

**ANÁLISE DO PERFIL TRANSCRICIONAL DE RNAS LONGOS
NÃO CODIFICANTES EM PACIENTES PEDIÁTRICOS COM
INFECÇÃO POR VÍRUS SINCICIAL RESPIRATÓRIO**

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ATA DA DEFESA PÚBLICA DA DISSERTAÇÃO DE MESTRADO DE ANA BEATRIZ RODRIGUES, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA), DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.

Aos 18 dias do mês de julho do ano de 2025, às 8h30min, por meio de Videoconferência, realizou-se a defesa de DISSERTAÇÃO DE MESTRADO de ANA BEATRIZ RODRIGUES, intitulada **Análise do Perfil Transcricional de RNAs Longos Não Codificantes em Pacientes Pediátricos com Infecção por Vírus Sincicial Respiratório**, sob orientação do Prof. Dr. Guilherme Targino Valente. A Comissão Examinadora foi constituída pelos seguintes membros: Prof. Dr. GUILHERME TARGINO VALENTE (Orientador(a) - Participação Virtual) do(a) Núcleo de Inteligência Artificial / Hospital das Clínicas da Faculdade de Medicina de Botucatu (HC- FMB - UNESP), Profa. Dra. TATIANA DE CAMPOS MELO (Participação Virtual) do(a) Departamento de Pediatria / Hospital das Clínicas da Faculdade de Medicina de Botucatu (HC- FMB) - UNESP, Dr. LEONARDO NAZARIO DE MORAES (Participação Virtual) do(a) Laboratório de Biotecnologia Aplicada (LBA) / Hospital das Clínicas da Faculdade de Medicina de Botucatu (HCFMB) - UNESP. Após a exposição pela mestrandia e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: Aprovada . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Prof. Dr. GUILHERME TARGINO VALENTE



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RESUMO

O vírus sincicial respiratório (VSR) é uma das principais causas de infecções respiratórias graves em crianças menores de cinco anos, responsável por milhões de hospitalizações e milhares de mortes anualmente, especialmente em países de baixa e média renda. Globalmente, as infecções respiratórias agudas (IRAs) representam um grande problema de saúde pública, sendo uma das principais causas de mortalidade, morbidade e internações hospitalares em crianças. Embora o VSR tenha sido amplamente estudado, os mecanismos moleculares que regulam a resposta à infecção ainda são pouco compreendidos. Os RNAs longos não codificantes (*lncRNAs*), transcritos com mais de 200 nucleotídeos que não codificam proteínas, emergem como reguladores essenciais da expressão gênica, desempenhando papéis cruciais em diversos processos biológicos, como imunidade antiviral, inflamação e resposta celular. Esses *lncRNAs* apresentam potencial como biomarcadores e alvos terapêuticos em doenças respiratórias virais. Este estudo reanalisou dados transcriptômicos de pacientes pediátricos infectados por VSR, integrando análises de *bulk RNA-Seq*, sequenciamento de RNA de células únicas (*scRNA-Seq*) e redes moleculares para elucidar os mecanismos regulatórios envolvidos na infecção e na resposta imune. Foram identificados centenas de *lncRNAs* diferencialmente expressos em resposta ao VSR, incluindo *SOX2-OT*, *C5orf64* e *MEG3*, que podem estar associados à regulação de genes relacionados a doenças pulmonares. Além disso, os *lncRNAs* *OIP5-AS1* e *LINC00342* mostraram associação com a menor gravidade da infecção. Análises de *scRNA-Seq* indicaram que certos *lncRNAs* apresentam maior expressão em células *NK CD16* e células T reguladoras (*Tregs*), enquanto outros estão reduzidos em células *ILC3* de pacientes infectados. A integração de dados de redes moleculares revelou uma reorganização significativa das interações entre *lncRNAs* e genes/proteínas em pacientes infectados, sugerindo que o VSR provoca uma reestruturação funcional nos sistemas regulatórios. Este é o primeiro estudo a demonstrar de forma abrangente o impacto do VSR sobre os *lncRNAs* em crianças, fornecendo novas perspectivas sobre os mecanismos regulatórios envolvidos na resposta à infecção. Esses achados destacam o potencial dos *lncRNAs* como biomarcadores da infecção, abrindo novas possibilidades para o desenvolvimento de estratégias diagnósticas e terapêuticas mais eficazes.

Palavras-chave: Vírus sincicial respiratório (VSR), RNAs longos não codificantes (*lncRNAs*), Redes moleculares, RNA-Seq, *scRNA-Seq*.

ABSTRACT

Respiratory syncytial virus (RSV) is one of the leading causes of severe respiratory infections in children under five years of age, responsible for millions of hospitalizations and thousands of deaths annually, particularly in low- and middle-income countries. Globally, acute respiratory infections (ARIs) represent a major public health issue, being one of the primary causes of mortality, morbidity, and hospital admissions in children. Although RSV has been widely studied, the molecular mechanisms regulating the host response remain poorly understood. Long non-coding RNAs (lncRNAs), transcripts longer than 200 nucleotides that do not encode proteins, emerge as essential regulators of gene expression, playing crucial roles in various biological processes such as antiviral immunity, inflammation, and cellular response. These lncRNAs hold potential as therapeutic targets in viral respiratory diseases. In this context, this study reanalyzed transcriptomic data from pediatric patients infected with RSV, integrating bulk RNA-Seq, single-cell RNA sequencing (scRNA-Seq), and molecular networks analyses to elucidate the regulatory mechanisms involved in the infection and immune response. Hundreds of differentially expressed lncRNAs in response to RSV were identified, including SOX2-OT, C5orf64, and MEG3, which may be associated with the regulation of genes related to lung diseases. Additionally, lncRNAs OIP5-AS1 and LINC00342 were associated with less severe infection. Single-cell RNA analyses indicated that certain lncRNAs are more highly expressed in NK CD16 cells, while others are reduced in ILC3 cells in infected patients. The integration of molecular network data revealed a significant reorganization of interactions between lncRNAs and genes/proteins in infected patients, suggesting that RSV induces functional restructuring in regulatory systems. This is the first study to comprehensively demonstrate the impact of RSV on lncRNAs in children, providing new insights into the regulatory mechanisms involved in the response to infection. These findings open new possibilities for the development of more effective diagnostic and therapeutic strategies.

Keywords: Respiratory syncytial virus (RSV), Long non-coding RNAs (lncRNAs), Molecular networks, RNA-Seq, scRNA-Seq.

SUMÁRIO

1. INTRODUÇÃO	7
1.1 Infecções Respiratórias Agudas	7
1.2 Vírus Sincicial Respiratório	8
1.3 RNAs Longos Não Codificantes (<i>lncRNAs</i>)	9
1.4 <i>LncRNAs</i> e Infecções Respiratórias.....	10
2. OBJETIVOS	13
2.1 Objetivo geral.....	13
2.2 Objetivos específicos	13
3. ARTIGO CIENTÍFICO PUBLICADO	14
4. CONSIDERAÇÕES FINAIS.....	51
5. REFERÊNCIAS BIBLIOGRÁFICAS	52

1. INTRODUÇÃO

1.1 Infecções Respiratórias Agudas

As infecções respiratórias agudas (IRAs) compreendem um conjunto de processos infecciosos que acometem o sistema respiratório, sendo categorizadas em dois grupos principais conforme a localização anatômica afetada: as que impactam as vias aéreas superiores, incluindo rinofaringite, faringite, amigdalite, sinusite e laringite, e as que comprometem as vias aéreas inferiores, como bronquite, bronquiolite e pneumonia (Rogan, 2017).

Globalmente, as infecções respiratórias agudas (IRAs) representam um importante problema de saúde pública, sendo uma das principais causas de mortalidade, morbidade e hospitalizações em crianças (Li *et al.*, 2021). Segundo estimativas da Organização Mundial da Saúde (OMS), as IRAs foram responsáveis por cerca de 3 milhões de óbitos em 2016, ocupando a quarta posição entre as principais causas de morte no mundo (Li *et al.*, 2021). Dentre essas, as infecções respiratórias agudas que acometem as vias aéreas inferiores destacam-se como as principais causas de internações e mortes hospitalares entre crianças menores de 5 anos (Troeger *et al.*, 2019). No estudo conduzido por Wang *et al.* (2021), estima-se que, anualmente, cerca de 5,5 milhões de admissões hospitalares foram atribuídas a infecções respiratórias agudas das vias aéreas inferiores, no período entre 1995 e 2019, em pacientes de 5 a 19 anos, culminando em 87.900 mortes entre os indivíduos hospitalizados nessa faixa etária.

As manifestações clínicas e a severidade dos sintomas relacionados às IRAs variam conforme o tipo específico de infecção e os patógenos envolvidos. Os principais agentes etiológicos dessas infecções incluem vírus, como o vírus sincicial respiratório, influenza, rinovírus, metapneumovírus humano, coronavírus, adenovírus e vírus da parainfluenza. Além disso, bactérias como *Streptococcus pneumoniae*, *Haemophilus influenzae* e *Staphylococcus aureus* são frequentemente identificadas como causadoras dessas infecções respiratórias (Assane *et al.*, 2018; Bhuyan *et al.*, 2017; Wolf *et al.*, 2006).

Os vírus respiratórios desempenham um papel central nas infecções respiratórias agudas em crianças, estando implicados em aproximadamente 60% das infecções das vias inferiores, o que evidencia sua significativa contribuição para as taxas de morbidade e mortalidade nessa faixa etária (Bhuyan *et al.*, 2017; Walker; Khurana; Tilden, 1994). Em um estudo abrangente envolvendo 233.037 pacientes diagnosticados com IRAs, Li *et al.* (2021) reportaram que crianças menores de 5 anos e aquelas em idade escolar (5 a 17 anos) apresentam as maiores taxas de positividade para infecções respiratórias agudas virais. Adicionalmente, os resultados indicaram que os principais patógenos virais identificados entre as crianças menores de 5 anos foram o vírus sincicial respiratório, seguido pelo rinovírus humano, o vírus parainfluenza

humano e o vírus da influenza, com o vírus sincicial respiratório sendo o patógeno mais prevalente, encontrado em 25,7% dos casos (Li *et al.*, 2021).

1.2 Vírus Sincicial Respiratório

O vírus sincicial respiratório (VSR) da família *Paramyxoviridae* é um vírus envelopado com genoma constituído por *RNA* de fita simples, de polaridade negativa e não segmentado (Borchers *et al.*, 2013; Kaler *et al.*, 2023). Classificado em dois subtipos, A e B, com base na reatividade da proteína de superfície G aos anticorpos monoclonais, o subtipo A é considerado mais virulento e, em geral, mais prevalente, embora ambos os subtipos circulem simultaneamente (Agoti *et al.*, 2015; Jallow *et al.*, 2023; Munro *et al.*, 2023).

O genoma do vírus sincicial respiratório (VSR) tem um comprimento de 15,2 kilobases (Kb) e é composto por 10 genes responsáveis pela codificação de 11 proteínas. No *RNA* M2, existem duas *open reading frames* (ORFs) sobrepostas, que resultam na tradução de duas proteínas matriciais distintas: M2-1 e M2-2. O envelope viral contém três proteínas essenciais: a glicoproteína G, que permite a adesão às células hospedeiras; a glicoproteína de fusão (F), que facilita a fusão do vírus com a célula e sua entrada subsequente; e a proteína SH (do inglês, *small hydrophobic*) (Agoti *et al.*, 2015; Anderson *et al.*, 2021; Borchers *et al.*, 2013). O VSR é também composto por cinco proteínas estruturais: a proteína de matriz (M), juntamente com os componentes do complexo ribonucleoproteico, que incluem a nucleoproteína (N), a fosfoproteína (P), a proteína M2-1 e a unidade principal da polimerase viral, a proteína L. Além disso, o vírus apresenta duas proteínas não estruturais, NS1 e NS2, bem como a proteína M2-2, que se acumula durante a infecção e está envolvida na transição da transcrição de *RNA* para a síntese de *RNA* genômico (Bermingham; Collins, 1999; Borchers *et al.*, 2013; Collins; Melero, 2011).

O vírus sincicial respiratório humano é o patógeno mais comumente identificado em crianças menores de 5 anos com infecção aguda do trato respiratório inferior. Nessa faixa etária, a vulnerabilidade a complicações graves causadas pelo VSR é especialmente alta, resultando em diversas infecções, tanto no trato respiratório superior quanto inferior, incluindo bronquiolite e pneumonia (Jallow *et al.*, 2023; Zhao *et al.*, 2022). Globalmente, o VSR afeta 33,1 milhões de crianças, sendo responsável por 3,2 milhões de internações hospitalares e 59.600 mortes anuais em hospitais (Shi *et al.*, 2017). Além disso, 99% das mortes relacionadas ao VSR ocorrem em países de baixa e média renda, evidenciando a gravidade dessas infecções nesse contexto (Jallow *et al.*, 2023; Jarju *et al.*, 2020). Apesar desses dados, o tratamento

continua a ser predominantemente de suporte, uma vez que não existem antivirais amplamente eficazes contra o VSR (Feng *et al.*, 2023).

1.3 RNAs Longos Não Codificantes (*lncRNAs*)

Estima-se que apenas cerca de 2% do genoma humano seja composto por éxons codificadores de proteínas, enquanto a maior parte é transcrita em *RNAs* não codificantes (*ncRNAs*) (Wu *et al.*, 2023). Os *ncRNAs* podem ser classificados em duas categorias principais com base no tamanho: *RNAs* longos e *RNAs* curtos e médios. Apesar de não codificarem proteínas, esses transcritos desempenham importantes funções regulatórias e estruturais (Meseure *et al.*, 2015).

Os *RNAs* longos não codificantes (*lncRNAs*) formam uma classe de transcritos com mais de 200 nucleotídeos que, geralmente, não são traduzidos em proteínas, embora alguns possam codificar pequenos peptídeos (Wu *et al.*, 2020). Com base em sua localização genômica e relação com genes codificadores de proteínas, os *lncRNAs* podem ser classificados como intergênicos, *sense*, *antisense*, bidirecionais ou intrônicos. Esses transcritos desempenham papéis essenciais no transcriptoma de mamíferos, atuando em diversos processos biológicos, como modificadores epigenéticos, ativadores ou repressores transcricionais, componentes estruturais, capturadores de *microRNAs* e outros *RNAs*, além de contribuírem para a organização nuclear. Além disso, em comparação aos *mRNAs*, os *lncRNAs* são mais frequentemente encontrados no núcleo do que no citoplasma. Embora sejam menos abundantes, os *lncRNAs* são amplamente expressos em tecidos humanos e apresentam uma especificidade tecidual em relação aos transcritos codificadores de proteínas. Essa especificidade destaca o potencial dos *lncRNAs* como biomarcadores promissores (Iyer *et al.*, 2015; Senmatsu; Hirota, 2020; Quinn; Chang, 2016).

Considerando suas funções em nível molecular, os *lncRNAs* podem atuar como *signals*, *decoys*, *guides* ou *scaffolds*. Como *signals*, eles estabelecem interações específicas com proteínas, formando complexos que modulam a atividade de fatores de transcrição e influenciam modificadores de cromatina, sinalizando eventos determinantes para a regulação gênica. Como *decoys*, os *lncRNAs* se ligam a proteínas-alvo, como fatores de transcrição, modificadores de cromatina ou pequenos RNAs regulatórios, desviando-os de seus sítios de ação, modulando negativamente a atividade de tais efetores. Uma subclasse específica, conhecida como competidores endógenos (*competing endogenous RNAs*, *ceRNAs*), atua como "esponjas" de *microRNAs*, sequestrando esses transcritos e permitindo que seus alvos sejam traduzidos. Como *guides*, os *lncRNAs* ligam-se a proteínas e direcionam os complexos

formados para alvos específicos, podendo regular genes em *cis*, próximos ao local de sua transcrição, ou em *trans*, alcançando genes distantes, até mesmo em outros cromossomos. Por fim, os *lncRNAs* também podem atuar como *scaffolds*, sobre os quais importantes componentes moleculares, com atividades repressoras ou ativadoras da transcrição, são montados. Dessa forma, esses *lncRNAs* conectam-se simultaneamente a diversas moléculas efetoras, por meio de diferentes domínios, combinando-as de forma coordenada no tempo e no espaço (Gao *et al.*, 2020; Ramón Y Cajal; Segura; Hümmer, 2019; Wang; Chang, 2011).

1.4 *LncRNAs* e Infecções Respiratórias

Os *lncRNAs* desempenham um papel significativo durante as infecções virais. *LncRNAs* provenientes dos genomas virais podem promover resistência à atividade antiviral celular e favorecer a replicação do vírus; enquanto *lncRNAs* do hospedeiro impedem a replicação viral (Kesheh; Mahmoudvand; Shokri, 2022). Esses *lncRNAs* influenciam as respostas de defesa imunológica ao interagir diretamente com sensores, adaptadores, efetores e fatores de transcrição (Wang, 2019). Tanto os *lncRNAs* humanos quanto aqueles codificados por vírus têm a capacidade de modular a expressão de genes do hospedeiro, sendo essenciais para processos como replicação viral, latência, ativação de vias de sinalização, produção de citocinas e quimiocinas, processamento de *RNAi*, expressão de interferon (*IFN*) e genes estimulados por interferon (*ISGs*) (Kesheh; Mahmoudvand; Shokri, 2022). Embora a função e os mecanismos dos *lncRNAs* não tenham sido completamente definidos, revelar a relação entre esses *lncRNAs* e os vírus proporcionaria uma nova perspectiva sobre as doenças virais e poderia indicar os *ncRNAs* como potenciais alvos para o desenvolvimento de novos medicamentos antivirais (Ding *et al.*, 2016).

Modelos *in vitro* e *in vivo* evidenciaram que, após a infecção pelo vírus da influenza, particularmente em células pulmonares, a ativação da via de sinalização dependente do gene *RIG-I* (do inglês, *retinoic acid-inducible gene I*) e a subsequente indução de interferon beta (*IFN-β*) resultaram em um aumento significativo do *lncRNA ISR* (do inglês, *Interferon-stimulated lncNA*). Este *lncRNA ISR*, por sua vez, mostrou-se capaz de suprimir efetivamente a replicação viral (Kesheh; Mahmoudvand; Shokri, 2022; Pan *et al.*, 2019).

O *ISG20* exibe atividade antiviral contra diversos vírus RNA, incluindo o vírus da Influenza A (IAV). Ele atua inibindo a replicação do IAV mediante interação com a nucleoproteína viral (NP) e da supressão da atividade da polimerase viral. A expressão de *ISG20* é regulada pelo *miRNA-326*, que se associa ao 3'UTR de seus *mRNAs*. Nesse contexto, a infecção IAV induz a expressão do *lnc-ISG20*, que exerce um papel inibitório sobre a replicação

do IAV ao se associar ao *miR-326*, aumentando assim os níveis da proteína *ISG20* (Kesheh; Mahmoudvand; Shokri, 2022).

Outro *lncRNA* de destaque na infecção por IAV é o *IVRPIE*, identificado como parte da resposta imunológica inata antiviral. Durante a infecção, o *lnc-IVRPIE* apresentou um aumento significativo em sua expressão. Dados *in vitro* demonstraram que esse *lncRNA* desempenha sua atividade antiviral ao potencializar o *IFN β 1* e vários genes estimulados por interferon (*ISGs*), como o fator regulador de interferon 1 (*IRF1*). Esses resultados destacam, portanto, o papel do *IVRPIE* na defesa inata do hospedeiro, atuando como um regulador positivo da expressão de *IFN β 1* e *ISGs* durante a infecção por IAV (Kesheh; Mahmoudvand; Shokri, 2022).

Na infecção por IAV, são identificados ainda *lncRNAs* associados à promoção da replicação viral, como o *IPAN*, que é sequestrado pelo vírus para estabilizar a proteína *PBI* e, assim, favorecer a replicação viral. Esse *lncRNA*, de fato, forma o complexo *IPAN/PBI*, impedindo a degradação do vírus pelo sistema imunológico do hospedeiro. Nesse mesmo contexto, o *lncRNA EGOT* promove a replicação viral ao modular os níveis do coativador transcricional *TBXL1* e de genes estimulados por interferon (*ISGs*), resultando no bloqueio da resposta antiviral mediada pelo interferon (*IFN*). A inibição de *EGOT* leva ao aumento dos níveis de diversos *ISGs* e à redução da replicação viral (Kesheh; Mahmoudvand; Shokri, 2022).

No que tange à infecção pelo vírus sincicial respiratório (RSV), a proteína de transporte intracelular *RAB5C* é um alvo direto do *miR-509* e pode ser utilizada para a entrada viral e a transmissão intracelular. O *lnc-NRAV* regula a expressão de *RAB5C* ao se ligar ao *miR-509*. Dessa forma, o *lnc-NRAV* promove a proliferação do RSV e acelera o transporte intracelular de vesículas, aumentando a expressão de diversos genes, como a proteína F, e intensificando a expressão do gene *RAB5C* por meio da captura do *miR-509*. Logo, o silenciamento do *lnc-NRAV* resulta na inibição da propagação do VSR (Kesheh; Mahmoudvand; Shokri, 2022).

Outros *lncRNAs* se destacam na infecção por VSR. A superexpressão do *lnc-MEG3* exerce um papel protetor contra a ativação de citocinas inflamatórias em células epiteliais das vias aéreas humanas. Em um modelo de camundongo asmático, o aumento da expressão do *lnc-n337374* resultou no alívio dos sintomas da asma. De maneira geral, a infecção por VSR provoca a maturação das células dendríticas, e há indícios de que o *lnc-n337374* inibe essa maturação por meio da regulação negativa de *CD86* e *pERK1/2*. Além disso, a interação entre o *lnc-PVT1* e o *miR-2031* aumenta a entrada de células quiescentes na fase G1/S do ciclo celular (Kesheh; Mahmoudvand; Shokri, 2022).

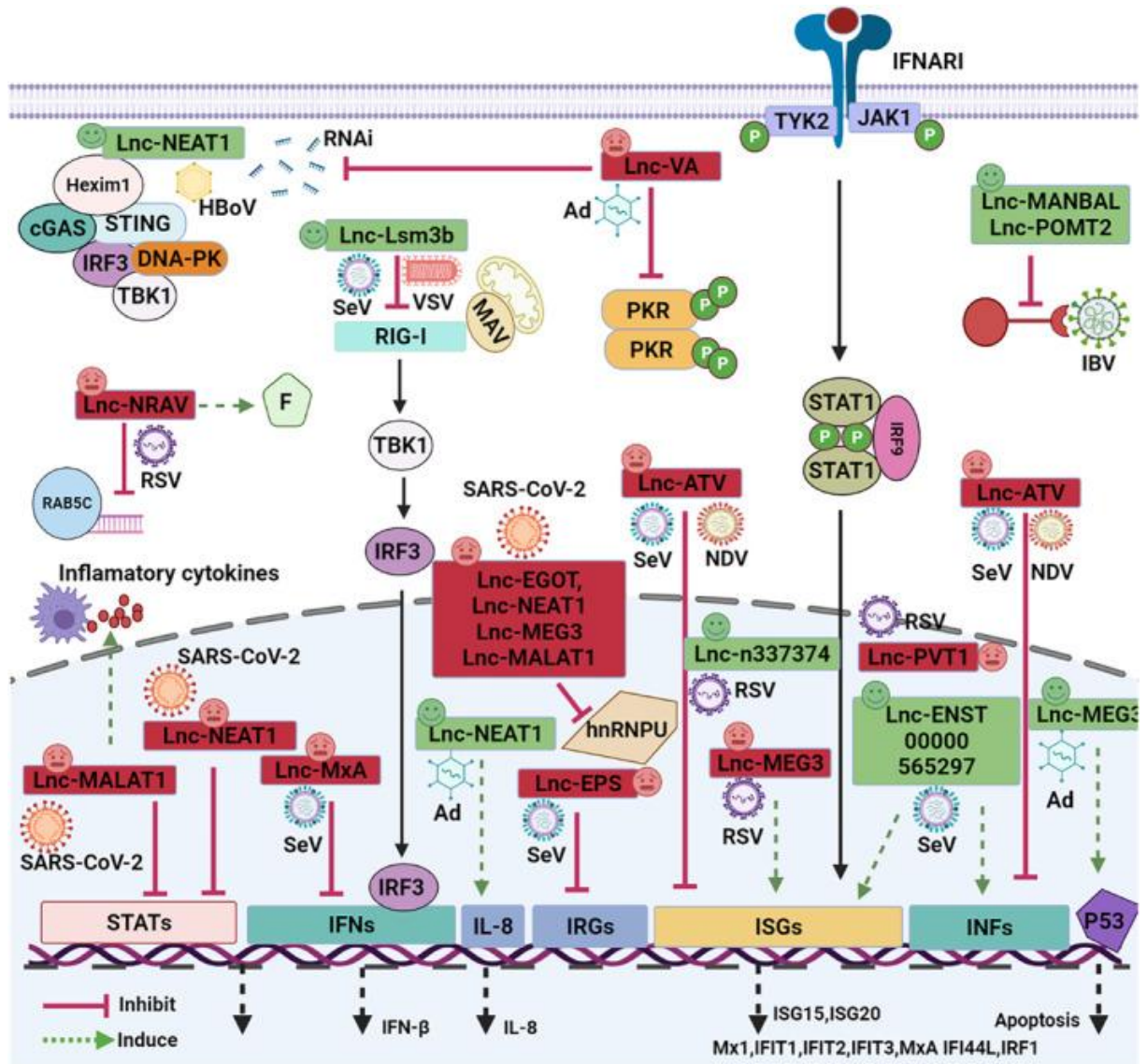


Figura 1. Representação esquemática dos papéis dos lncRNAs durante infecções virais respiratórias. A maioria desses lncRNAs regula a resposta imunológica do hospedeiro em diferentes estágios para inibir (em verde) ou promover (em vermelho) as infecções virais. Fonte: (Kesheh; Mahmoudvand; Shokri, 2022).

2. OBJETIVOS

2.1 Objetivo geral

Avaliar o perfil transcricional de RNAs longos não codificantes (*lncRNAs*) em pacientes pediátricos infectados pelo vírus sincicial respiratório (VSR), integrando dados de *bulk RNA-Seq*, sequenciamento de RNA de células únicas (*scRNA-Seq*) e redes moleculares, a fim de investigar mecanismos regulatórios envolvidos na infecção e na resposta imune.

2.2 Objetivos específicos

- Reanalisar dados de *bulk RNA-Seq* de amostras de sangue total de pacientes pediátricos infectados pelo vírus sincicial respiratório (VSR) para *lncRNAs* diferencialmente expressos.
- Construir e comparar redes moleculares associadas a *lncRNAs* em condições saudáveis e infectadas pelo VSR, destacando interações críticas relacionadas à infecção.
- Identificar *lncRNAs* presentes exclusivamente nas redes moleculares de pacientes infectados pelo VSR, associados a doenças respiratórias, e suas interações com genes ou proteínas relacionados a essas condições.
- Inferir o papel funcional dos *lncRNAs* exclusivos da rede VSR, realizando uma análise de enriquecimento de ontologia gênica para seus interatores.
- Realizar a deconvolução dos dados de *bulk RNA-Seq* para avaliar o enriquecimento de tipos celulares imunológicos em pacientes saudáveis e infectados por VSR.
- Avaliar o perfil de expressão de *lncRNAs* em diferentes tipos celulares utilizando dados *scRNA-Seq* provenientes de aspirados traqueais de pacientes pediátricos.

3. ARTIGO CIENTÍFICO

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Analysis of lncRNAs in children infected with respiratory syncytial virus using the transcriptome, single-cell RNA-Seq, and networks

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Abstract

The respiratory syncytial virus (RSV) is one of the leading causes of severe respiratory illness in children, responsible for millions of hospitalizations and thousands of deaths annually. Despite extensive research on RSV in pediatric patients, the molecular mechanisms underlying the host response to this infection remain poorly understood. Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that do not encode proteins; they regulate gene expression, participating in antiviral immunity, inflammation, cellular responses, and others. Here, we analyzed bulk transcriptome data from pediatric RSV-infected patients and integrated it with single-cell RNA-Seq analysis, focusing on molecular networks involving lncRNAs. We built a network comprising 3,085,980 experimentally validated molecular interactions, from which we created networks to model the molecular systems of healthy and RSV-infected patients. Network analysis revealed hundreds of lncRNAs responsive to RSV infection, some of which interact with infection-related genes and proteins. The lncRNA interactors in the RSV network were associated with mRNA metabolism and the regulation of nucleobase-containing compound metabolic processes. Network analysis highlighted SOX2-OT, C5orf64, and MEG3 as candidate lncRNAs potentially involved in pediatric RSV infection through regulation or interaction with genes associated with respiratory diseases. Furthermore, the lncRNAs OIP5-AS1 and LINC00342 were associated with mild RSV infections. Single-cell RNA-Seq data revealed lncRNA markers with elevated expression in CD16- NK and regulatory T cells, and reduced expression in ILC3 cells during infection. We offered insight into the RSV-induced remodeling of lncRNA networks, with implications for the development of novel biomarkers and therapeutic strategies in viral respiratory diseases.

Keywords Systems Biology, Data integration, RSV, Pediatrics, Long non-coding RNAs

Introduction

The human respiratory syncytial virus (RSV) is the most common cause of severe respiratory disease in infants, young children, and the elderly (1). Globally, RSV accounts for a significant proportion of morbidity and mortality, particularly in infants under 6 months of age (2). By the age of 2, nearly all children have been infected with RSV (3). This virus is responsible for more than 3 million hospitalizations and 100,000 deaths annually in children under 5 years of age; 99% of cases occur in low- and middle-income countries (4).

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that do not encode proteins. LncRNAs are involved in various biological processes by regulating gene expression both in *cis* and *trans* across multiple species (5–9). LncRNAs act as epigenetic modulators, transcriptional activators or repressors, scaffolds for chromatin-modifying complexes, molecular sponges for microRNAs, and work as structural components of the nuclear architecture. These transcripts can also interact with DNA, several classes of RNA, proteins, lipids, and other cellular components to modulate chromatin dynamics, DNA accessibility, transcription, RNA processing, and signal transduction (10,11).

During respiratory infections, lncRNAs exhibit altered expression patterns that modulate the host immune response (12,13). Viral genome-derived lncRNAs may facilitate immune evasion and support viral replication, whereas host-derived lncRNAs often play protective roles by restricting viral proliferation. LncRNAs also influence the immune response through interactions with RNA sensors, adaptor proteins, effector molecules, and transcription factors. Both host and viral lncRNAs regulate the expression of interferons (IFNs), interferon-stimulated genes (ISGs), cytokines, chemokines, and components of the RNA interference machinery, thereby impacting viral replication, latency, and host-pathogen interactions (14).

The role of specific lncRNAs in RSV infection has been reported in the literature. For example, the lncRNA *NRAV* promotes RSV replication by sponging *miR-509*, resulting in the derepression of *RAB5C*, enhanced intracellular vesicle transport, and increased expression of the F protein. Interestingly, *NRAV* silencing has been shown to inhibit RSV propagation (14,15). Conversely, *MEG3*

appears to exert a protective effect on airway epithelial cells by suppressing the activation of pro-inflammatory cytokines (16). Furthermore, *Inc-n337374* has been reported to mitigate asthma-like symptoms in murine models and inhibit dendritic cell maturation by downregulating the expression of *CD86* and *pERK1/2* (14).

Despite advances in understanding the clinical features and epidemiology of RSV in pediatric patients, comprehensive transcriptomic analyses of lncRNA regulation in this group remain limited. To date, only three RNA sequencing-based transcriptomic studies have investigated RSV infection in children. One study analyzed bulk RNA-seq data from whole blood samples of hospitalized patients and reported the upregulation of genes involved in neutrophil activation and inflammation, alongside the downregulation of T cell-associated genes (17). Transcriptomic analysis of sputum samples from RSV-infected pediatric patients identified differentially expressed genes enriched for cilium organization, immune system processes, interferon response, and T cell subtypes, including CD8⁺ and follicular helper T cells (18). A third study analyzed tracheal aspirates from children with acute respiratory distress syndrome using single-cell RNA-seq (scRNA-seq), revealing 15 transcriptionally distinct cell clusters and significant alterations in the expression profiles of various immune cell types in RSV-infected patients (19). Notably, none of these studies mentioned evaluated lncRNAs in response to RSV infection.

In light of these knowledge gaps, we investigated the role of lncRNAs in pediatric RSV infection by analyzing publicly available bulk and single-cell transcriptomic datasets, incorporating network analysis. We identified hundreds of lncRNAs responsive to RSV infection and observed that RSV altered the relevance of network connections involving lncRNAs. Six lncRNAs (*CASC2*, *LINC00882*, *MEG3*, *MIR100HG*, *SNHG4*, and *SOX2-OT*) identified in networks specific to RSV-infected patients were associated with respiratory diseases. Moreover, *SOX2-OT*, *C5orf64*, and *MEG3* appear to be directly related to RSV infection, while *OIP5-AS1* and *LINC00342* were associated with milder RSV severity. Based on scRNA-seq data, we found that lncRNAs may be marker genes for RSV infection. Some of these marker lncRNAs showed higher expression in CD16⁺ NK cells and regulatory T cells (Tregs), while others exhibited reduced expression in ILC3 cells in infected patients. Altogether, these

results underscore the potential of lncRNAs as regulators of RSV infection and biomarkers of RSV infection in pediatric patients.

Materials and Methods

Bulk transcriptome analysis

First, we searched in the Gene Expression Omnibus (GEO) Illumina-based transcriptomics datasets related to respiratory syncytial virus (RSV) infection in pediatric patients. The dataset GSE155925 was selected for our analysis because it contains RNA-Seq data from whole blood samples of 31 RSV-infected children and 10 healthy controls (17) (**Supplementary Table 1**). Raw sequencing reads were downloaded from the European Nucleotide Archive (ENA) under the accession number PRJNA656180. The quality of the reads was assessed using FASTQC (20), which showed a desirable Phred score (≥ 30).

For differential gene expression, we mapped the reads against the human genome (GRCh38.p14) using STAR (default) (21), and gene-level counts were generated using featureCounts (default settings) (22). Differential expression was analyzed using DESeq2 (default settings) (23) to compare healthy controls and RSV-infected patients. A heatmap and hierarchical clustering (using one minus Pearson correlation) of the top 300 upregulated and downregulated lncRNAs comparing healthy vs. infected patients were generated using Morpheus (<https://software.broadinstitute.org/morpheus>). Additional pairwise comparisons were performed between mild and severe infected cases, mild cases and healthy patients, and severe cases and healthy patients.

Bulk transcriptome deconvolution was performed to evaluate the enrichment of immune cell types in healthy and RSV-infected pediatric patients. For this purpose, the read count matrix generated by DESeq2 was analyzed using the Immune Estimation module of TIMER 2.0 (24). Predicted xCell cell type proportions were visualized using a dot plot generated with the ggplot2 package in R. A two-tailed t-test was used to compare the abundance of each cell type between healthy controls and RSV-infected patients. Group differences were further explored by calculating log₂ fold change values based on the mean proportion of xCell-predicted cell types to determine which immune cell populations were enriched or depleted in RSV-infected patients.

Network analysis

We constructed a human scaffold molecular network by integrating gene regulatory interactions, ncRNA interactions, and protein-protein interactions (PPI) from public databases. Regulatory interactions were obtained from RegNetwork (25) and from an aggregated dataset containing regulatory interactions from 14 distinct databases (26). PPI data were downloaded from BioGRID (v. 4.4.233) (27), and ncRNA interactions were retrieved from NPInter v5.0 (28). Interactions based on co-expression, expression correlation, and DNA-DNA interactions were excluded from regulatory network data. Only experimentally validated PPIs were included, supported by techniques such as affinity capture-luminescence, affinity capture-MS, affinity capture-Western, co-crystal structure, far-Western, FRET, PCA, reconstituted complex, and two-hybrid assays. The resulting scaffold network consisted of nodes representing genes, RNAs, or proteins, and edges (links) representing regulatory or physical interactions.

We integrated multiple datasets into the scaffold network matrix for further analysis (<https://figshare.com/s/3a671bb8a2877ac0239f>): 1- we calculated edge weights independently for healthy and RSV-infected patients, based on significantly differentially expressed genes ($p_{adj} < 0.05$) identified by DESeq2. The edge weight for each interaction was calculated as the product of the average expression levels of interactor 1 and interactor 2; 2- Gene ID, transcript ID, gene name, transcript name, and transcript type information were retrieved from Ensembl using Biomart and incorporated into the network; 3- DESeq2-derived data were integrated, including log2 fold change, adjusted p-values, and normalized mean expression values for healthy and RSV-infected patients.

We generated the pre-healthy network (related to healthy patients) and the pre-RSV network (related to RSV-infected patients) from the scaffold network according to Equation 1:

$$SE = E_i \geq C \mid C = \overline{Ew} \quad \text{eq. 1}$$

where SE represents the selected edges, E_i is a specific edge under analysis, C is the cutoff, and $\overline{Ew}$ corresponds to the average edge weight. For example, if the cutoff is 4, all edges with weights greater than or equal to four were selected for further analysis. This procedure was applied independently to each condition (healthy and RSV-infected patients) to construct the pre-healthy and the pre-RSV networks. Statistical differences in edge weights between these networks were

evaluated using the Mann-Whitney U test (unpaired, two-tailed, with a 95% confidence interval).

We selected edges present only in the pre-RSV net compared to pre-healthy net to generate the RSV-net, from which we extracted the lncRNA interactions. Thus, the interactions analyzed here are specific to RSV-infected patients. A similar procedure was applied to construct the healthy-net, which consists of interactions exclusive to the pre-healthy net.

The aforementioned pipeline was also employed to compare mild vs. severe RSV-infected cases, mild cases vs. healthy patients, and severe cases vs. healthy patients.

To elucidate the biological processes potentially regulated by lncRNAs exclusively present in the RSV-net, we implemented a guilt-by-association strategy. For this purpose, gene ontology enrichment analysis of lncRNA interactors was performed using g:Profiler (default settings) (29). Additionally, we conducted a literature review to identify lncRNA interactions previously associated with respiratory diseases, aiming to prioritize the most relevant interactions.

The initial analysis of RSV-net highlighted SOX2-OT as a lncRNA potentially relevant to RSV infection in the analyzed patients. To investigate whether SOX2-OT interactions differed between the pre-healthy net and pre-RSV net, we applied the diffusion algorithm (default parameters) (30) to the SOX2-OT-centered subnetworks derived from the pre-healthy net and the pre-RSV net. Then, we selected the top 100 most relevant nodes ranked by diffusion and constructed two new subnetworks ("pre-healthy net SOX2-OT diffusion" and "pre-RSV net SOX2-OT diffusion"). These subnetworks were compared using the DyNet tool (default parameters) (31), implemented in Cytoscape, to identify the most variable nodes. To determine whether these network differences reflected functional changes, we conducted gene ontology enrichment analysis of the lncRNA interactors from both diffusion-based subnetworks using g:Profiler (default parameters) (29). Finally, according to the DyNet comparison, we focused on the interactions involving HNRNPA1, a protein exhibiting extensive edge rewiring (discussed further below), to assess condition-specific interaction patterns.

Single-cell analysis

The relationship between tracheal aspirates and whole blood samples in the context of viral infections is essential for understanding the presence and dynamics of pathogens in respiratory illnesses. Tracheal aspirates are fundamental for detecting respiratory viral infections at the primary site of infection, whereas whole blood samples provide insights into systemic viral dissemination and overall viral load during various infections (32). We analyzed a single-cell RNA-Seq (scRNA-Seq) data from tracheal aspirates (ENA database PRJNA971535, **Supplementary Table 1**) (19) to generate a landscape of cell types expressing lncRNAs in healthy pediatric patients and those infected with RSV.

First, the original BAM files were downloaded and converted to FASTQ format using the bamtofastq tool (<https://github.com/10XGenomics/bamtofastq>; accessed on 11/11/2024). The resulting FASTQ files were processed and mapped to the human genome (GRCh38) using Cell Ranger v8.0.1 (33).

The outputs generated by Cell Ranger were analyzed using the Scanpy package (34). H5 files containing four control samples and four RSV-infected samples (**Supplementary Table 1**) were loaded and concatenated into a single AnnData object. Cells with fewer than 200 detected genes, genes expressed in fewer than 3 cells, and cells with a high proportion of mitochondrial genes (>20%) were excluded. Gene expression data were normalized to a total of 10,000 counts per cell and subsequently log-transformed. Highly variable genes ($n = 2000$) were selected for downstream analysis. Dimensionality reduction was performed using principal component analysis (PCA). UMAP visualization was generated to highlight the distribution of cells by condition (RSV vs. control). Cell clusters were annotated based on gene expression data using the pretrained "Immune_All_Low" model from the CellTypist package (35), assigning the dominant cell type per cluster.

LncRNAs were identified in the scRNA-Seq data, and differential marker genes for each cluster and condition were determined using the Wilcoxon rank-sum test. LncRNAs were prioritized by cross-referencing Ensembl IDs and gene names with reference databases, including the Homo_sapiens.GRCh38.112 annotation and LNCipedia (36). Only identified lncRNAs were retained in the

expression matrix and differential expression was analyzed between RSV and healthy conditions.

Results

Bulk transcriptome analysis

We detected a total of 3,968 significantly differentially expressed genes (adjusted p-value < 0.05), including 684 lncRNAs. Among these, 538 lncRNAs were significantly downregulated, and 146 were significantly upregulated (**Supplementary Table 2; Supplementary Figure 1**). The heatmap of 300 upregulated and downregulated lncRNAs, followed by hierarchical clustering, enabled the identification of lncRNA expression patterns that distinguish healthy individuals from RSV-infected patients (**Supplementary Figure 2**).

Deconvolution analysis of the bulk transcriptomic data revealed differences in immune cell types between healthy and RSV-infected patients (**Figure 1**). Specifically, fourteen cell types were significantly more abundant and three were less abundant in RSV-infected patients. We observed an overabundance of regulatory T cells (Tregs) and CD4⁺ T cells (non-regulatory), along with a lower abundance of eosinophils, neutrophils, and NK cells (**Table 1**).

Network analysis

The scaffold network comprised 71,650 nodes (including proteins, genes, and ncRNAs) and 3,085,980 interactions, encompassing 109,527 protein-protein interactions (PPIs), 2,351,464 regulatory interactions, and 624,988 lncRNA interactions (<https://figshare.com/s/3a671bb8a2877ac0239f>). The degree distribution (number of connections per node) followed a power-law pattern, consistent with the Barabási-Albert model (37) (**Supplementary Figure 3**), supporting the reliability and scale-free topology of the scaffold network.

The pre-healthy net and pre-RSV net (**Supplementary Data 1A-B**) shared 64,910 interactions (**Supplementary Figure 4**). Differences in edge weights between the two networks were statistically significant (p-value < 0.0001). Based on these edge weights, we constructed the healthy-net and RSV-net. A total of 2,464 interactions were exclusive to the RSV-net, predominantly involving upregulated genes, while 1,681 interactions were exclusive to the healthy-net,

with most genes downregulated (**Supplementary Data 1C-D; Supplementary Figure 4**).

From the RVS-net, 72 nodes and 67 interactions were related to ncRNA interactions, of which 33 and 39 nodes corresponded to genes (including lncRNAs) and proteins, respectively (**Figure 2; Supplementary Data 1E**). The lncRNA interactors in the RSV-net were significantly enriched in genes associated with the molecular function “mRNA binding” (adjusted p-value = 1.163×10^{-20}), and the biological processes “regulation of mRNA metabolic process” (adjusted p-value = 3.476×10^{-17}), and “positive regulation of nucleobase-containing compound metabolic process” (adjusted p-value = 4.401×10^{-14}) (**Supplementary Figure 5**). Therefore, these terms can be considered potential general functions of the lncRNAs exclusive to the RSV-net. From the healthy-net, 43 nodes and 32 interactions were related to ncRNA interactions, of which 13 nodes were lncRNAs (**Supplementary Data 1F**).

Regarding the relationship between lncRNA interactions in the RSV-net and respiratory diseases, a total of 8 genes/proteins were associated with viral respiratory diseases, and 6 lncRNAs were linked to respiratory diseases (**Figure 2**).

The network propagation analysis using the diffusion algorithm enabled the construction of two subnetworks: the pre-healthy net SOX2-OT diffusion network and pre-RSV net SOX2-OT diffusion network (**Supplementary Data 1G and H**). A comparison of these subnetworks revealed extensive rewiring within the SOX2-OT community. Notably, the connections involving the *HNRNPA1* gene exhibited pronounced changes between the two subnetworks (**Figure 3A; Supplementary Data 1I**). Furthermore, *HNRNPA1* was densely connected to lncRNAs, all of which were exclusively present in the RSV-derived network (**Figure 3B; Supplementary Data 1J**).

Gene ontology enrichment analysis of the pre-healthy net SOX2-OT and pre-RSV net SOX2-OT diffusion subnetworks revealed no significant differences in overall functional terms. Both subnetworks were enriched for the molecular function “RNA binding” (or “mRNA binding”), the cellular component “cytoplasmic stress granules” (or “cytoplasmic ribonucleoprotein granule”), and the biological process “regulation of mRNA metabolic process” (**Supplementary Figure 6-7**).

Additionally, we performed a network analysis comparing mild vs. severe, healthy vs. mild, and healthy vs. severe cases to assess whether lncRNAs were involved in RSV severity. Among these, only the mild vs. healthy comparison revealed a differential network with lncRNA interactions (**Figure 4**).

Single cell analysis

A total of 10,033 cells from 8 selected samples (4 controls and 4 RSV-infected patients) were analyzed. First, we generated a UMAP plot to visualize the distribution of the conditions (RSV vs. control), allowing the distinction between cells from RSV-infected and healthy patients (**Figure 5A**). Clustering and cell-type annotation identified 9 distinct dominant cell types, including alveolar macrophages, CD16⁺NK cells, classical monocytes, double-positive thymocytes, epithelial cells, fibroblasts, ILC3, intermediate macrophages, and NK cells (**Figure 5B**). The cell clusters from RSV-infected patients (**Figure 5A**) were enriched in classical monocytes, intermediate macrophages, and NK cells. Furthermore, RSV-infected patients showed a general depletion of fibroblasts (**Figure 5B**). The expression filter retained 18,390 lncRNAs, of which 20 (hereafter referred to as marker lncRNAs) were differentially expressed between RSV-infected and healthy patients (**Figure 5C-D**). These 20 marker lncRNAs were also differentially expressed across annotated cell types. In general, these 20 marker lncRNAs exhibited higher expression in all annotated cell types from RSV-infected patients, with ILC3 being the only exception. For instance, increased expression of some marker lncRNAs was observed in CD16⁺NK cells and regulatory T cells (Tregs) exclusively in infected patients (**Figure 6**).

Discussion

Overview and cell types involved in RSV infection

Deconvolution analysis of the bulk transcriptome revealed an increase in regulatory T cells (Tregs) in pediatric patients infected with RSV. This finding contrasts with previous studies that reported reduced levels of activated Tregs in the peripheral blood of infants with RSV, suggesting a less effective immune response against the virus (38). Nevertheless, Tregs may suppress RSV progression and appear to contribute to effective viral control (39).

Our reanalysis of scRNA-Seq revealed an enrichment of classical monocytes, intermediate macrophages, and NK cells in the cell clusters of RSV-infected patients.

RSV alters monocyte function, which correlates with increased disease severity (40,41). RSV can infect monocytes, potentially reducing the protective immune response against the virus (40). After RSV infection, classical monocytes can differentiate into dendritic cells that are less effective at activating the adaptive immune response (42). In infants with RSV infection, monocytes produce lower levels of TNF- α (43) and display impaired capacity to produce IL-10 (41), suggesting that the infection negatively interferes with the immune response to RSV (41,44). Despite the enrichment of monocytes in patients with RSV infection, these cells may have impaired function, thereby hindering an effective immune response.

Mainly in infants, the polarization of intermediate macrophages during RSV infection has been associated with long-term airway hyperresponsiveness. The balance between M1 and M2 macrophage responses can influence viral clearance and long-term respiratory outcomes, potentially predisposing patients to asthma (45).

Although NK cells provide protective immunity during RSV infection, their activation can also contribute to lung injury. In severe RSV cases, the accumulation of NK cells in the airways is correlated with increased disease severity (46). Furthermore, NK cells can be infected by RSV, contributing to the inflammatory environment (47,48).

The reanalysis of scRNA-Seq data also revealed a depletion of fibroblasts and ILC3 cells in RSV-infected patients. RSV infection in fibroblasts promotes inflammation through mechanisms mediated by hyaluronic acid, which enhances the expression of some mast cell proteins. These effects contribute to microenvironmental inflammation (49). Fibroblasts can differentiate into myofibroblasts in response to RSV, inducing fibrosis. The depletion of fibroblasts may lead to long-term respiratory problems such as asthma (50,51).

Altogether, our findings suggest that the RSV-infected patients analyzed through scRNA-Seq of tracheal aspirates were experiencing difficulties in coping with RSV infection or may have been exposed to additional health complications. In contrast, the deconvolution analysis of whole blood samples indicates that the

pediatric patients with RSV infection might be enhancing their ability to cope with the virus.

Cell types with lncRNA marker genes

Differential expression analysis of lncRNAs in healthy and RSV-infected patients using bulk transcriptomic data identified hundreds of differentially expressed lncRNAs. Sets of these lncRNAs were able to distinguish between healthy and infected patients, a finding not previously reported. Furthermore, several lncRNAs appear to act as marker genes for RSV infection, as also observed in the scRNA-Seq reanalysis.

In the scRNA-Seq data, we observed that some of the 20 marker lncRNAs were more highly expressed in CD16⁺NK cells and Tregs from infected patients. Conversely, some marker lncRNAs exhibited reduced expression in ILC3 cells in these patients.

Activated NK cells expressing CD16 may exacerbate lung inflammation, contributing to acute pulmonary immune injury, particularly in severe cases of RSV infection (52). Infants with severe RSV infection show an increased number of NK cells in their airways. NK cell dysregulation has been associated with adverse outcomes such as acute respiratory distress (46).

Treg cells play a crucial role in regulating the immune response during primary RSV infection (53). Children with severe RSV infection often exhibit reduced levels of circulating Tregs (39,54). Moreover, this reduction has been linked to increased disease severity and respiratory distress in infants with RSV (38).

RSV infection in STAT1-deficient mice leads to an increased number of ILC3 cells (55). Similarly, RSV-infected mice show elevated levels of ILC3 compared to controls (56).

RSV-infected patients presented lncRNA interactions involved in respiratory diseases

RSV infection caused significant changes in the relevance of network connections, allowing the modeling of specific interactions in healthy individuals and RSV-infected patients. We identified a set of 33 lncRNAs exclusive to the RSV-net, most of which are involved in DNA/RNA regulatory processes. Among

these, six lncRNAs were related to respiratory diseases, such as chronic obstructive pulmonary disease (COPD), pulmonary hypertension, pulmonary fibrosis, and RSV infection. Notably, RSV infection during the first months of life increases the risk of developing COPD and asthma (57). Exacerbation of COPD in children appears to be associated with respiratory virus outbreaks, including RSV (58). RSV can also cause pulmonary hypertension in infants, particularly those with severe bronchiolitis (59,60). Altogether, our data indicate that the strategy used to model the RSV-net was effective in recovering relevant lncRNA interactions related to RSV infection. We highlight the importance of the lncRNAs *SOX2-OT*, *C5orf64*, and *MEG3* in this context (discussed further below).

The lncRNA *SOX2-OT* has been associated with pulmonary hypertension (61). In RSV-net, *SOX2-OT* interacts with *MEX3C* and three proteins implicated in respiratory viral diseases (*NPM1*, *RBM15*, *HNRNPA1*). *MEX3C* plays a critical role in antiviral immune responses (62), although it has not been directly linked to respiratory viral diseases. *NPM1* affects RSV protein expression (63). *RBM15* expression is elevated during SARS-CoV-2 infection and is related to disease severity (64), while *HNRNPA1* knockdown has been reported to increase Influenza A virus replication (65).

The *TCF12* gene has a regulatory relationship with the lncRNA *C5orf64* in our RSV-net. Additionally, *TCF12* has been implicated in the regulation of gene expression during SARS-CoV-2 infection (66).

The levels of lncRNA *MEG3* are reduced in nasopharyngeal samples from patients infected with RSV. Overexpression of this lncRNA has been associated with suppression of inflammatory responses induced by RSV (16). In our RSV-net, *MEG3* interacts with the protein *TARDBP*. However, to our knowledge, *TARDBP* has not been previously linked to respiratory diseases.

Altogether, our data suggest that at least the upregulated lncRNAs *SOX2-OT*, *C5orf64*, and *MEG3* may be related to RSV infection in pediatric patients, although *C5orf64* has never been associated with respiratory diseases.

The SOX2-OT community is rewired, with HNRNPA1 playing an essential role

Although the subnetworks generated by signal diffusion from *SOX2-OT* in pre-healthy and pre-RSV networks exhibited extensive interaction rewiring, they did not differ in terms of functionality, particularly regarding RNA-binding activity.

These findings indicate that the *SOX2-OT* community is functionally robust enough to cope with RSV infection.

The *HNRNPA1* protein interacts with *SOX2-OT*, as evidenced in our RSV-net. In RSV-infected patients, *HNRNPA1* underwent pronounced interaction rewiring within the *SOX2-OT* community, becoming densely connected to lncRNAs. *HNRNPA1* is involved in RNA processing, transcriptional regulation, and cellular stress responses (67,68). As previously mentioned, it has also been implicated in influenza A virus replication (65).

Altogether, RNA metabolism appears to be preserved in both healthy and RSV-infected subnetworks, with a specific enrichment in the RSV-net. Therefore, we speculate that mechanisms related to RNA metabolism may be intensified in RSV-infected patients due to the rewiring of *HNRNPA1* interactions.

LncRNAs seem to be related to mild RSV severity

Comparing the mild and healthy networks, we identified a set of lncRNAs potentially related to mild RSV infection. The lncRNA *OIP5-AS1* interacts with the proteins *METTL14*, *NOLC1*, *DHX36*, *UCL5*, *YTHDF2*, *METAP2*, and *NCBP2*, most of which are involved in responses to infection. *METTL14* influences RSV replication in infected patients (69,70). Suppression of the *NOLC1* protein reduces H3N2 influenza virus proliferation (71). *DHX36* appears to play a role in the antiviral response (72). *UCL5* may contribute to the pathogenesis of idiopathic pulmonary fibrosis (73). *YTHDF2* is involved in the response to DNA viruses (74). *METAP2* is associated with autoimmunity (75). Additionally, the lncRNA *LINC00342* interacts with the genes *HNRNPU* and *DDX6*. *DDX6* binds to viral RNA, promoting an effective antiviral response against respiratory viruses such as influenza (76).

The present study involved the reanalysis of bulk transcriptome and scRNA-Seq data, which were integrated with network analysis to identify lncRNAs responsive to RSV infection. This is the first report of a systematic and integrative analysis of lncRNAs in pediatric patients with RSV infection. We identified a set of lncRNAs that may serve as molecular markers of RSV infection, opening new avenues for further research. In this case, it is essential to conduct experimental validation in a larger and more genetically diverse cohort for the purpose of translational medicine based on these makers.

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Conflict of interest

The authors declare no conflict of interests.

Authors contribution

Project conceptualization: GTV, ABR, and TCM; Data collection: ABR, and MRS; Bulk differential expression analysis and scRNA-Seq analysis: ABR, and MRS; Deconvolution analysis: JSS; Network analysis: GTV, and AMSK; Data interpretation: GTV, ABR, TCM, and RMTG; Draft: GTV, ABR, and TCM; Final version: all authors.

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Figures and Tables

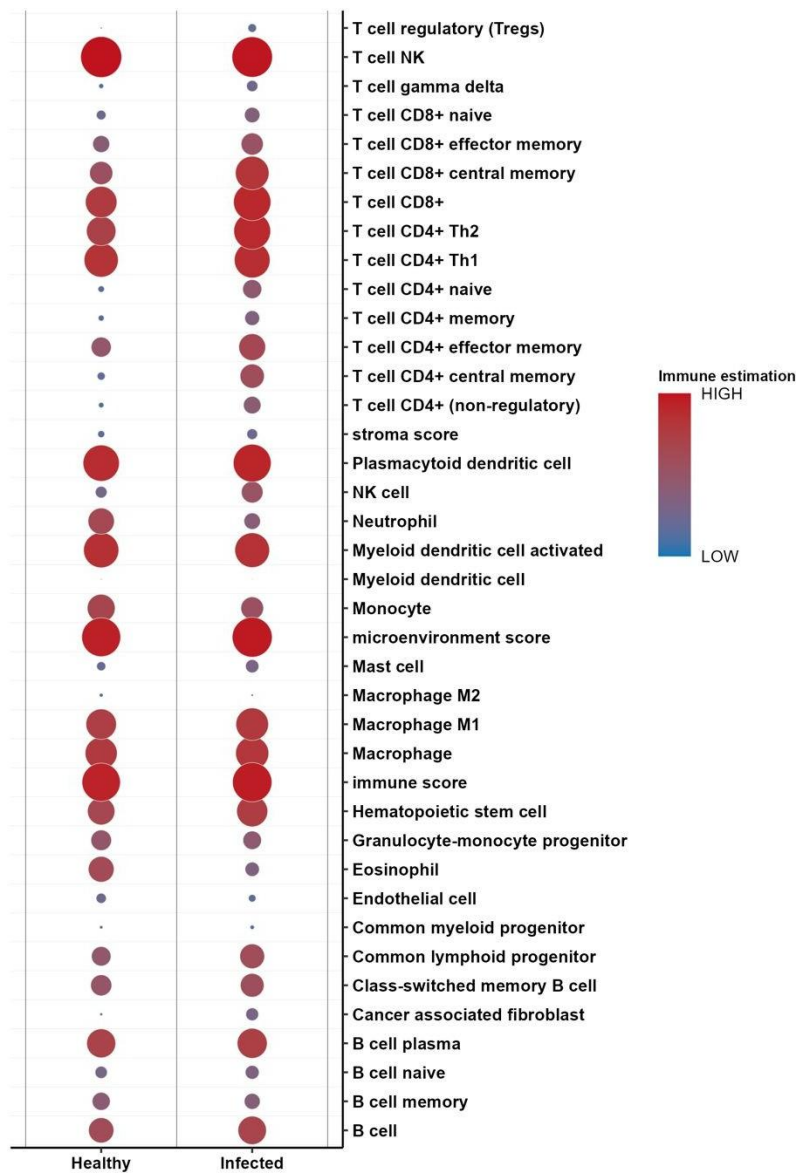


Figure 1: Deconvolution analysis of the bulk transcriptome.

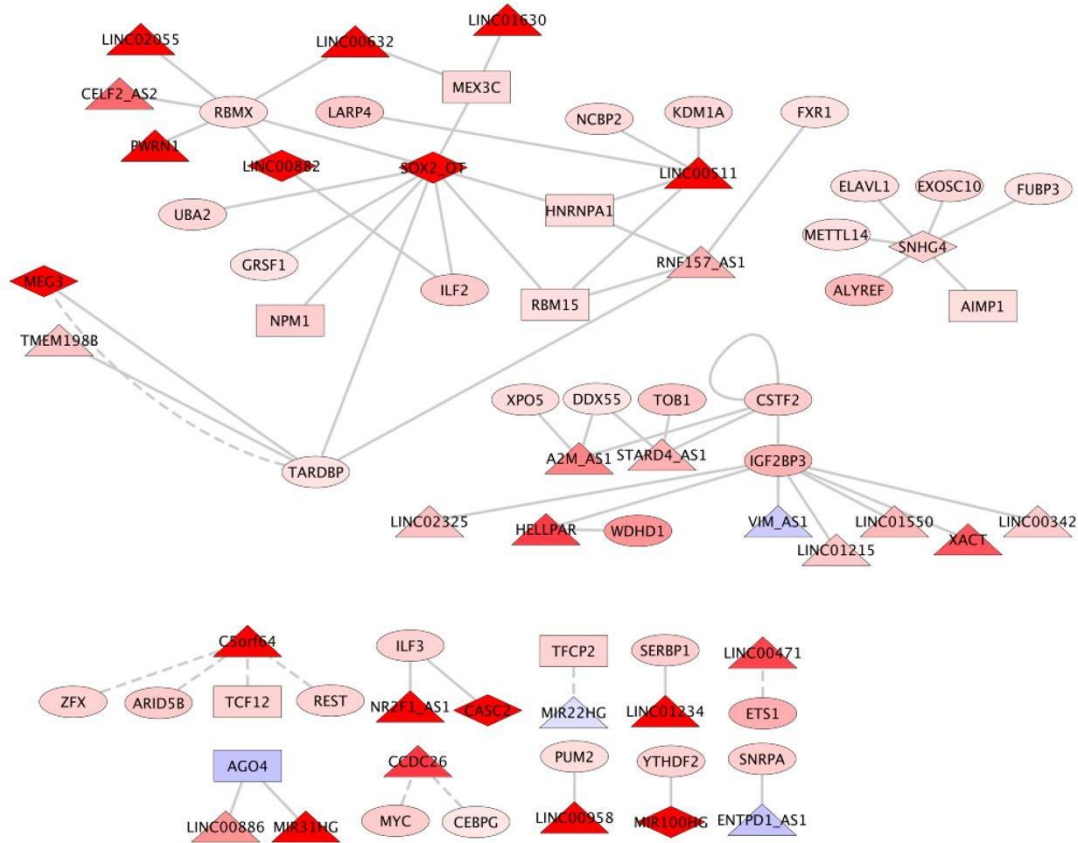


Figure 2: The lncRNA-interaction network present only in the network of patients infected with RSV (RSV-net). Triangle- and diamond-shaped nodes represent lncRNAs. Squared nodes are genes/proteins related to respiratory virus diseases or antiviral activity (**Supplementary Table 3**). Diamond-shaped nodes indicate lncRNAs associated with respiratory virus diseases or antiviral responses (**Supplementary Table 4**). The color of the node corresponds to the log2 fold change from the differential gene expression data of the bulk transcriptome, where red and blue are the genes up- and down-regulated, respectively. Solid and dashed edges are regulatory and physical interactions, respectively.

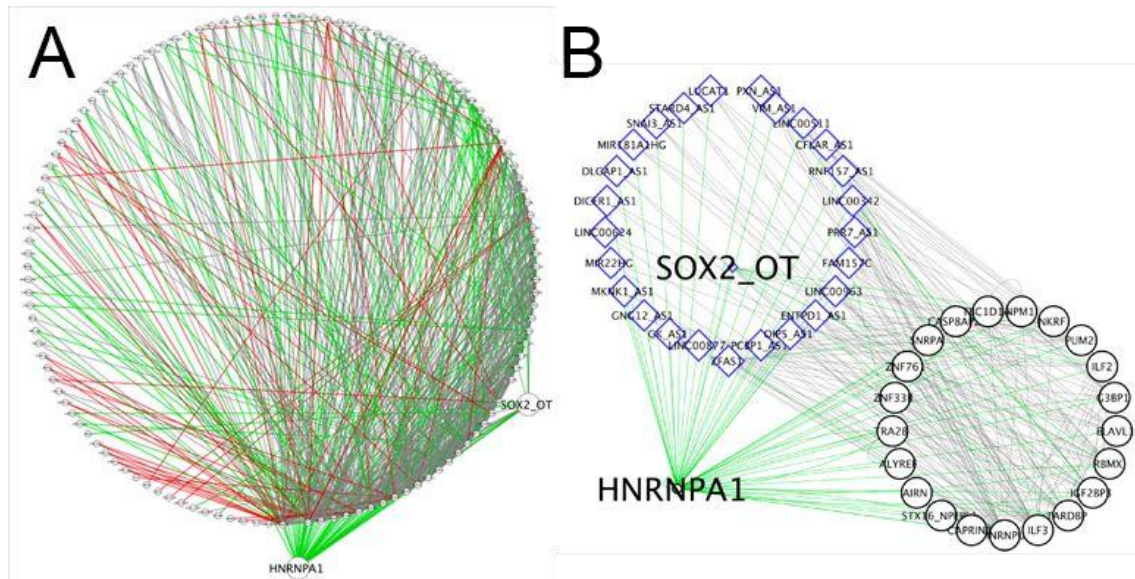


Figure 3: Comparison of propagation analysis using the SOX2-OT gene diffusion algorithm. **A:** comparison between the diffusions of the pre-healthy net SOX2-OT and the pre-RSV net SOX2-OT; **B:** the subnetwork of HNRNPA1 interactions derived from 'A'. The blue nodes are lncRNAs. For both 'A' and 'B', the red and green links are those present only in the pre-healthy net SOX2-OT and pre-RSV net SOX2-OT diffusion, respectively.

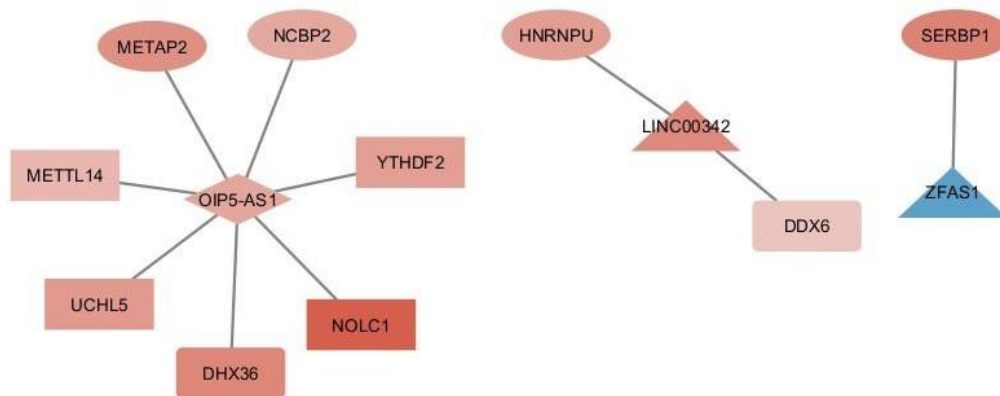


Figure 4: LncRNA-interaction network comparing the mild vs. healthy networks. Triangle- and diamond-shaped nodes are the lncRNAs. Rectangles are proteins related to respiratory diseases or antiviral responses. The node color reflects the log2-fold change from the differential gene expression data of the bulk transcriptome: red and blue are the genes up- and down-regulated, respectively.

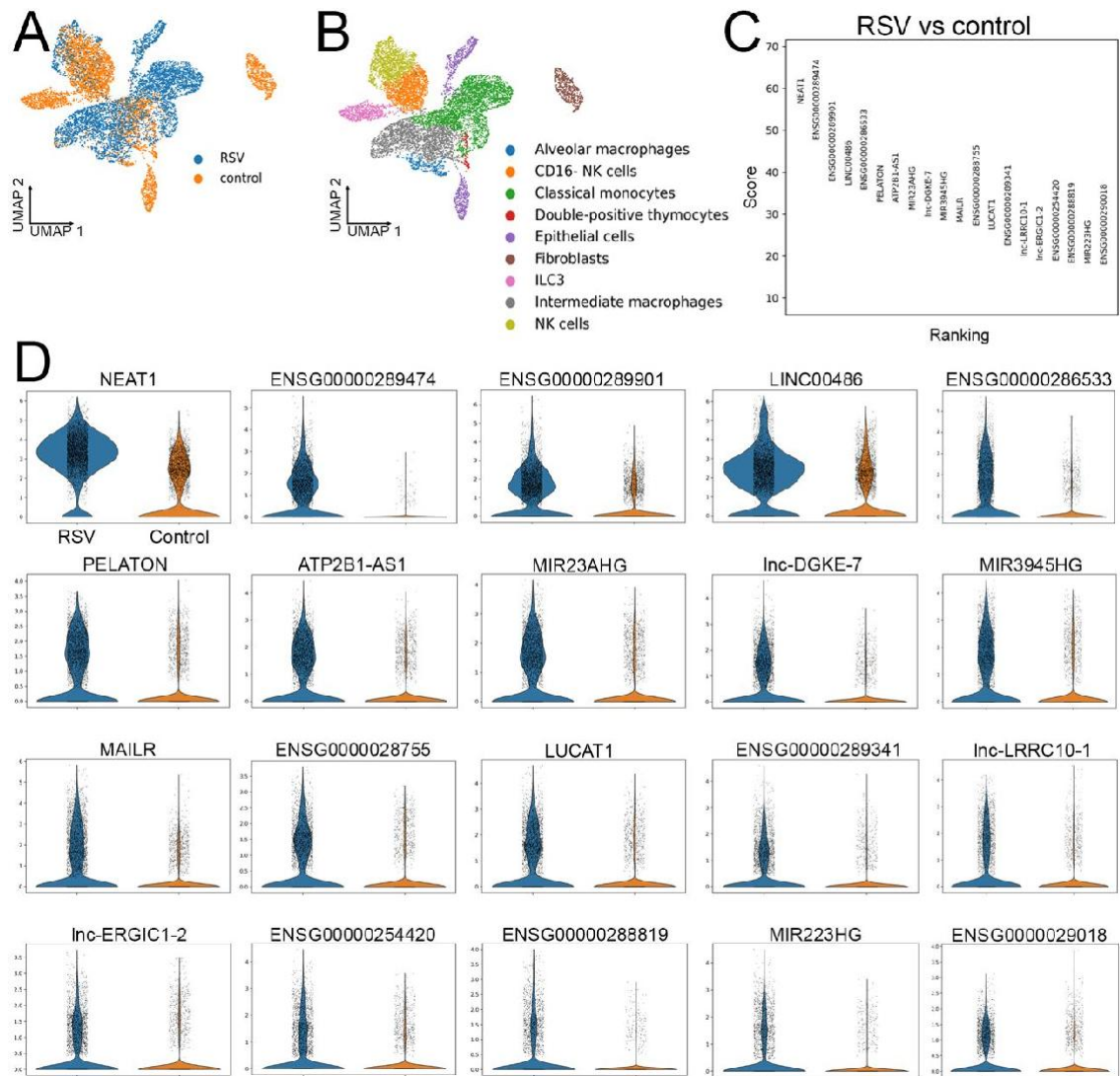


Figure 5: ScRNA-Seq analysis of RSV-infected and healthy patients. **A:** UMAP of clusters according to group condition (infection status); **B:** UMAP highlighting the annotated cell types; **C:** 20 most highly variable genes in terms of expression comparing RSV vs. control samples; **D:** violin plots of the 20 marker genes reported in “C”.

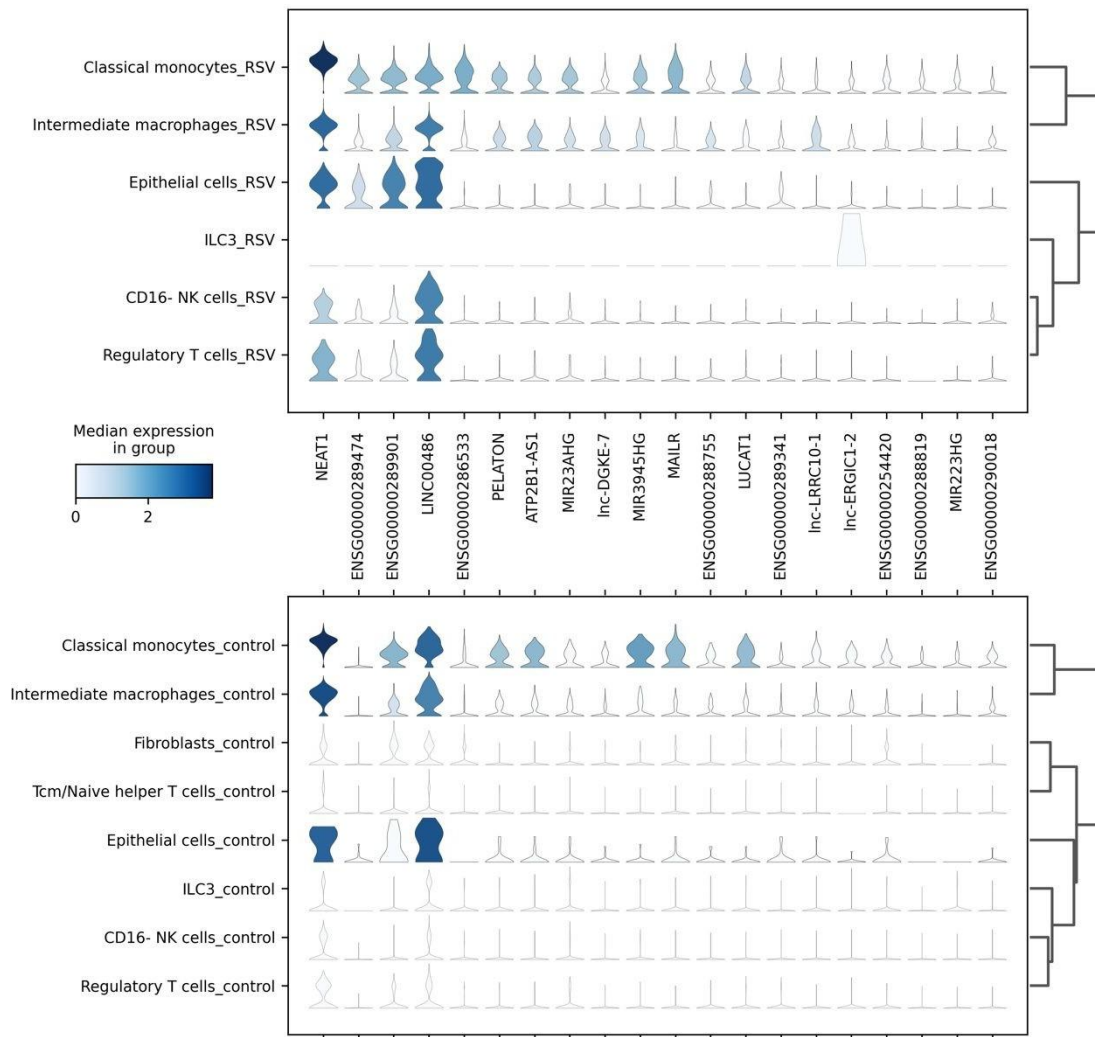


Figure 6: Stacked violin plots showing the expression distribution of the top 20 marker lncRNAs from RSV vs. control comparison across cell subpopulations (**Figure 5C**). The upper plot represents the RSV sample, and the bottom plot represents the control sample.

Cell type	mean Healthy	mean RSV	Log2 fold change	p-value
Eosinophil	0.0102750	0.0033208	-1.63	4.62E-02
Neutrophil	0.0109793	0.0038853	-1.50	2.82E-04
T cell NK	0.2117407	0.1220602	-0.79	5.85E-04
immune score	0.0539029	0.0691742	0.36	5.39E-03
microenvironment score	0.0548081	0.0711244	0.38	3.06E-03
T cell CD4+ Th1	0.0246542	0.0401319	0.70	1.35E-02
Common lymphoid progenitor	0.0054460	0.0096475	0.82	6.92E-03
T cell CD8+	0.0166688	0.0432586	1.38	6.24E-04
T cell CD8+ central memory	0.0086002	0.0233699	1.44	4.83E-03
T cell CD4+ Th2	0.0149078	0.0422396	1.50	1.39E-03
NK cell	0.0021124	0.0073937	1.81	5.36E-04
T cell CD4+ memory	0.0007229	0.0034897	2.27	4.46E-03
T cell CD4+ naive	0.0007283	0.0049580	2.77	1.54E-04
T cell gamma delta	0.0002891	0.0019694	2.77	2.65E-03
T cell CD4+ central memory	0.0012844	0.0088162	2.78	3.38E-05
T cell CD4+ (non-regulatory)	0.0003059	0.0046397	3.92	2.97E-04
T cell regulatory (Tregs)	0.0000000	0.0013349	50.07	4.53E-03

Table 1: Results of the T-test analysis comparing immune cell types between healthy patients and RSV-infected patients.

Abbreviations

COPD: chronic obstructive pulmonary diseases

GEO: gene expression omnibus

lncRNAs: long noncoding RNAs

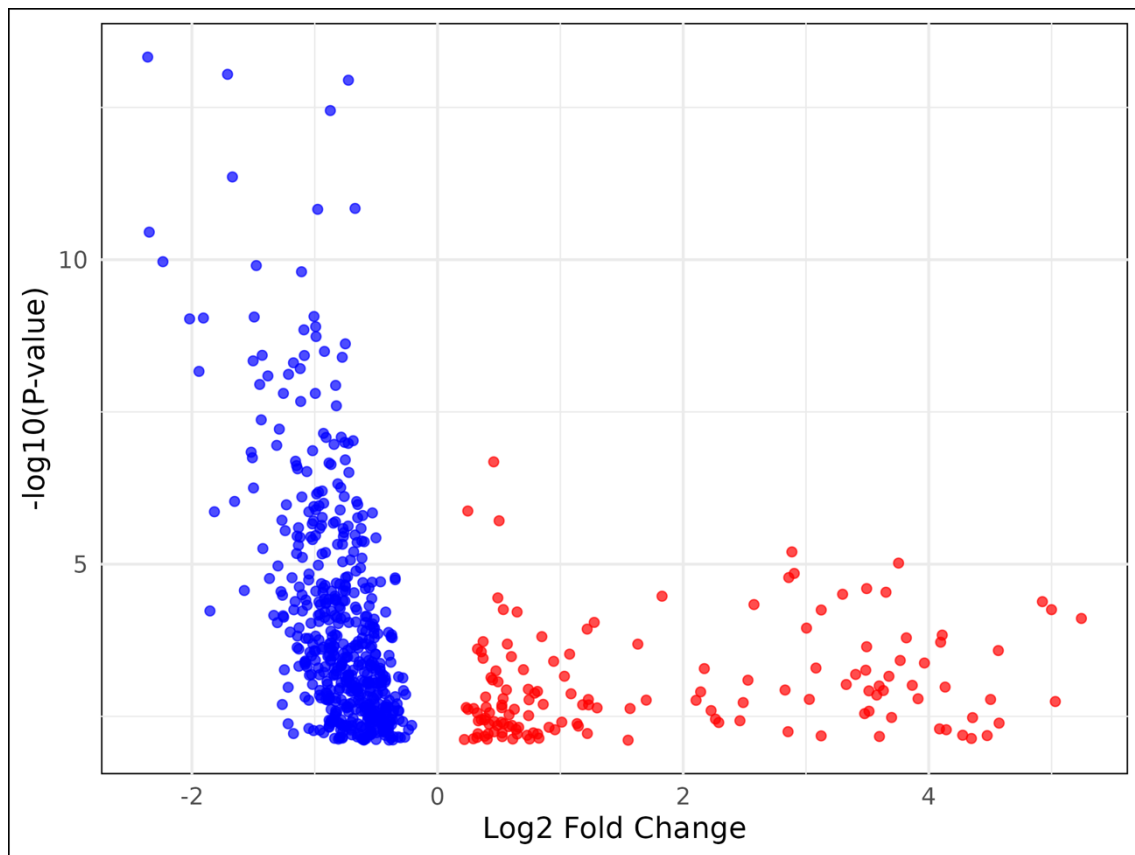
padj: p-value adjusted

PPI: protein-protein interaction

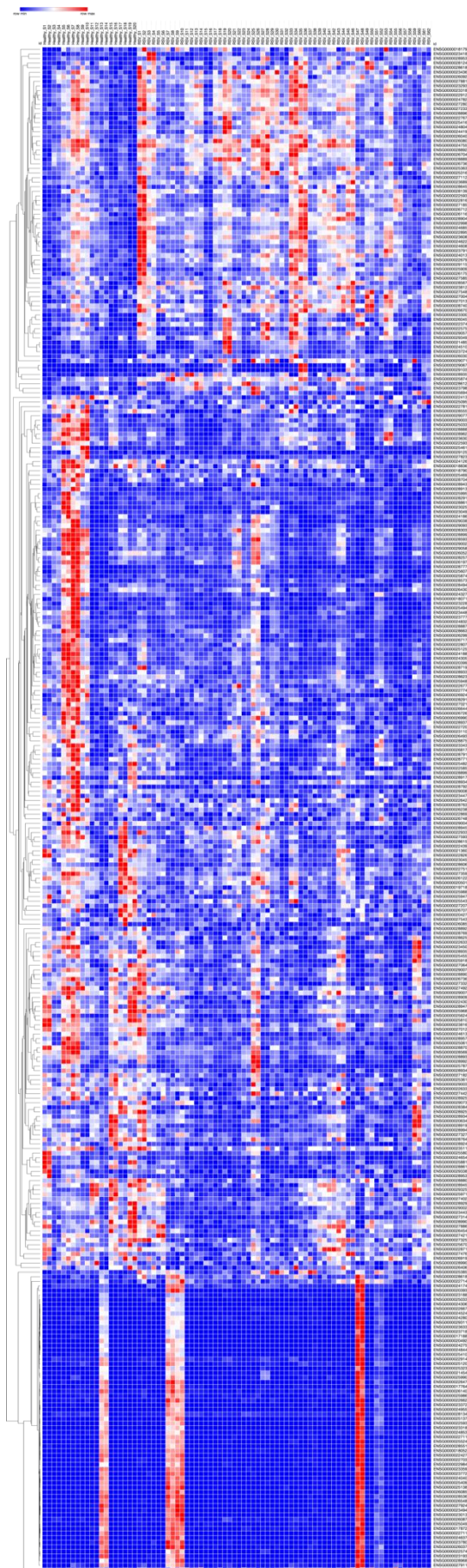
RSV: respiratory syncytial virus

scRNA-Seq: Single-cell RNA-Seq

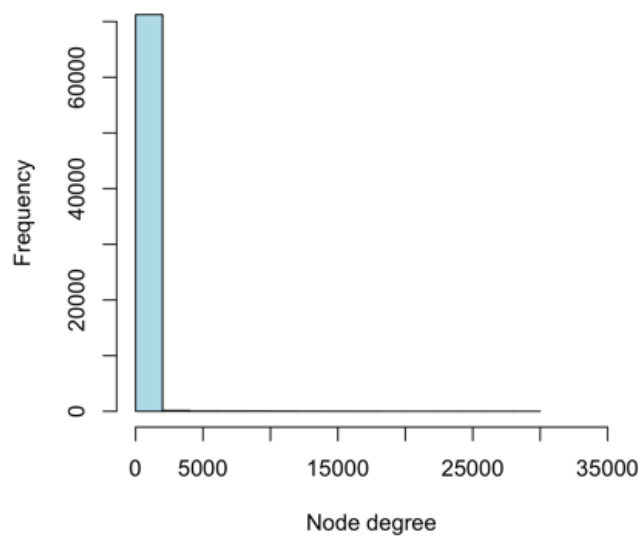
Tregs: T cell regulatory

Supplementary Figures

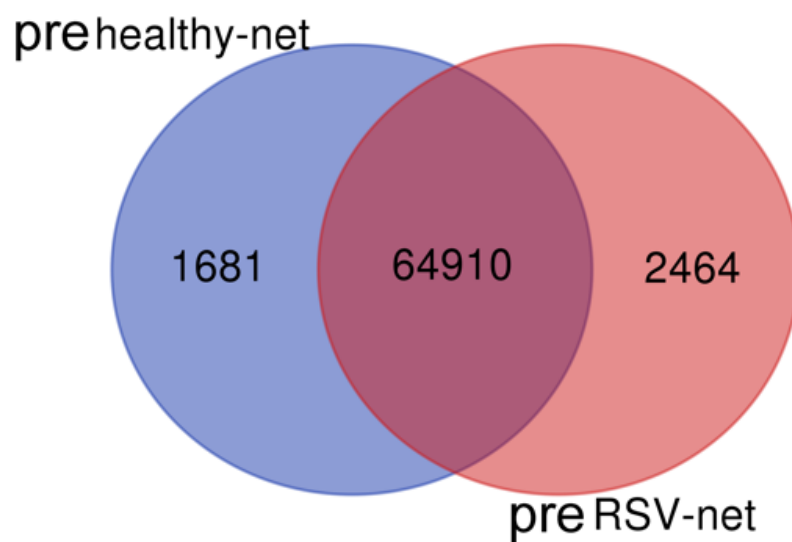
Supplementary Figure 1. Vulcan plot of differentially expressed lncRNAs.



Supplementary Figure 2. Heatmap of ~300 differentially expressed lncRNAs.



Supplementary Figure 3. Degree distribution of scaffold network.



Supplementary Figure 4. Venn diagram comparing the interactions of pre-healthy net and pre-RSV net. The Venn diagram was drawn using <https://bioinformatics.psb.ugent.be/webtools/Venn/>.



Supplementary Figure 5. Gene ontology enrichment analysis using the g:profiler concerning the RSV-net. MF: molecular function; BP: biological process; CC: cellular component.



Supplementary Figure 6. Gene ontology enrichment analysis using the g:profiler concerning pre-healthy net SOX2_OT diffusion. MF: molecular function; BP: biological process; CC: cellular component.



Supplementary Figure 7. Gene ontology enrichment analysis using the g:profiler concerning pre-RSV net SOX2_OT diffusion. MF: molecular function; BP: biological process; CC: cellular component.

4. CONSIDERAÇÕES FINAIS

O presente estudo proporcionou uma análise abrangente dos *lncRNAs* responsivos à infecção por vírus sincicial respiratório (VSR) em pacientes pediátricos, utilizando dados de *bulk RNA-Seq* e *scRNA-Seq*, integrados a uma análise de redes moleculares. Este é o primeiro estudo a realizar uma análise sistemática e integrativa dos *lncRNAs* em crianças infectadas pelo VSR. Através dessa abordagem, foram identificados *lncRNAs*, como *SOX2-OT*, *C5orf64* e *MEG3*, que podem atuar como marcadores moleculares para a infecção, além de *OIP5-AS1* e *LINC00342*, que se associam a uma menor gravidade da infecção.

As redes moleculares revelaram uma reconfiguração significativa nas redes de *lncRNAs* durante a infecção por VSR, sugerindo que o vírus modula a rede de *lncRNAs* e afeta a resposta imunológica. Além disso, a análise de *scRNA-Seq* destacou a expressão diferencial de *lncRNAs* em tipos celulares específicos, como células NK e Tregs, fornecendo uma visão mais detalhada da resposta imune.

Esses achados abrem novas perspectivas para o desenvolvimento de biomarcadores e alvos terapêuticos para o VSR, além de contribuir para o entendimento dos mecanismos moleculares envolvidos na infecção. A identificação de *lncRNAs* associados à infecção oferece um caminho promissor para futuras pesquisas, que podem resultar em novas estratégias diagnósticas e terapêuticas mais eficazes no tratamento da infecção por VSR em pacientes pediátricos.

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