL3. A associação direta entre a carga bacteriana e carga viral, reforçam a interação entre as cargas virais e microbianas para a dinâmica do microbioma na

nasofaringe. Na verdade, a presença viral e bacteriana simultânea pode perturbar o equilíbrio existente aumentando a complexidade como na otite média, por exemplo (Ruohola, et al., 2013). A rede Bayesiana também mostrou que sintomas como rouquidão, espirros, tosse e oclusão nasal foram dissociadas a da carga microbiana tanto a partir de bacterias ou virus, reforçando a necessidade de identificar marcadores mais específicos de infecção virai ou bacteriano (Heinonen et al., 2015; Hu, et al., 2013). Embora RSV e RV têm sido implicados na respiração ofegante e da presença viral é um preditor significativo de desenvolvimento de asma em crianças (Jackson et al., 2008), mas não encontramos uma associação entre a carga viral e chiado, possivelmente devido ao número limitado de NPAs classificados como viral. observou-se também uma associação directa entre rouquidão e CD19 e entre oclusão nasal e CD79a, que sao proteínas associadas com a ativação do receptor de célula B, indicando uma ligação entre estes dois sintomas clínicos e a resposta imune humoral.

Em resumo, este estudo exploratório transversal identificou os agentes microbianos da IRA e a resposta imune presente no ANF. A análise transcriptômicados ANFs revelou perfis imunológicos para apresença viral, bacteriana ou para a presençade ambos os transcritos. Nossas análises integrativas revelaram um subconjunto de genes relacionados à resposta imune que interagem diretamente com a carga microbiana e com os sintomas clínicos. Essa abordagem pode ajudar a elucidar como cada fator contribui para a IRA nos pacientes pediátricos e levar à identificação de moléculas candidatas a uma investigação mais aprofundada.

VI. PROPOSTAS DE ESTUDO

Avaliação de biomarcadores imunes associados com a carga bacteriana em crianças com infecção respiratória aguda

Kiyoshi Ferreira Fukutani

Salvador- BA, 2016

Justificativa

Durante o projeto de doutorado intitulado de “Avaliação da carga viral e da resposta imune em crianças portadoras de infecção respiratória aguda”, encontramos 5genes (*THY1* , *LIF* , *IL1RL1* , *MRC1* , *IFNL3* e *AICDA*) associados com a carga bacteriana em pacientes com infecção respiratória aguda. Esse projeto pretendevalidar essas moléculas como biomarcadores de carga bacteriana,medindo a sua concentraçãono plasma/soro de crianças com infecção respiratória aguda.

Objetivos

Objetivo 1: Identificar sorologicamente as proteínascandidatas em crianças com infecção respiratória aguda.

1. Realizar ensaios imuno enzimáticos, empregando kits comerciais específicos para as moléculas selecionadas (*THY1* , *LIF* , *IL1RL1* , *MRC1* , *IFNL3* e *AICDA*).
2. Associar os níveis séricos dos marcadores a carga de infecção dos 600 pacientes com carga bacteriana previamente estabelecida.

Objetivo 2: Integrar os dados em um modelo matemático/algoritmo clinicamente aplicávelpara selecionar biomarcadores de infecção respiratória.

1. Analisar de forma uni variada, multivariada e por data mining os genes selecionados.
2. Estabelecendo um classificador com as concentrações desses marcadores no plasma/soro de crianças com infecção respiratória aguda.
3. Validar essa classificação em uma outra coorte.

Infra-estrutura

O Laboratório de Imunoparasitologia (LIP) possui todos os equipamentos necessários para a parte laboratorial assim como três servidores para as análises de bioinformática.

Metodologia

Amostras

Pacientes residentes na comunidade que preenchem critérios de inclusão (febre, tosse, espirro ou obstrução nasal por um período de até sete dias e sem episódio anterior de sibilância) foram recrutadose o plasma foi coletado individualmente. Após a coleta , as amostras foram congeladas a -70 ° C.O presente estudo foi submetido ao Comité de Ética em Pesquisa da Maternidade Climério de Oliveira da Universidade Federal da Bahia – Bahia, obtendo aprovação do mesmo para realização da pesquisa, sobre o número (067/2009).O estudo foi realizado com os pacientes que assentirem a participação na pesquisa a partir da assinatura do Termo de Consentimento Livre e Esclarecido.As diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos constantes na resolução 466/2012 do Ministério da Saúde e Conselho Nacional de Saúde foram respeitadas.

Quantificação de marcadores no soro/plasma

Os marcadores selecionados serão quantificados no soro ou plasma de crianças com IRA, empregando kits comerciais.

Análise estatística

Para a análise dos dados, utilizaremos o GraphPad6.0, JMP SAS 11, R statistical e WEKA.

Resultados preliminares

Ainda durante no período de doutoramento com as 60 amostras iniciais, encontramos por *dimensional reduction*, 20 genes (*KIT, TNFRSF13C, IL1RL1, AICDA, THY1, LIF, MRC1, TNFSF12, IL19, TRAF5, ITGA4, C1R, VCAM1, BLNK, CD79B, CD19, CCBP2, IFNL3, CD5*e*CD22*)diferencialmente expressos e capazes de distinguir a infecção respiratória com presença de vírus ou de bactérias. Uma análise da rede de correlação demonstrou que dentre esses 20 genes,há cincogenes (*THY1, LIF ,IL1RL1, MRC1, IFNL3* e *AICDA*) que se correlacionam com a carga viral ou bacteriana (Figura 1A). Além disso, a intensidade dessascorrelaçõestambém foi diferente para carga viral e bacteriana (Figura 1B). Por fim, o perfil de expressão desses cinco genes também diferem em aspirados com transcritos de bactérias comparados a aspirados com transcritos virais (Figura1C).

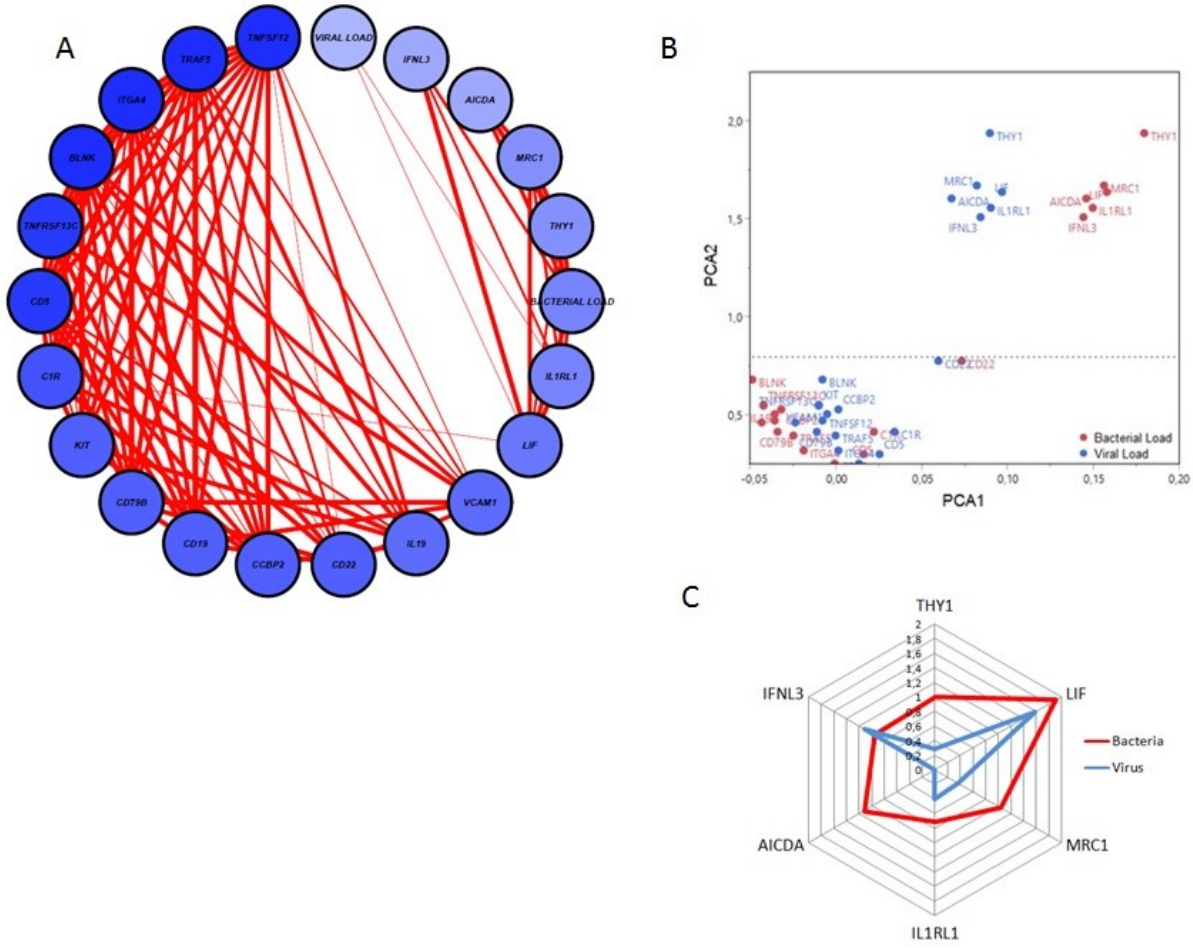


Figura 1. Rede de correlação dos biomarcadores do sistema imunológico e da carga microbiana em crianças com IRA. (A) As linhas exibem interações significantes (p < 0,002, teste de Pearson corrigido pelo número de moléculas) entre os genes relacionados à resposta imune e carga microbiana. A estabilidade da rede foi estabelecida através dobootstrapcom 100 × repetições. Somente arcos com valor de bootstrap acima de 50% foram retratados. A intensidade da cor de nodos indica o número de interações observadas para cada marcador. (B) PartialLeast Square Model"PLS" prever a presença da correlação dos transcritos virais ou bacterianos. Variável latente (LV) 1 e LV2 mostram um módulo (valor de p Indicar) consistindo de genes imunológicos (THY1 , LIF , IL1RL1 , MRC1 , IFNL3 e AICDA) que estão associados com a presença viral e bacteriana. (C) Expressão dos genes (média geométrica) descritos em (B). Todos os dados foram transformados de log 10 .

Apartir desse resultado estratificamos a nossa população pelos quartis da carga bacteriana e começamos a descrevê-los. O primeiro quartil é constituído pelos 25% da população que não apresentou ou apresentou carga baixa de bactéria, o segundo quartil o intervalo entre os 25% e a media do numero de bactéria, o terceiro quartil a media ate 25% superior da media e o ultimo quartil todos os pacientes com carga superior a 25% da media. O nosso primeiro resultado mostra uma diferença significativa na quantidade de transcritos imunes*versus* os quartis definindo a carga bacteriana (Figura 2).

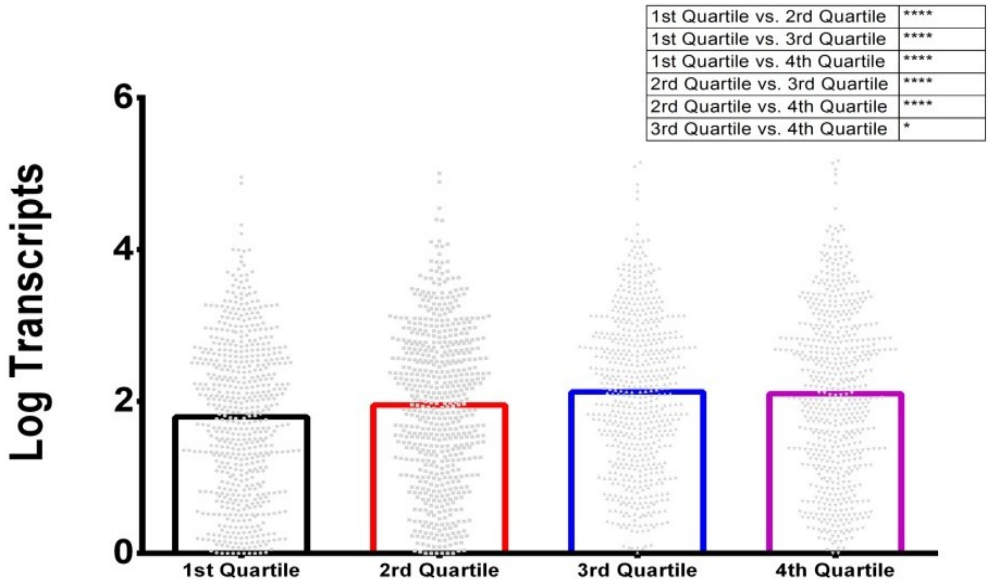


Figura 2. Presença de transcritos imunespor quartil de carga bacteriana presente no aspirado de nasofaringe de crianças com IRA O número total de transcritos foram mensurados de acordo com a carga bacteriana que foi dividida em quartis. As significâncias estatísticas quanto às diferenças no número de transcritos imunes foram calculadaspelo teste ANOVA e corrigido pela comparação múltiplas de Dunn. *, $p<0,05$.

Embora tenhamos observado uma diferença significante no número de transcritos imunes, não observamos diferenças nos sintomas clínicos avaliados, após a estratificação por quartil de carga bacteriana (Tabela 1).

Tabela1. Sinais clínicos porquartil de carga bacteriana presente no aspirado de nasofaringe de crianças com IRA.

	Total (n=60)		Quartil 1 (n=16)		Quartil 2 (n=14)		Quartil 3 (n=13)		Quartil 4 (n=17)	
Tosse (n, %)	60	100%	16	100%	14	100%	13	100%	17	100%
Espirro (n, %)	9	15%	1	6%	1	7%	3	23%	4	24%
Coriza (n, %)	7	12%	2	12%	2	14%	1	8%	2	12%
Obs. Nasal (n, %)	12	20%	2	12%	4	29%	1	8%	5	29%
Febre (n, %)	11	18%	3	19%	2	14%	2	15%	4	24%
Rouquidão (n, %)	19	32%	4	25%	5	36%	6	46%	4	24%
Otalgia (n, %)	6	10%	3	19%	1	7%	1	8%	1	6%
Secreção no ouvido (n, %)	2	3%	0	0%	1	7%	0	0%	1	1%
Chiado (n, %)	22	37%	6	38%	6	43%	5	38%	5	29%

A análisedos transcritos imunes por quartil demonstrou queos mesmos podem ser utilizados para distinguir o grupo contendo a maior carga bacteriana (Figura 2A), o que foi confirmado pela análise de grupamentos hierárquico (Figura 2B). Especulamos então que esses genes possam servir como marcadores de infecção bacteriana já que a carga bacteriana é o critério comumente aplicado para se diferenciar infecção e colonização.

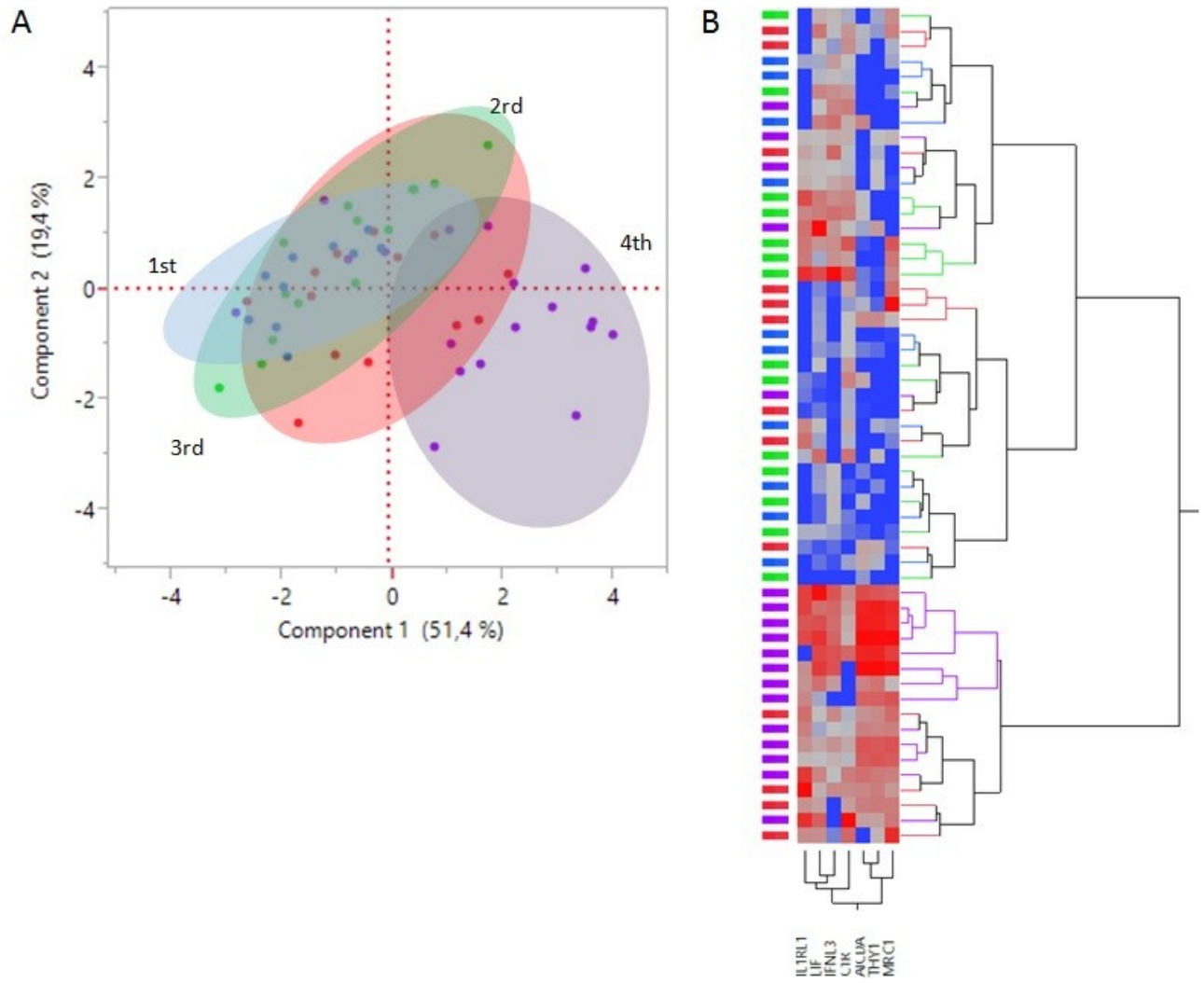


Figura 3. Análise de componente principal e clusterização hierárquica de genes da resposta imune expressos em ANF de crianças com IRA, por carga de bactéria.. A. Análise de componente principal utilizando 2 eixos (Componentes 1 e 2) com a variação de 51,4% e 19,4% respectivamente. B. Heatmapmostrando a expressão de *THY1*,*LIF*,*IL1RL1*, *MRC1*, *IFNL3 e A*ICDAem amostras de ANF. As cores representam os quartis de carga bacteriana.Primeiro quartil (azul), segundo (verde), terceiro (vermelho) e quarto (roxo). Todas as variáveis foram transformadas em Log de 10.

VII. CONCLUSÃO

O aspirado de nasofaringe de crianças com infecção respiratória aguda, recrutadas em Salvador, BA, apresentam transcritos bacterianos, transcritos virais e bacterianos (presença mista) ou transcritos exclusivamente virais. A presença desses transcritos modulam determinados genes da resposta imune e essa modulação esta correlacionada com a carga microbiana.

VIII. SUMMARY

Introduction: Acute respiratory infections (ARI) have high morbidity and represent an important public health problem. Objectives: To detect, via the presence of RNA, viral and bacterial agents present in nasopharyngeal aspirate (NPA) in children aged 6-23 months, suffering from IRA and detect the immune response profile associated. Methodology: NPAs were subjected to RNA extraction and this was used in nCounter assays using probes designed for viral and bacterial detection and employing standard probes for the detection of the immune response. Results: in the cohort of 60 NPAs obtained from children with ARF, we detected transcripts for Parainfluenza (1-3), Respiratory Syncytial Virus (A and B) (21%), human Metapneumovirus (5%), Bocavirus, Coronavirus and Influenza A Virus (3%), Rhinovirus (2%), *Staphylococcus aureus* (77%), *Haemophilusinfluenzae* (69%), *Streptococcus pneumoniae* (26%), *Moraxella catarrhalis* (8%), *Mycoplasma pneumoniae* (3%) and *Chlamydophilapneumoniae* (2 %). Among the 60 patients, 28 had single bacterial infection, 22 patients showed the presence of bacteria and viruses, and five patients had viral infection only. In five patients, detection of the transcripts was below the cutoff point and these were considered negative. We also observed a differential modulation of immune genes in NPAs containing the different types of transcripts; the number of modulated genes was reduced by dimensional reduction. Thus, there are 30 genes that are associated with microbial load. We used a Bayesian network and observed an association between markers of immune response and microbial load. Conclusion: nCounter is a sensitive technique for identifying the pathogen and the immune transcriptome in NPAs. The integrated analysis of the data revealed a subset of genes that directly interacts with microbial load.

IX.REFERÊNCIAS BIBLIOGRÁFICAS

1. Aino Ruohola, Matti Waris, Olli Ruuskanen Tobias Allander, Thedi Ziegler, Terho Heikkinen. “Viral Etiology of Common Cold in Children, Finland.” *Emerging Infectious Diseases* 15 (2): 344–46. 2009.

2. Andres-Terre, Marta, Helen M. McGuire, Yannick Pouliot, Erika Bongen, Timothy E. Sweeney, Cristina M. Tato, and Purvesh Khatri. “Integrated, Multi-Cohort Analysis Identifies Conserved Transcriptional Signatures across Multiple Respiratory Viruses.” *Immunity* 43 (6). 2015.

3. Ann R. Falsey, Kenneth L. Becker, Andrew J. Swinburne, Eric S. Nylen, Maria A. Formica, Patricia A. Hennessey, Mary M. Criddle, Derick R. Peterson, Andrea Baran, Edward E. Walsh. “Bacterial Complications of Respiratory Viral Illness: A Comprehensive Evaluation.” *Journal of Infectious Diseases Advance*.2013.

4. Barczak, Amy K, James E Gomez, Benjamin B Kaufmann, Ella R Hinson, Lisa Cosimi, Mark L Borowsky, Andrew B Onderdonk “RNA Signatures Allow Rapid Identification of Pathogens and Antibiotic Susceptibilities.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (16): 6217–22. 2012.

5. Bonfim, Caroline M, Maurício L Nogueira, Paulo Vítor M Simas, Luis Gustavo a Gardinassi, Edison L Durigon, Paula Rahal, and Fátima Pereira Souza. “Frequent Respiratory Pathogens of Respiratory Tract Infections in Children Attending Daycare Centers.” *Jornal de Pediatria* 87 (5): 439–44,2011.

6. Brealey, J. C., P. D. Sly, P. R. Young, and K. J. Chappell. “Viral Bacterial Co-Infection of the Respiratory Tract during Early Childhood.” *FEMS Microbiology Letters* 362 (10): 2015.

7. Bulla, a, and K L Hitze. “Acute Respiratory Infections: A Review.” *Bull World Health Organ* 56 (3): 481–98.1978.

8. Caliendo, Angela M. “Multiplex PCR and Emerging Technologies for the Detection of Respiratory Pathogens.” *Clinical Infectious Diseases* 2011.

9. Chaussabel, Damien, Virginia Pascual, and Jacques Banchereau. “Assessing the Human Immune System through Blood Transcriptomics.” *BMC Biology* 8 (1): 84. 2010.

10. Cumming, J.a., and D.a. Wooff. “Dimension Reduction via Principal Variables.” *Computational Statistics & Data Analysis* 52 (1): 550–65, 2007.

11. Durbin RK, Kotenko SV, Durbin JE. Interferon induction and function at the mucosal surface. Immunol Rev **2013**; 255(1): 25-39

12. Duttweiler, L, D Nadal, and B Frey. “Pulmonary and Systemic Bacterial Co-Infections in Severe RSV Bronchiolitis.” *Archives of Disease in Childhood* 89 (12): 1155–57. 2004.

13. Ellis, Joanna S, Joanne W Smith, Sharleen Braham, Matthew Lock, Katrina Barlow, and Maria C Zambon. “Design and Validation of an H5 TaqMan Real-Time One-Step Reverse Transcription-PCR and Confirmatory Assays for Diagnosis and Verification of Influenza A Virus H5 Infections in Humans.” *Journal of Clinical Microbiology* 45 (5): 1535–43. 2007.

14. Ermers, Marieke J J, Riny Janssen, N Charlotte Onland-Moret, Hennie M Hodemaekers, Maroeska M Rovers, Michiel L Houben, Jan L L Kimpen, and Louis J Bont. “IL10 Family Member Genes IL19 and IL20 Are Associated with Recurrent Wheeze after Respiratory Syncytial Virus Bronchiolitis.” *Pediatric Research* 70 (5): 518–23. 2011.

15. Faber, Tina E, Annemieke Schuurhof, Annelies Vonk, Gerard H Koppelman, Marije P Hennus, Jan L L Kimpen, Riny Janssen, and Louis J Bont. “IL1RL1 Gene Variants and Nasopharyngeal IL1RL-a Levels Are Associated with Severe RSV Bronchiolitis: A Multicenter Cohort Study.” *PloS One* 7 (5): 2012.

16. Fendrick, A. Mark, Arnold S. Monto, Brian Nightengale, and Matthew Sarnes. “The Economic Burden of Non-Influenza-Related Viral Respiratory Tract Infection in the United States.” *Archives of Internal Medicine* 163 (4): 487–94. 2003.

17. Foronjy, R F, A J Dabo, N Cummins, and P Geraghty. “Leukemia Inhibitory Factor Protects the Lung during Respiratory Syncytial Viral Infection.” *BMC Immunol* 15 (1): 41. 2014.

18. Geiss, Gary K, Roger E Bumgarner, Brian Birditt, Timothy Dahl, Naeem Dowidar, Dwayne L Dunaway, H Perry Fell, et al. “Direct Multiplexed Measurement of Gene Expression with Color-Coded Probe Pairs.” *Nature Biotechnology* 26 (3): 317–25. 2008.

19. Gillard, Geoffrey O, Maytal Bivas-Benita, Avi-Hai Hovav, Lauren E Grandpre, Michael W Panas, Michael S Seaman, Barton F Haynes, and Norman L Letvin. “Thy1+ NK [corrected] Cells from Vaccinia Virus-Primed Mice Confer Protection against Vaccinia Virus Challenge in the Absence of Adaptive Lymphocytes.” *PLoS Pathogens* 7 (8): 2011.

20. Girard, Marc P., Thomas Cherian, Yuri Pervikov, and Marie Paule Kieny. “A Review of Vaccine Research and Development: Human Acute Respiratory Infections.” *Vaccine* 23 (50): 5708–24. 2005.

21. Hallstrom T, Jarva H, Riesbeck K, Blom AM. Interaction with C4b-binding protein contributes to nontypeable Haemophilus influenzae serum resistance. J Immunol **2007**; 178(10): 6359-66.

22. Hallstrom T, Resman F, Ristovski M, Riesbeck K. Binding of complement regulators to invasive nontypeable Haemophilus influenzae isolates is not increased compared to nasopharyngeal isolates, but serum resistance is linked to disease severity. J Clin Microbiol **2010**; 48(3): 921-7.

23. Hartzell, Joshua D, Charles N Oster, and Joel C Gaydos. “How Contagious Are Common Respiratory Tract Infections?” *The New England Journal of Medicine* 349: 95. 2003.

24. Heinonen, Santtu, Tuomas Jartti, Carla Garcia, Silvia Oliva, Cynthia Smitherman, Esperanza Anguiano, Wouter A.A. de Steenhuijsen Piters, et al. “Rhinovirus Detection in Symptomatic and Asymptomatic Children: Value of Host Transcriptome Analysis.” *American Journal of Respiratory and Critical Care Medicine* 40 (5): 2015.

25. Inoue, Yuzaburo, and Naoki Shimojo. “Epidemiology of Virus-Induced Wheezing/asthma in Children.” *Frontiers in Microbiology* 4 (December): 391. 2013.

26. Jackson, Daniel J, Michael D Evans, Ronald E Gangnon, Christopher J Tisler, Tressa E Pappas, Wai-Ming Lee, James E Gern, and Robert F Lemanske. “Evidence for a Causal Relationship between Allergic Sensitization and Rhinovirus Wheezing in Early Life.” *American Journal of Respiratory and Critical Care Medicine* 185 (3): 281–85. 2012.

27. Jackson, Daniel J, Ronald E Gangnon, Michael D Evans, Kathy a Roberg, Elizabeth L Anderson, Tressa E Pappas, Magnolia C Printz, et al. “Wheezing Rhinovirus Illnesses in Early Life Predict Asthma Development in High-Risk Children.” *American Journal of Respiratory and Critical Care Medicine* 178 (7): 667–72. 2008.

28. Jacomy, Mathieu, Sebastien Heymann, T Venturini, and M Bastian. “Force Atlas 2, a Graph Layout Algorithm for Handy Network Visualization.” 2011.

29. Jamieson, Amanda M, Lesley Pasman, Shuang Yu, Pia Gamradt, Robert J Homer, Thomas Decker, and Ruslan Medzhitov. “Role of Tissue Protection in Lethal Respiratory Viral-Bacterial Coinfection.” *Science (New York, N.Y.)* 340 (6137): 2013.

30. Khaitov, M R, V Laza-Stanca, M R Edwards, R P Walton, G Rohde, M Contoli, A Papi, L a Stanciu, S V Kotenko, and S L Johnston. “Respiratory Virus Induction of Alpha-, Beta- and Lambda-Interferons in Bronchial Epithelial Cells and Peripheral Blood Mononuclear Cells.” *Allergy* 64 (3): 375–86. 2009.

31. Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol **2008**; 8(1): 22-33

32. Kupz, Andreas, Timothy A Scott, Gabrielle T Belz, Daniel M Andrews, Marie Greyer, Andrew M Lew, Andrew G Brooks, et al. “Contribution of Thy1+ NK Cells to Protective IFN-γ Production during Salmonella Typhimurium Infections.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (6): 2252–57. 2013.

33. Lambert, Stephen B, Kelly M Allen, Robert C Carter, and Terence M Nolan. “The Cost of Community-Managed Viral Respiratory Illnesses in a Cohort of Healthy Preschool-Aged Children.” *Respiratory Research* 9: 11.2008.

34. Costa, LF, Yokosawa J, OC Mantese, TFM Oliveira, HL Silveira, LL Nepomuceno, LS Moreira, G Dyonisio, LMG Rossi, RC Oliveira, LZG Ribeiro, DAO Queiroz. “Respiratory Viruses in Children Younger than Five Years Old with Acute Respiratory Disease from 2001 to 2004 in Uberlândia, MG, Brazil.” *Memorias Do Instituto Oswaldo Cruz* 101 (3): 2006.

35. Mahony, James B. “Detection of Respiratory Viruses by Molecular Methods.” *Clinical Microbiology Reviews* 21 (4): 716–47. 2008.

36. Malkov, Vladislav a, Kyle a Serikawa, Noel Balantac, James Watters, Gary Geiss, Afshin Mashadi-Hossein, and Thomas Fare. “Multiplexed Measurements of Gene Signatures in Different Analytes Using the Nanostring nCounter Assay System.” *BMC Research Notes* 2 (January): 80. 2009.

37. Maria, Filomena, Maria Helena Valente, Caroline Mami Tanaka, Leonardo

Daumas Passos, Paula Aguiar De Aragão, and Claudio Leone. n.d. “Incidencia de Infecciones Respiratorias Agudas En Niños Del Centro de Salud Escuela ‘ Profesor Samuel B . Pessoa ’, São Paulo Brasil,” 11–22.

38. Mayer-Barber, Katrin D, Bruno B Andrade, Sandra D Oland, Eduardo P Amaral, Daniel L Barber, Jacqueline Gonzales, Steven C Derrick, et al. “Host-Directed Therapy of Tuberculosis Based on Interleukin-1 and Type I Interferon Crosstalk.” *Nature* 511 (7507). 2014.

39. Mejias, Asuncion, Blerta Dimo, Nicolas M. Suarez, Carla Garcia, M. Carmen Suarez-Arrabal, Tuomas Jartti, Derek Blankenship, et al. “Whole Blood Gene Expression Profiles to Assess Pathogenesis and Disease Severity in Infants with Respiratory Syncytial Virus Infection.” Edited by Rosalind Louise Smyth. *PLoS Medicine* 10 (11): 2013.

40. Mishra, Pramod Kumar, Elizabeth G. Morris, Jenny a. Garcia, Astrid E. Cardona, and Judy M. Tealea. “Increased Accumulation of Regulatory Granulocytic Myeloid Cells in Mannose Receptor C Type 1-Deficient Mice Correlates with Protection in a Mouse Model of Neurocysticercosis.” *Infection and Immunity* 81 (4): 2013.

41. Nascimento-Carvalho, Cristiana Maria. “The Role of Respiratory Viral Infections among Children Hospitalized for Community Acquired Pneumonia in Developing Country.” *The Pediatric Infectious Disease Journal*. Vol. 27. 2008.

42. Nascimento-Carvalho, Cristiana Maria, and Olli Ruuskanen. “Clinical Significance of Multiple Respiratory Virus Detection Cristiana.” *The Pediatric Infectious Disease Journal* 44 (8): 2016.

43. Noguchi, E, M Shibasaki, M Inudou, M Kamioka, Y Yokouchi, K Yamakawa-Kobayashi, H Hamaguchi, a Matsui, and T Arinami. “Association between a New Polymorphism in the Activation-Induced Cytidine Deaminase Gene and Atopic Asthma and the Regulation of Total Serum IgE Levels.” *The Journal of Allergy and Clinical Immunology* 108 (3): 382–86. 2001.

44. Nordstrom T, Blom AM, Forsgren A, Riesbeck K. The emerging pathogen Moraxella catarrhalis interacts with complement inhibitor C4b binding protein through ubiquitous surface proteins A1 and A2. J Immunol **2004**; 173(7): 4598-606.

45. Oved, Kfir, Asi Cohen, Olga Boico, Roy Navon, Tom Friedman, Liat Etshtein, Or Kriger, et al.. “A Novel Host-Proteome Signature for Distinguishing between Acute Bacterial and Viral Infections.” *PloS One* 10 (3): 2015a

46. ———. “A Novel Host-Proteome Signature for Distinguishing between Acute Bacterial and Viral Infections.” Edited by Oliver Schildgen. *PLOS ONE* 10 (3)2015b.

47. Pavia, Andrew T. “Viral Infections of the Lower Respiratory Tract: Old Viruses, New Viruses, and the Role of Diagnosis.” *Clinical Infectious Diseases* 52,2011.

48. Payton, Jacqueline E, Nicole R Grieselhuber, Li-wei Chang, Mark Murakami, Gary K Geiss, Daniel C Link, Rakesh Nagarajan, Mark A Watson, and Timothy J Ley. “Technical Advance High Throughput Digital Quantification of mRNA Abundance in Primary Human Acute Myeloid Leukemia Samples” 119 (6).2009.

49. Pettigrew, Melinda M., Janneane F. Gent, Richard B. Pyles, Aaron L. Miller, Johanna Nokso-Koivisto, and Tasnee Chonmaitree. “Viral-Bacterial Interactions and Risk of Acute Otitis Media Complicating Upper Respiratory Tract Infection.” *Journal of Clinical Microbiology* 49 (11): 3750–55. 2011.

50. Pitrez, Paulo M C, and José Lb Pitrez. “Infecções Agudas Das Vias Aéreas Superiores – Diagnóstico E Tratamento Ambulatorial.” *Jornal de Pediatria* 79 (1): 77–86. 2003.

51. Prokopec, Stephenie D, John D Watson, Daryl M Waggott, Ashley B Smith, Alexander H Wu, Allan B Okey, Raimo Pohjanvirta, and Paul C Boutros. “Systematic Evaluation of Medium-Throughput mRNA Abundance Platforms.”

RNA (New York, N.Y.) 19 (1): 51–62. 2013.

52. Rabie, Tamer, and Valerie Curtis. “Handwashing and Risk of Respiratory Infections: A Quantitative Systematic Review.” *Tropical Medicine & International Health : TM & IH* 11 (3): 258–67.2006.

53. Ren, L, R Gonzalez, Z Wang, Z Xiang, Y Wang, H Zhou, J Li, et al. “Prevalence of Human Respiratory Viruses in Adults with Acute Respiratory Tract Infections in Beijing, 2005-2007.” *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 15 (12): 1146–53. 2009.

54. Robinson, Joan. “Colonization and Infection of the Respiratory Tract: What Do We Know?” *Paediatrics & Child Health* 9 (1): 21–24. 2004.

55. Shirey, K A, W Lai, L M Pletneva, C L Karp, S Divanovic, J C G Blanco, and S N Vogel. “Role of the Lipoxygenase Pathway in RSV-Induced Alternatively Activated Macrophages Leading to Resolution of Lung Pathology.” *Mucosal Immunology* 7 (3). 2014.

56. Shirey, K A, L M Pletneva, A C Puche, A D Keegan, G A Prince, J C G Blanco, and S N Vogel. “Control of RSV-Induced Lung Injury by Alternatively Activated Macrophages Is IL-4R Alpha-, TLR4-, and IFN-Beta-Dependent.” *Mucosal Immunology* 3 (3). 2010.

57. Shu Mei Teo, Authors, Danny Mok, Kathryn E Holt, Michael Inouye Correspondence, Shu Mei Teo, Kym Pham, Merci Kusel, et al. 2015. “The Infant Nasopharyngeal Microbiome Impacts Severity of Lower Respiratory Infection and Risk of Asthma Development.” *Cell Host & Microbe* 17. 2015.

58. Silva, Maíra Domingues Bernardes, Márcia Barbosa de Paiva, Leila Rangel da Silva, and Maria Aparecida de Luca Nascimento. “Doença Respiratória Aguda Na Criança: UMA Revisão Integrative.” *Revista Enfermagem* 20 (2): 260–66.2012.

59. Silva, Rosangela de Castro, Marilda Agudo Mendonça Siqueira, Eduardo Martins Netto, Jacione Silva Bastos, Cristiana Maria Nascimento-Carvalho, Ana Luisa Vilas-Boas, Maiara Lana Bouzas, Fernando do Couto Motta, and Carlos Brites. “Epidemiological Aspects of Influenza A Related to Climatic Conditions during and after a Pandemic Period in the City of Salvador, Northeastern Brazil.” *Memorias Do Instituto Oswaldo Cruz* 109 (2): 229–35.2014.

60. Smith, Steven B, William Dampier, Aydin Tozeren, James R Brown, and Michal Magid-Slav. “Identification of Common Biological Pathways and Drug Targets across Multiple Respiratory Viruses Based on Human Host Gene Expression Analysis.” *PloS One* 7 (3): e33174.2012.

61. Soediono, Budi. “No Title No Title.” *Journal of Chemical Information and Modeling* 53: 160.1989.

62. Stein, Renato T, and Paulo José Cauduro Marostica. “Community-Acquired Pneumonia: A Review and Recent Advances.” *Pediatric Pulmonology* 42 (12): 1095–1103. 2007.

63. Suarez, N. M., E. Bunsow, a. R. Falsey, E. E. Walsh, a. Mejias, and O. Ramilo. “Transcriptional Profiling Is Superior to Procalcitonin to Discriminate Bacterial vs Viral Lower Respiratory Tract Infections in Hospitalized Adults.” *Journal of Infectious Diseases*,2015.

64. Thorburn, K, S Harigopal, V Reddy, N Taylor, and H K F van Saene. “High Incidence of Pulmonary Bacterial Co-Infection in Children with Severe Respiratory Syncytial Virus (RSV) Bronchiolitis.” *Thorax* 61 (7): 611–15.2006.

65. Tregoning, John S., and Jürgen Schwarze.. “Respiratory Viral Infections in Infants: Causes, Clinical Symptoms, Virology, and Immunology.” *Clinical Microbiology Reviews* 23 (1): 74–98. 2010

66. Uitti, Johanna M., Paula a. Tähtinen, Miia K. Laine, Pentti Huovinen, Olli

Ruuskanen, and Aino Ruohola. “Role of Nasopharyngeal Bacteria and Respiratory Viruses in Acute Symptoms of Young Children.” *The Pediatric Infectious Disease Journal* 34 (10): 2015.

67. Wiertsema, Chidlow, Kirkham, Corscadden, Mowe, Harnett Vijayasekaran, Coates, and Richmond. “A Clinically Relevant, Syngeneic Model of Spontaneous, Highly Metastatic B16 Mouse Melanoma.” *Anticancer Research* 30 (12): 4799–4804. 2010.

68. Yang, Samuel, and Richard E Rothman. “PCR-Based Diagnostics for Infectious Diseases : Uses , Limitations , and Future Applications in Acute-Care Settings.” *The Lancet* 4: 337–48.2004.

69. Zhu, Min, Jing Xia, Molei Yan, Guolong Cai, Jing Yan, and Gangmin Ning. “Dimensionality Reduction in Complex Medical Data : Improved Self-Adaptive Niche Genetic Algorithm” 2015.
70. Spann KM, Tran KC, Chi B, Rabin RL, Collins PL. Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. J Virol **2004**; 78(8): 4363-9.