



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto



UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCÊNCIAS, LETRAS E CIÊNCIAS EXATAS (IBILCE/UNESP)
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA

Rafaela dos Santos Peinado

**Perfis metabólicos de células Vero infectadas com
Chikungunya e inibição por Wedelolactona investigados por
Metabolômica de RMN**

São José do Rio Preto

2024

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas – UNESP, Campus de São José do Rio Preto, como requisito para a obtenção do título de Doutora em Microbiologia.

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Agência financiadora: CAPES e FAPESP (2020/03639-6)

São José do Rio Preto

2024

P377p

Peinado, Rafaela dos Santos

Perfis metabólicos de células Vero infectadas com Chikungunya e inibição por Wedelolactona investigados por Metabolômica de RMN / Rafaela dos Santos Peinado. -- São José do Rio Preto, 2024
158 p.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto

Orientador: Raghuvir Krishnaswamy Arni

Coorientadora: Mônica Aparecida Coronado

1. Virologia. 2. Chikungunya. 3. Metabolômica. 4. Wedelolactona. I.
Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca do Instituto de Biociências Letras e Ciências Exatas, São José do Rio Preto. Dados fornecidos pelo autor(a).

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12 de Janeiro de 2024

Dedicatória

Gostaria de dedicar este trabalho a todos que desempenharam papéis significativos ao longo do meu curso de Doutorado. Inicialmente, expresso minha gratidão a Deus, cuja força me sustentou ao longo do caminho que escolhi trilhar. Aos meus pais, minha irmã e meu cunhado, dedico especial reconhecimento, pois sempre estiveram ao meu lado, oferecendo apoio incondicional em todas as minhas escolhas e decisões, tornando-se meu refúgio e fonte de conforto nos momentos mais desafiadores.

Dedico também aos meus amigos mais próximos, cuja presença e suporte, tanto presencial quanto remoto, foram fundamentais. Seus ouvidos atentos, apoio constante e a capacidade de trazer leveza aos meus dias foram elementos essenciais para minha jornada. E, por fim, dedico este trabalho a mim mesma, reconhecendo minha persistência diante das dificuldades, meu aprendizado contínuo e o crescimento que alcancei, culminando na superação de mais uma etapa significativa nesta trajetória acadêmica.

Agradecimentos

Gostaria de expressar minha profunda gratidão a Deus pela oportunidade e força concedida durante todo o período do doutorado. Sua presença e amparo foram fundamentais, proporcionando-me a perseverança e a determinação necessárias para enfrentar os desafios encontrados. Agradeço de coração por essa jornada sob Sua proteção e por ser a fonte constante de inspiração e motivação ao longo desse caminho acadêmico.

Ao meu orientador, Prof. Dr. Raghuvir Krishnaswamy Arni, e às minhas coorientadoras, Dra. Monika Aparecida Coronado e Dra. Carolina Colombelli Pacca, expresso minha gratidão pelas constantes manifestações de apoio, incentivo, disponibilidade e compreensão ao longo desta etapa crucial da minha formação. Suas orientações e comprometimento foram fundamentais para o desenvolvimento deste trabalho.

Agradeço especialmente à Profa. Dra. Ljubica Tasic do Instituto de Química da UNICAMP pela colaboração e cedência da estrutura necessária para conduzir os experimentos e pesquisas. Sua disponibilidade e apoio foram cruciais em cada etapa do processo, desde a concepção das ideias até a conclusão do projeto, assim como sua experiência, dedicação e generosidade, que foram fundamentais para o sucesso deste trabalho. Também expresso minha gratidão ao Prof. Dr. Maurício Lacerda Nogueira do Laboratório de Pesquisas em Virologia da Faculdade de Medicina de São José do Rio Preto pela colaboração firmada e generosa cedência de infraestrutura, apoio técnico/científico e amparo metodológico que possibilitaram a execução deste projeto.

Aos meus colaboradores e colegas que conheci nessa jornada, também manifesto minha gratidão pelo trabalho conjunto no desenvolvimento deste projeto. Expresso minha profunda gratidão ao Dr. Lucas Gelain Martins, Dr. Raphael Eberle, à Dra. Nathalia Zini, Me. Clarita Secco Avilla, Me. Marielena Vogel Saivish, Dra. Ana Beatriz Bortolozo de Oliveira, Mônica Mayumi Akinaga e ao Thyerre Santana da Costa por suas substanciais contribuições e auxílio sem medir esforços no decorrer deste doutorado. Seu suporte técnico, conhecimento agregado e comprometimento foram indispensáveis para a realização deste trabalho e para o meu desenvolvimento acadêmico.

Gostaria de expressar minha profunda gratidão à minha amiga e companheira de doutorado Kelly Cristine Borsatto, por todo o apoio inestimável que me proporcionou ao longo deste doutorado. Sua presença constante, compreensão e colaboração foram fundamentais para o sucesso dessa jornada, oferecendo seu tempo, conhecimento e incentivo. Sua generosidade e amizade fizeram toda a diferença, tornando esse período mais leve e significativo. Acredito

sinceramente que a conclusão deste doutorado não teria sido a mesma sem a sua ajuda valiosa.

Minha sincera gratidão à comunidade do Instituto de Biociências, Letras e Ciências Exatas (IBILCE/UNESP), seus docentes e funcionários, que desde meu ingresso no curso de graduação em Ciências Biológicas em 2014, participaram desta jornada acadêmica, contribuindo significativamente para a minha formação como bióloga.

Agradeço também aos órgãos que financiaram o projeto. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001, e da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Processo 2020/03639-6), que viabilizou a realização desta tese. O apoio financeiro dessas instituições foi fundamental para o sucesso e conclusão deste trabalho.

Manifesto meu especial agradecimento a meus pais, Rafael Agudo Peinado e Ilícia Pereira dos Santos Peinado, minha irmã Ariane dos Santos Peinado e meu cunhado Rafael Augusto Sampaio, minhas amigas Alessandra Savraz, Aniele Estevo, Carla Yasmin Dias Marçal, Danielly Leonel de Oliveira, Elisângela Soares, Jéssica Fernanda Paixão e meu amigo Nicolas Toledo que foram presenças fundamentais ao longo do meu doutorado. Seu incentivo, ajuda, conforto, leveza e suporte foram pilares essenciais que me impulsionaram durante esse desafiador percurso acadêmico. Em momentos de incerteza, suas palavras de ânimo e gestos de solidariedade foram como uma luz que iluminou meu caminho. O conforto que encontrei em vocês tornou os dias mais difíceis mais suportáveis e as vitórias mais significativas. Agradeço por ouvirem minhas preocupações, por compartilharem risadas que aliviaram o peso das responsabilidades acadêmicas e por estarem presentes, mesmo que à distância. Cada um de vocês contribuiu de maneira única para tornar essa jornada mais rica em experiências e mais gratificante. Este doutorado não foi apenas um desafio acadêmico, mas uma jornada compartilhada, e estou imensamente grata por ter pessoas como vocês ao meu lado.

***“Confie em si mesmo quando estiver em um labirinto. Quando o
inverno passar, a primavera chegará.”***

Sonyeondan, Bangtan. Answer: Love Myself.

Resumo

Apesar dos danos causados em seres humanos por arbovírus como o Chikungunya, os tratamentos atuais focam-se principalmente em medidas e tratamentos para controlar os mosquitos vetores e proporcionar alívio sintomático por meio de medicamentos analgésicos, os quais, no entanto, têm eficácia limitada em complicações crônicas. Assim, compreender os mecanismos da patogênese viral e as interações e modificações induzidas pelo vírus no organismo é crucial para o desenvolvimento de terapias inovadoras. As pesquisas em metabolômica possibilitam a investigação dos efeitos que esses patógenos exercem sobre seus hospedeiros, analisando metabólitos e suas respectivas vias que sofrem alterações durante a infecção. Além disso, permitem a análise dos efeitos metabólicos de moléculas inibitórias, tanto na infecção viral quanto no metabolismo celular, gerando resultados promissores para estudos voltados à modulação ou inibição de vias essenciais para a replicação viral, visando não prejudicar as células hospedeiras. O foco deste trabalho residiu no uso da metabolômica pela Espectroscopia de Ressonância Magnética Nuclear de Alta Resolução com Giro no Ângulo Mágico (^1H RMN HR-MAS) para investigar os efeitos metabólicos provocados pelo vírus Chikungunya em células Vero E6, bem como os efeitos causados pelo inibidor da protease viral (nsP2): Wedelolactona (WDL). Os resultados obtidos por meio da técnica de ^1H RMN HR-MAS incluem a caracterização metabólica das células Vero E6, nas quais foram identificados um total de 29 metabólitos. Dentre estes, utilizando-se métodos estatísticos e quimiométricos destacaram-se 12 metabólitos diferenciais entre as condições analisadas: aspartato, betaína, creatina, fenilalanina, fosfocolina, glicose, isoleucina, lactato, mio-inositol, prolina e valina. Com base nesses resultados, discutimos as potenciais alterações metabólicas induzidas pela infecção das células com o CHIKV e pela introdução da WDL, tanto em células saudáveis quanto infectadas, fundamentando-nos em dados previamente publicados na literatura. No entanto, é fundamental ressaltar a necessidade premente de estudos adicionais para aprofundar a compreensão dos efeitos sobre o metabolismo, devido a limitações em nosso estudo e também na literatura. Além disso, a integração das diversas áreas ômicas se apresenta como um passo crucial, fornecendo novos *insights* para o desenvolvimento de moléculas voltadas para a modulação de vias metabólicas como mecanismos antivirais.

Palavras-chave: Chikungunya. Metabolômica. RMN HR-MAS. Wedelolactona.

Abstract

Despite the damage caused in humans by arboviruses such as Chikungunya, current treatments primarily focus on prophylactic measures to control vector mosquitoes and provide symptomatic relief through analgesic medication. However, these have limited efficacy in chronic complications. Thus, understanding viral pathogenesis mechanisms and the interactions and modifications induced by the virus is crucial for the development of innovative therapies. Research in metabolomics enables the investigation of the effects these pathogens exert on their hosts by analyzing metabolites and their respective pathways undergoing alterations during infection. Additionally, it allows the analysis of the metabolic effects of inhibitory molecules, both in viral infection and cellular metabolism, generating promising results for studies aimed at modulating or inhibiting essential pathways for viral replication, without harming host cells. The focus of this work was the use of High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy (^1H NMR HR-MAS) metabolomics to investigate the metabolic effects induced by the Chikungunya virus in Vero E6 cells, as well as the effects of a viral protease inhibitor (nsP2): Wedelolactone (WDL). Results obtained through ^1H NMR HR-MAS include the metabolic characterization of Vero E6 cells, identifying a total of 29 metabolites. Among these, 12 differential metabolites were highlighted using statistical and chemometric methods between the analyzed conditions: aspartate, betaine, creatine, phenylalanine, phosphocholine, glucose, isoleucine, lactate, myo-inositol, proline and valine. Based on these results, we discuss potential metabolic alterations induced by CHIKV infection and WDL introduction in both healthy and infected cells, grounding our discussion in previously published literature. However, it is crucial to emphasize the urgent need for additional studies to deepen the understanding of these effects on metabolism due to limitations of our study as well as on available literature. Furthermore, the integration of various omics areas emerges as a crucial step, providing new insights for the development of molecules aimed at modulating metabolic pathways as antiviral mechanisms.

Keywords: Chikungunya. Metabolomics. HR-MAS NMR. Wedelolactone.

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Lista de Abreviaturas e Siglas

2D ou RMN 2D - Ressonância Magnética Nuclear Bidimensional

BMRB - *Biological Magnetic Resonance Data Bank*

CC - Controle celular

CHIKV - Vírus Chikungunya

CV - Controle viral

CW - Controle da Wedelolactona

CWV - Células infectadas e tratadas

COSY - *Correlation Spectroscopy*

CPMG- Sequência de pulso cpmgpr1d (Carr-Purcell-Melboom -Gill)

D₂O - Água deuterada

EM - Espectrometria de Massas

FID – Decaimento de indução livre

HCA- Análise Hierárquica de Clusters

HMDB - *Human Metabolome Database*

HR-MAS - *High Resolution Magic Angle Spinning*

HSQC - *Heteronuclear single quantum coherence*

Hz - Hertz

J – Constante de acoplamento escalar

KEGG - *Kyoto Encyclopedia of Genes and Genomes*

LOOCV - *Leave-One-Out-Cross-Validation*

NOESY – Sequência de pulso noesygppr1d

OMS - Organização Mundial da Saúde

PCA - *Principal Component Analysis*

PLS-DA - *Partial Least Squares - Discriminant Analysis*

ppm - Partes Por Milhão

RMN - Ressonância Magnética Nuclear

TMS - Tetrametilsilano

TOCSY - *Total Correlation Spectroscopy*

VIP - *Variable Importance in Projection*

WDL - Wedelolactona

δ - Deslocamento Químico

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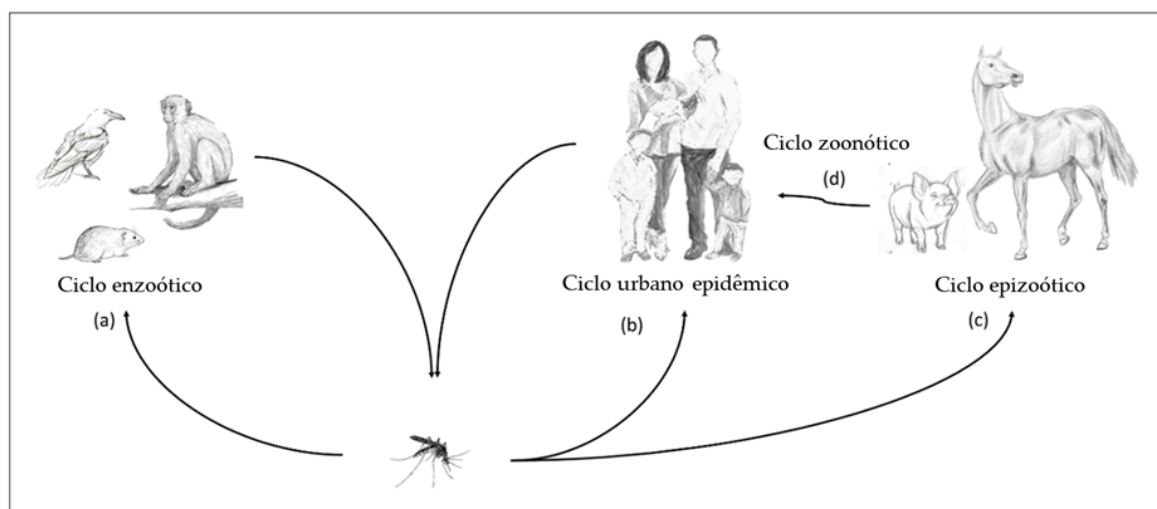
1. Introdução

1.1. Arbovírus – aspectos gerais

Os arbovírus constituem um grupo diversificado de vírus cuja característica principal é a ocorrência de parte de seu ciclo replicativo em vetores artrópodes hematófagos (“**Arthropod-borne viruses**”), que são responsáveis pela sua transmissão em animais e humanos através da picada do mosquito contaminado. Esses artrópodes são infectados ao ingerirem sangue de um hospedeiro em fase de viremia, ocasionando a replicação viral no trato gastrointestinal do artrópode. Esta replicação resulta em alta concentração de partículas virais infectantes, principalmente nas glândulas salivares do vetor favorecendo a transmissão (YOUNG, 2018). Os arbovírus que mais afetam a saúde humana são transmitidos por mosquitos hematófagos dos gêneros *Culex* e *Aedes*, embora outros artrópodes como flebotomíneos e carrapatos também sejam vetores bem conhecidos (DONALISIO et al., 2017).

Em sua maioria, as doenças causadas pelos arbovírus são zoonoses, mantidas em um ciclo de infecção silvestre de animais vertebrados na natureza como reservatórios e hospedeiros amplificadores, e reinfecção dos artrópodes transmissores. Contudo, eventuais contatos com humanos, principalmente devido a efeitos antropogênicos, como mudanças climáticas, desmatamentos, altas taxas de migração populacional e urbanização, facilitam a adaptação dos vetores a esses novos ambientes em escala global (BELLONE; FAILLOUX, 2020). Com isso, favorecem a transmissão e amplificação viral em novos hospedeiros, resultando na ocorrência de ciclos urbanos com áreas endêmicas e eventuais epidemias causadas por esses vírus em humanos (**Figura 1**) (LOPES, et al. 2014; YOUNG, 2018). A maioria dos vírus clinicamente relevantes pertence às famílias *Flaviviridae*, *Togaviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Reoviridae* e *Rhabdoviridae*. Embora esses vírus sejam mais prevalentes em regiões tropicais, onde as condições são propícias para a manutenção dos vetores e do ciclo viral, as atuais mudanças climáticas e o aumento da temperatura global têm contribuído para a sua disseminação em escala mundial. Esse fenômeno transforma esses vírus em um desafio significativo para a saúde pública em todo o mundo (GUBLER, 2001; HUANG et al., 2019).

Figura 1. Ilustração dos ciclos dos arbovírus na natureza e em meios urbanos, envolvendo seus respectivos hospedeiros.



Fonte: Adaptada de (PEINADO et al., 2022b).

O Brasil, que apresenta uma extensão territorial de mais de 8.500.000 km² em uma área geográfica com clima predominantemente tropical, torna-se um local apropriado para a existência dessas arboviroses e para a ocorrência de epidemias (LOPES et al., 2014). A cocirculação de arbovírus como Dengue (DENV), Zika (ZIKV), Chikungunya (CHIKV), Febre Amarela (YFV), Mayaro (MAYV), além de outros arbovírus como o Vírus do Nilo do Oeste (WNV), Oropouche (OROV), Encefalite de Saint Louis (SLEV), Rocio (ROCV) e Ilhéus (ILHV) é reconhecida e documentada no Brasil. Devido às prevalências, estabelecem recorrentes ameaças de circulação epidêmica, além da dificuldade de diagnóstico e manejo clínico devido a semelhança de sintomas causados em humanos, principalmente na fase aguda da doença (DONALISIO et al., 2017; FIGUEIREDO, 2007).

De modo geral, as arboviroses são caracterizadas como doenças febris acompanhadas de sintomas como dores articulares e musculares, cefaleia e exantemas. Contudo, em quadros mais complicados, os pacientes podem desenvolver febre hemorrágica (MELTZER, 2012), doenças articulares (como artrite e artrose) (AMDEKAR et al., 2017), além de complicações neurocognitivas como encefalite, meningite, mielite, paralisia aguda, microcefalia em recém-nascidos (devido à transmissão vertical), e síndromes como a de Guillain-Barré e outras, que podem perdurar por longos períodos ou até mesmo se tornarem doenças crônicas a longo prazo (CLÉ et al., 2020; GUBLER, 2001).

Recentemente, o FDA (*U.S. Food and Drug Administration*) aprovou a IXCHIQ, uma nova vacina desenvolvida pela Valneva SE. Essa vacina inovadora é administrada em dose

única e destinada à prevenção da doença causada pelo vírus Chikungunya em adultos com idade superior a 18 anos, especialmente aqueles que apresentam maior risco de exposição e desenvolvimento da enfermidade (SCHNEIDER et al., 2023). Na ausência de uma vacina, o tratamento dos pacientes em fase de viremia tem como foco amenizar os sintomas e consiste em repouso e uso de medicamentos como acetaminofeno, popularmente conhecido como paracetamol, que aliviam a febre alta na fase aguda da doença, e ibuprofeno ou outros antiinflamatórios não hormonais que amenizam as dores articulares. Narcóticos como a morfina ou corticosteroides de curto prazo podem ser considerados em casos de dor articular severa com avaliação minuciosa do risco-benefício do tratamento pelo profissional da saúde (MINISTÉRIO DA SAÚDE, 2014).

A principal forma de controle e prevenção da disseminação dos arbovírus constitui-se em medidas de controle de vetor e medidas individuais de proteção. O combate aos mosquitos vetores do gênero *Aedes*, que se encontram altamente adaptados a ambientes urbanos, pode ser realizado por meio de medidas coletivas relativamente simples. Uma maneira bastante efetiva é evitar quaisquer possíveis depósitos de água, que servem como principal veículo de reprodução e criadouros dos mosquitos, além do uso de roupas de mangas compridas, calças e de repelentes e inseticidas (adulticidas e larvicidas) em ambiente domiciliar (MINISTÉRIO DA SAÚDE, 2014; VAIRO et al., 2019).

1.2. Vírus Chikungunya

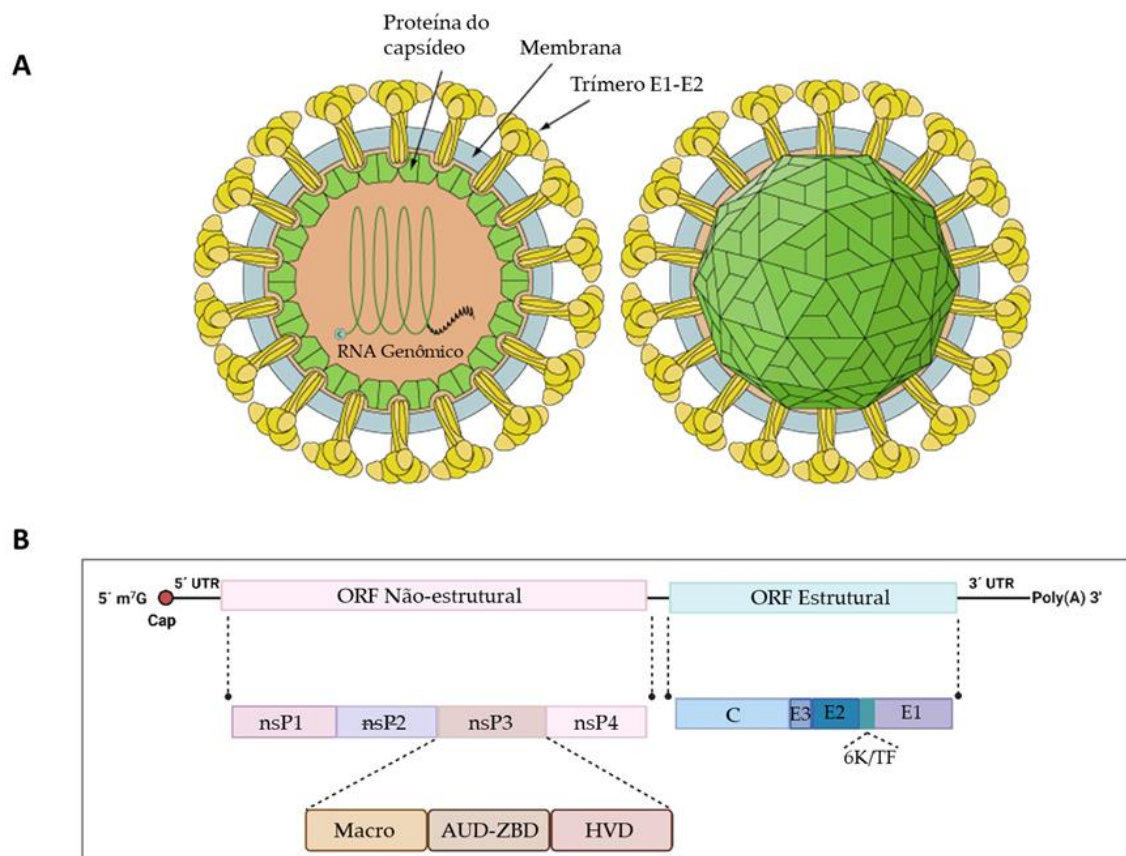
O vírus da Chikungunya foi isolado e descrito pela primeira vez em 1952 em Newala, distrito de Tanganica, localizado no leste da África. O vírus foi nomeado de acordo com o dialeto local, que significa “aquele que se curva”, referindo-se à postura dos acometidos em função da dor articular severa (CASTRO et al., 2016; ROBINSON, 1955).

Pertencente à família *Togaviridae*, gênero *Alphavirus* (ICTV, 2023), o vírus Chikungunya é caracterizado por partículas virais de aproximadamente 70 nanômetros (nm) de diâmetro, com um capsídeo esférico de simetria icosaédrica circundado por um envoltório lipídico com espículas glicoproteicas em sua superfície – proteínas E1 e E2, que promovem a ligação aos receptores celulares específicos (E2) e fusão com a membrana celular (E1), e estão associadas em estrutura de heterodímero com a proteína E3 em sua extremidade, que mantém a estabilidade da estrutura (BURT et al., 2017; GRIFFIN, 2013; HOLMES et al., 2020).

O nucleocapsídeo é composto por múltiplas cópias da proteína do capsídeo (C), e internamente contém o genoma viral, que é constituído por RNA (42S) de fita única, linear e de polaridade positiva com aproximadamente 12.000 pares de bases. O genoma viral apresenta

duas regiões de leitura aberta (*Open Reading Frame – ORF*) separadas por uma região curta não codificante, e contém duas extremidades não codificantes: a extremidade 5', que é “capeada” (5'cap), e a extremidade 3' que contém uma cauda poliadenilada (poly-A 3'). (**Figura 2A**). A primeira ORF, situada próxima à extremidade 5' codifica poliproteína que dá origem às proteínas não estruturais após sucessivas clivagens (nsP1, nsP2, nsP3 e nsP4), essenciais para a patogênese e evasão do sistema imune, além de outras funções elucidadas, assim como o processo de replicação do genoma viral e transcrição do RNA subgenômico, que consiste na segunda ORF, situada próxima à extremidade 3' e que codifica as proteínas estruturais (C, E3, E2, 6K/TF e E1) a partir de clivagens da poliproteína formada (**Figura 2B**). (RUPP et al., 2015).

Figura 2.(A) Estrutura da partícula viral dos Alphavirus. **(B)** Organização do genoma do Alphavirus.



Fonte: Adaptada de (PEINADO et al., 2022a) e ViralZone, Swiss Institute of Bioinformatics, (HULO et al., 2011).

A ligação do vírus às células é mediada por uma variedade de receptores específicos

expressos em muitos tipos celulares, e o processo de endocitose pode ser dependente ou independente de proteínas clatrina (BERNARD et al., 2010). Após a adsorção à membrana, as partículas virais são transportadas pela via endocítica, e através da acidificação do compartimento endocítico, ocorre o processo de fusão das membranas do endossomo com a partícula viral, que permite a liberação e desnudamento do nucleocapsídeo viral no citoplasma, e promove o início do processo de replicação viral (SILVA; DERMODY, 2017).

A tradução do RNA genômico gera uma poliproteína não-estrutural P1234, que é então processada e clivada por proteases virais e celulares, dando origem às proteínas P123 e nsP4. Ambas se associam a membranas e proteínas celulares para formar os complexos de replicação viral, que promovem a síntese do RNA viral de polaridade negativa. As outras proteínas não estruturais (nsP1 – nsP3) são produzidas por clivagens sucessivas da poliproteína P123, e juntamente à nsP4 promovem a síntese do RNA genômico, assim como a transcrição do mRNA 26S para produção da poliproteína (C-p62-6K-E1) que dará origem às proteínas estruturais (GRIFFIN, 2013; RUPP et al., 2015; STRAUSS; STRAUSS, 1994).

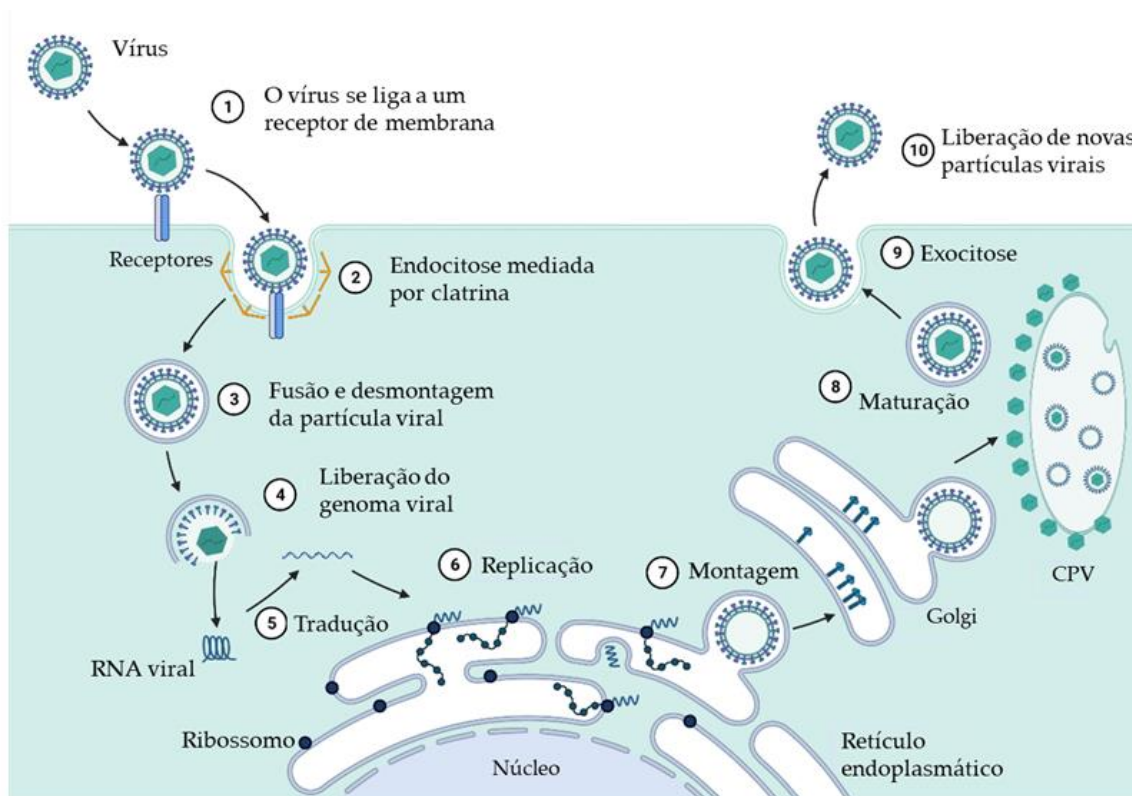
A proteína do capsídeo (C) é clivada da poliproteína por sua função de autoprotease e liberada no citoplasma, enquanto as glicoproteínas do envelope (E) E2 e E3 são traduzidas como uma porção da poliproteína inicial denominada p62 que se associa à proteína E1 no retículo endoplasmático e posteriormente é clivada e processada na rede trans-Golgi gerando as proteínas E2 e E3 maduras. As glicoproteínas são translocadas pelo sistema secretório e depositadas na membrana plasmática, para posteriormente serem associadas ao nucleocapsídeo viral (GAROFF et al., 2004; SILVA; DERMODY, 2017).

Além das proteínas do envelope (E) e capsídeo (C), também é traduzida a proteína 6K, um peptídeo sinal que atua no desdobramento e maturação das glicoproteínas, na montagem e liberação da partícula viral, e a proteína *Transframe* (TF) que também participa do processo de maturação das novas partículas virais e encontra-se associada às glicoproteínas E1 e E2 na partícula viral madura (HOLMES et al., 2020; RAMSEY et al., 2017).

Durante a maturação, as unidades proteicas do capsídeo são processadas e montadas juntamente a uma molécula de RNA genômico de polaridade positiva formando nucleocapsídeos no citoplasma, que migram para a membrana plasmática e interagem com as glicoproteínas do envelope, especificamente com o domínio citoplasmático da glicoproteína E2, para o processo de brotamento da membrana celular. Nesse processo, a partícula viral adquire um envelope lipídico derivado da membrana celular hospedeira que contém as glicoproteínas E1, E2 e E3, e novas partículas virais infectantes – vírions – são liberadas no meio extracelular (**Figura 3**) (GAROFF et al., 2004; SCHWARTZ; ALBERT, 2010; SILVA;

DERMODY, 2017; STRAUSS; STRAUSS, 1994).

Figura 3. Ciclo replicativo do Alphavirus. (1) Ligação e adsorção do vírus a um receptor de membrana; (2) Endocitose da partícula viral mediada por clatrina; (3) Fusão do nucleocapsídeo com o endossomo e (4) liberação do genoma viral no citoplasma; (5) Tradução do genoma viral e replicação viral (6) nas membranas do retículo endoplasmático; (7) montagem e (8) maturação das partículas virais pela via trans-Golgi, e por fim, (9) exocitose e (10) liberação de novos vírions das células.



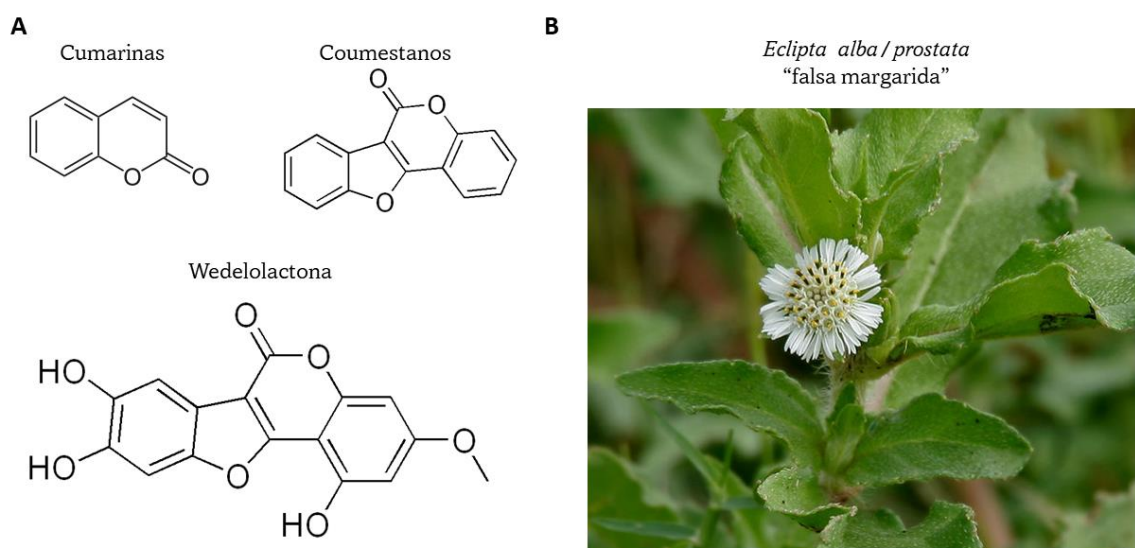
Fonte: Adaptada de (PEINADO et al., 2022a).

1.4 Wedelolactona

Na busca por antivirais eficazes contra vírus patogênicos, incluindo os arbovírus, compostos e produtos naturais derivados de plantas medicinais como polifenóis e extratos de plantas têm sido estudados intensivamente e demonstrado efeitos promissores em uma grande variedade de estudos (ATANASOV et al., 2021). Os polifenóis são metabólitos secundários produzidos por plantas que apresentam inúmeras funções, como, por exemplo, proteção contra a radiação UV, contra microorganismos e defesa contra insetos e outros animais. Em diversas patologias, esses compostos atuam como antioxidantes, antibacterianos, antivirais, antifúngicos, antiinflamatórios e anticancerígenos, dentre uma variedade de outras funções, e geralmente apresentam efeitos colaterais mínimos ou até mesmo ausentes (SHI et al., 2020).

Dentre os mais de 4.000 tipos de polifenóis conhecidos estão os coumestanos, compostos orgânicos derivados da oxidação de cumarinas pertencentes a uma subclasse de isoflavonoides, com estrutura química caracterizada pela presença do esqueleto das cumarinas e uma unidade benzofurano (**Figura 4A**) (HA; HOP; SON, 2023). São encontrados em plantas como *Wedelia calendulacea* e *Eclipta alba/prostata* (*Asteraceae*), herbácea invasora conhecida popularmente como “falsa margarida” (**Figura 4B**), e apresentam inúmeras propriedades fitoterápicas que são aproveitadas na medicina tradicional asiática e sul-americana. Os coumestanos mais conhecidos são a Wedelolactona e a Deimetilwedelolactona (FENG et al., 2019; VINYAGAM et al., 2021).

Figura 4. (A) Estrutura química das cumarinas, coumestanos e Wedelolactona. (B) *Eclipta alba/prostata* (*Asteraceae*), popularmente conhecida como “falsa margarida”.



Fonte: (A) Autoria própria, gerada pelo software ChemDraw; (B) Janglan et al., 2013.

A Wedelolactona (“WDL”; 7-methoxy 5,11,12-trihydroxy-coumestan; $C_6H_{10}O_7$) (**Figura 4A**) é o princípio bioativo presente nas partes aéreas de *E. alba* e apresenta uma diversidade de propriedades, tais como: hepatoprotetora (QINGQIONG et al., 2018; XIA et al., 2013), antioxidante (LI et al., 2020; MAO-MAO et al., 2019a), antimicrobiana (BOREGOWDA et al., 2019; DALAL et al., 2010; PANDEY et al., 2011), anticancerígena (HSIEH et al., 2015; NEHYBOVÁ et al., 2017; SARVESWARAN et al., 2016; SARVESWARAN et al., 2012),

antidiabética (SHAHAB; AHMAD, 2018), anti-inflamatória (MAO-MAO et al., 2019b; YUAN et al., 2013), neuro, cardio e osteoprotetora (LIU et al., 2016; PENG et al., 2017; SHARMA et al., 2021), além de benefícios dermatológicos, como por exemplo, no tratamento de doenças de pele e em casos de alopecia e queda de cabelo promovendo o crescimento capilar (LEE et al., 2019).

Na virologia, os efeitos inibitórios da WDL e outras cumarinas e coumestanos foram estudados com diversos vírus (COLLEGE; BENGAL, 2018). Esses compostos apresentaram atividade antiviral contra o vírus da imunodeficiência humana (HIV), com inibição da integrase viral (TEWTRAKUL et al., 2007). Também apresentaram inibição de diferentes etapas do ciclo replicativo do citomegalovírus humano (HCMV) (SVRLANSKA et al., 2020), e potencial inibitório da RNA polimerase dependente de RNA (NS5B) do vírus da hepatite C (HCV) (KAUSHIK-BASU et al., 2008). Apesar da ausência de estudos dos efeitos da WDL com arbovírus, o potencial inibitório de outras cumarinas extraídas da *Mammea americana* e *Tabernaemontana* foi investigado e resultados promissores com mais de 50% de inibição da infecção por Dengue (DENV) e Chikungunya (CHIKV) foram obtidos (GÓMEZ-CALDERÓN et al., 2017), demonstrando que a WDL e coumestanos no geral são compostos naturais promissores para o estudo de antivirais.

A escolha da Wedelolactona neste trabalho, deu-se devido a estudos em nosso grupo de pesquisa que evidenciaram seu potencial inibitório da protease do CHIKV, a nsP2. Esses resultados de inibição da nsP2 fizeram parte do artigo científico publicado, apresentado nos resultados e discussões desta tese.

1.5 Metabolômica: uma breve introdução e suas aplicações

A metabolômica é um campo de estudo relativamente recente dentre as outras ômicas (genômica, proteômica e transcriptômica) que utiliza tecnologias analíticas juntamente a métodos estatísticos e multivariados para a interpretação dos dados oriundos de um sistema biológico (ROESSNER; BOWNE, 2009). Os metabólitos são os produtos finais derivados dos processos bioquímicos celulares como a expressão gênica e proteica, e, quando analisados em conjunto com resultados das outras ômicas, podem ser considerados como assinaturas bioquímicas altamente correlacionadas ao fenótipo, que podem ser utilizadas para avaliar diferentes condições de biofluidos, tecidos, células ou organismos, inclusive sob processos patológicos, possibilitando a investigação de processos biológicos e biomarcadores patológicos, assim como o diagnóstico de doenças (JOHNSON et al., 2016; PATTI et al., 2012).

Apesar de inovadora, interpretar dados de metabolômica ainda gera um grande desafio, devido às moléculas analisadas representarem produtos em diferentes estágios de reações metabólicas e processos celulares (WANG et al., 2020). Esses produtos podem ter funções distintas em diversos organismos ou até mesmo em diferentes células de um mesmo organismo. A complexidade química dos metabólitos, que apresentam heterogeneidade estrutural e uma variedade extensa de propriedades físicas adiciona uma camada extra de desafio analítico à metabolômica em comparação com outras abordagens ômicas. Atualmente, o desenvolvimento técnico está em andamento para alcançar uma cobertura mais abrangente de metabólitos dentro de um único método analítico, e a integração das ômicas torna-se crucial para avaliar condições e patologias integralmente (WANG et al., 2020; GRAW et al., 2021).

O uso da metabolômica pode ser feito através de duas abordagens distintas: a metabolômica não direcionada (*Untargeted Metabolomics*) e a direcionada (*Targeted Metabolomics*), sendo que a combinação de ambas também pode ser realizada para uma maior precisão de resultados (PATTI et al., 2012). A metabolômica não direcionada permite a análise abrangente de todos os metabólitos mensuráveis presentes na amostra em estudo, o que possibilita a descoberta de biomarcadores, contudo, essa abordagem também gera uma enorme quantidade de dados que devem ser minuciosamente tratados através de metodologias analíticas adequadas para a identificação de uma ampla variedade de metabólitos, além de requerer protocolos viáveis para a preparação de amostras aliados ao uso de técnicas sensíveis e específicas para a coleta de dados (JOHNSON et al., 2016; ROBERTS et al., 2012). Na abordagem direcionada, utiliza-se conjuntos de metabólitos quimicamente pré-definidos e conhecidos, baseados em hipóteses bioquímicas previamente estabelecidas, conferindo um alto nível de precisão e exatidão das análises, além de possibilitar a otimização dos protocolos de preparação de amostras, reduzindo-se a presença de metabólitos que não são de interesse para o estudo direcionado de vias e processos bioquímicos específicos (ROBERTS et al., 2012).

O metaboloma de células, tecidos e organismos compreende uma variedade de moléculas, incluindo lipídios, aminoácidos, açúcares, ácidos orgânicos, entre outros. Enquanto a metabolômica concentra-se primariamente nas classes hidrofílicas de metabólitos, a lipidômica surge como uma disciplina independente devido à complexidade dos lipidomas nos organismos, porém complementar à metabolômica (WANG et al., 2020). Os potenciais papéis dos lipídios e pequenos metabólitos na patogênese de doenças têm sido extensivamente explorados em diversas condições e patologias humanas (YANG et al., 2016). A integração da metabolômica e lipidômica está se consolidando como uma abordagem emergente, proporcionando um atlas abrangente da paisagem metabólica. Essa integração viabiliza uma

análise de redes metabólicas completas, identificando metabólitos cruciais na patologia das doenças e facilitando a investigação das interconexões entre lipídios e outros metabólitos durante a progressão das doenças (HAN, 2016; YANG et al., 2016; HU, et al., 2009).

Além disso, a metabolômica é uma disciplina científica com amplas aplicações, desempenhando um papel crucial em diversas áreas de pesquisa e prática clínica. Seu uso abrange a descoberta de biomarcadores, identificando moléculas indicadoras de estados fisiológicos, doenças ou respostas a tratamentos, sua contribuição significativa para a compreensão das bases metabólicas de condições de saúde e doenças, fornecendo insights valiosos para o desenvolvimento de terapias direcionadas (YAN, XU, 2018; JOHNSON, IVANISEVIC, SIUZDAK, 2016). Na farmacometabolômica, investiga-se o impacto dos medicamentos no metabolismo, permitindo a personalização de tratamentos (KADDURAH-DAOUK, WEINSHILBOUM, 2015). A relação entre dieta e metabolismo é explorada na nutrimetabolômica, enquanto estudos em microbioma beneficiam-se da análise dos perfis metabólicos associados a microorganismos (ULASZEWSKA, et al. 2019; POSMA, et al., 2020; BAUERMEISTER, et al., 2022.). A metabolômica também desempenha um papel crucial na monitorização de processos de produção de produtos biológicos, estudos sobre estresse, toxicidade, saúde materna e neonatal, bem como avanços na pesquisa oncológica e em saúde cardiovascular (NOCTOR, LELARGE-TROUVERIE, MHAMDI, 2015.; FANUS, PINTUS, DESSI, 2018; SCHMIDT et al., 2021; KUMAR, MISRA, 2019; MCGARRAH et al., 2018, FORD et al., 2022). Em resumo, a metabolômica oferece uma visão abrangente dos metabólitos em sistemas biológicos, consolidando-se como uma ferramenta indispensável em diversas disciplinas científicas.

A aplicação da metabolômica ao estudo de infecções virais é bem difundida, utilizando-se uma variedade de técnicas, como a Espectroscopia de Ressonância Magnética Nuclear (RMN), a Cromatografia Líquida acoplada à Espectrometria de Massas (LC-MS) e a Cromatografia Gasosa acoplada à Espectrometria de Massas (GC-MS), Espectroscopia Vibracional (VS), entre outras, aplicadas a diferentes tipos de amostras (biofluidos, tecidos, órgãos e cultura celular) para avaliar as alterações metabólicas causadas pelos vírus nos alvos de estudo (MANCHESTER; ANAND, 2017). Como patógenos intracelulares obrigatórios, os vírus dependem de precursores biológicos e da maquinaria biossintética celular e afetam o metabolismo dos seus hospedeiros, visando sua replicação e geração de novas partículas virais infectantes. O estudo dessas interações utilizando-se a metabolômica, possibilita a identificação de metabólitos e vias alteradas pela infecção viral, de modo a compreender-se os mecanismos envolvidos, além de proporcionar novos alvos para o estudo de moléculas inibidoras e

antivirais, visando-se modular vias e produtos que podem ser essenciais para a patogênese viral (MANCHESTER; ANAND, 2017; NOTO et al., 2014; RABINOWITZ et al., 2011; TOUNTA et al., 2021).

Quanto às diferentes técnicas e suas aplicações, a RMN tem sido especialmente utilizada para a identificação de novos compostos e a elucidação de estruturas (EMWAS et al., 2019). O uso da MS está se tornando cada vez mais popular devido à sua versatilidade, facilidade de integração com técnicas de separação e alta sensibilidade (ZHOU et al., 2012; PAPADIMITROPOULOS et al., 2018). Por outro lado, a VS é extensivamente empregada em estudos *in situ*, aproveitando seu caráter não destrutivo (FRAGA-CORRAL et al., 2022). O campo da metabolômica está experimentando um aumento nas aplicações em diversas áreas científicas a cada ano, impulsionado pelos avanços em técnicas analíticas e pela integração com outras disciplinas ômicas, o que possibilita uma melhor compreensão dos processos metabólicos. É fundamental continuar o desenvolvimento constante de ferramentas analíticas para explorar plenamente todas as potencialidades da metabolômica (FRAGA-CORRAL et al., 2022).

1.6 Princípios Básicos de Ressonância Magnética Nuclear (RMN)

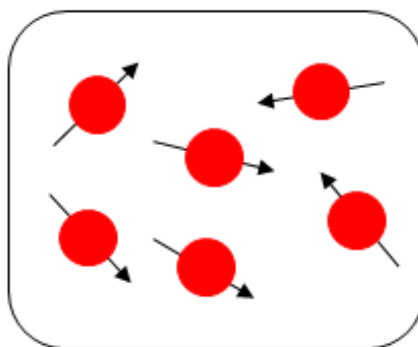
A escolha da técnica analítica para os estudos metabolômicos depende do foco do estudo, do tipo de amostra e de outros fatores como o custo, acessibilidade e *expertise* da técnica em si, sendo que cada plataforma apresenta suas vantagens e desvantagens. Atualmente, dentre as técnicas mais utilizadas está a RMN, uma técnica que se baseia na interação da radiação eletromagnética com a matéria, e que tem como vantagens a alta reprodutibilidade, ser não destrutiva e com protocolos relativamente simples para a preparação de amostras, além de possibilitar a identificação de uma variedade de compostos provenientes de diversos tipos de amostras (biofluidos, órgãos, tecidos e células em estado sólido ou semissólido) (EMWAS et al., 2019).

O princípio da RMN é a interação dos spins nucleares de átomos como o de hidrogênio (^1H) – o mais abundante e com alta razão giromagnética (γ), que resulta em maior sensibilidade de detecção – mas também a interação de outros spins nucleares como dos átomos de ^{13}C , ^{14}N , ^{15}N , ^{18}O e ^{31}P , com ondas de radiofrequências e sob presença de um campo magnético externo (B_0) (KEELER, 2010; SOLOMONS et al., 2018).

O spin nuclear é uma grandeza física e propriedade de determinados núcleos que é caracterizado pela combinação de todos os momentos angulares das partículas dentro do núcleo a partir de sua movimentação no espaço. O momento angular é capaz de gerar um momento

magnético (μ), pois, partículas elementares como os elétrons e os núcleos atômicos são como cargas em movimento que possuem propriedades magnéticas. Esse momento magnético é descrito em termos de número de spin (I), cujos valores podem variar de 0, $\frac{1}{2}$, 1, $\frac{3}{2}$, etc. Núcleos com massas e números atômicos pares, como o ^{12}C , ^{16}O , ^{32}S , entre outros, não possuem spin resultante por não apresentarem momentos angulares associados a eles, resultando na ausência de propriedades magnéticas ($I = 0$). Os núcleos detectáveis por RMN possuem spin $I \neq 0$. Um exemplo é o núcleo de hidrogênio ($I = \frac{1}{2}$), cuja combinação de seus momentos angulares no espaço dão origem a um momento magnético (μ), fazendo com que o próton se comporte como um pequeno ímã, e na ausência de um campo magnético externo (B_0), sua orientação é aleatória (**Figura 5**) (BOB; SILVA, 2001; MARTINS et al., 2015).

Figura 5. Representação dos spins nucleares em orientação aleatória na ausência de um campo magnético externo (B_0).

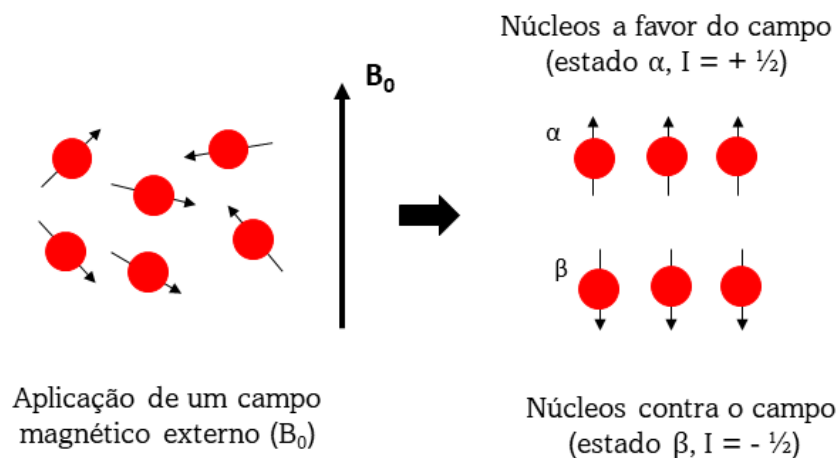


Fonte: Adaptada de Keeler et al., 2010.

Na presença de um campo magnético externo (B_0), a orientação desses núcleos pode variar, e o número de orientações que um núcleo pode assumir é determinado pelo número de spin (I) na relação: $2I + 1$. No caso do núcleo ^1H , cujo spin é $\frac{1}{2}$, existem somente duas orientações possíveis: contra ou a favor do campo magnético externo, e esses alinhamentos apresentam estados de energia diferentes. Quando a favor do campo magnético externo (ou parcialmente alinhado a ele), o estado de spin é $+\frac{1}{2}$, também chamado de estado α , e é o estado de menor nível energético. Quando contra o campo, o spin assume o estado $-\frac{1}{2}$, ou estado β , que é o de maior energia (**Figura 6**) (BOB; SILVA, 2001; SILVERSTEIN et al., 2019).

Figura 6. Representação da orientação dos spins nucleares na presença de um campo magnético externo (B_0). Há duas populações de spin, a de menor nível energético (estado α), e

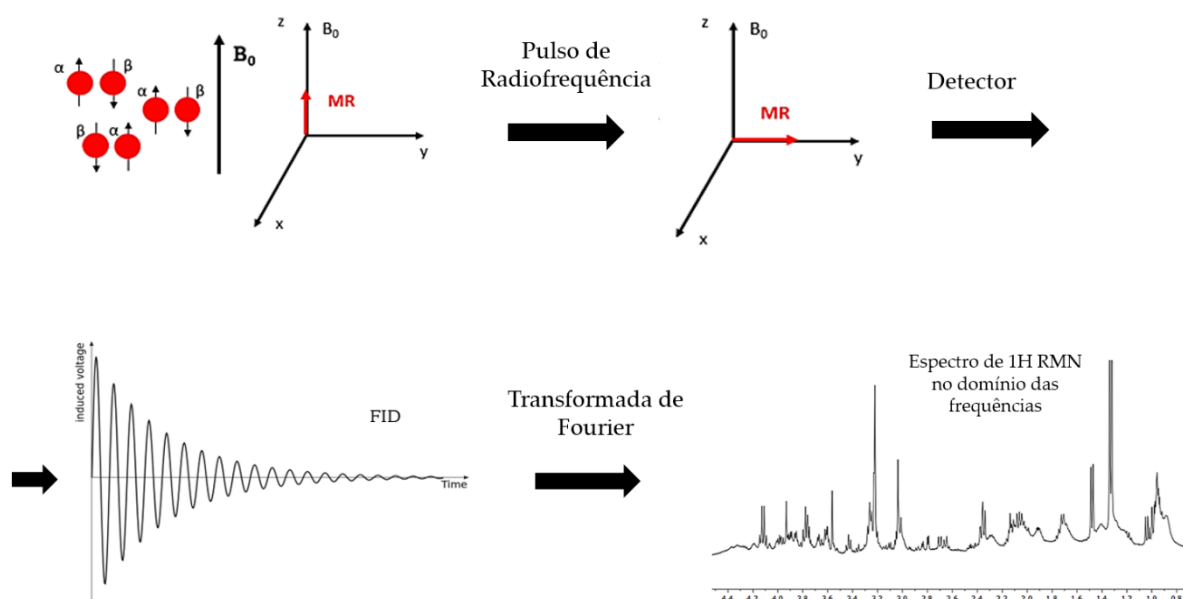
a de maior nível energético (estado β).



Fonte: Adaptada de Keeler et al., 2010.

A diferença entre os estados de spin resulta em uma magnetização resultante (M_R) que gera um movimento de precessão em torno do campo (B_0), porém sem coerência. A aplicação de um campo (B_1) em pulsos de radiofrequência perpendicularmente ao campo (B_0) faz com que os átomos absorvam energia, tornando o movimento de precessão dos spins nucleares coerente, unidos em torno de B_0 . A partir disso, a magnetização é detectada por um indutor e gera um sinal elétrico chamado decaimento livre de indução (FID – *Free Induction Decay*), que ocorre devido ao decaimento da magnetização, pois os pulsos de radiofrequência são controlados e aplicados em função do tempo, na ordem de micro a milissegundos. Nesse período, os núcleos excitados passam por um período de relaxação e tendem a retornar à sua posição de equilíbrio original, dando origem ao FID que decai no tempo. Esse sinal é registrado e digitalizado e passa por uma operação matemática chamada Transformada de Fourier que transforma os sinais do FID (uma função do tempo) para o domínio de frequências de ressonância, gerando um espectro de RMN de ^1H típico (**Figura 7**) (BOB; SILVA, 2001; MARTINS et al., 2015).

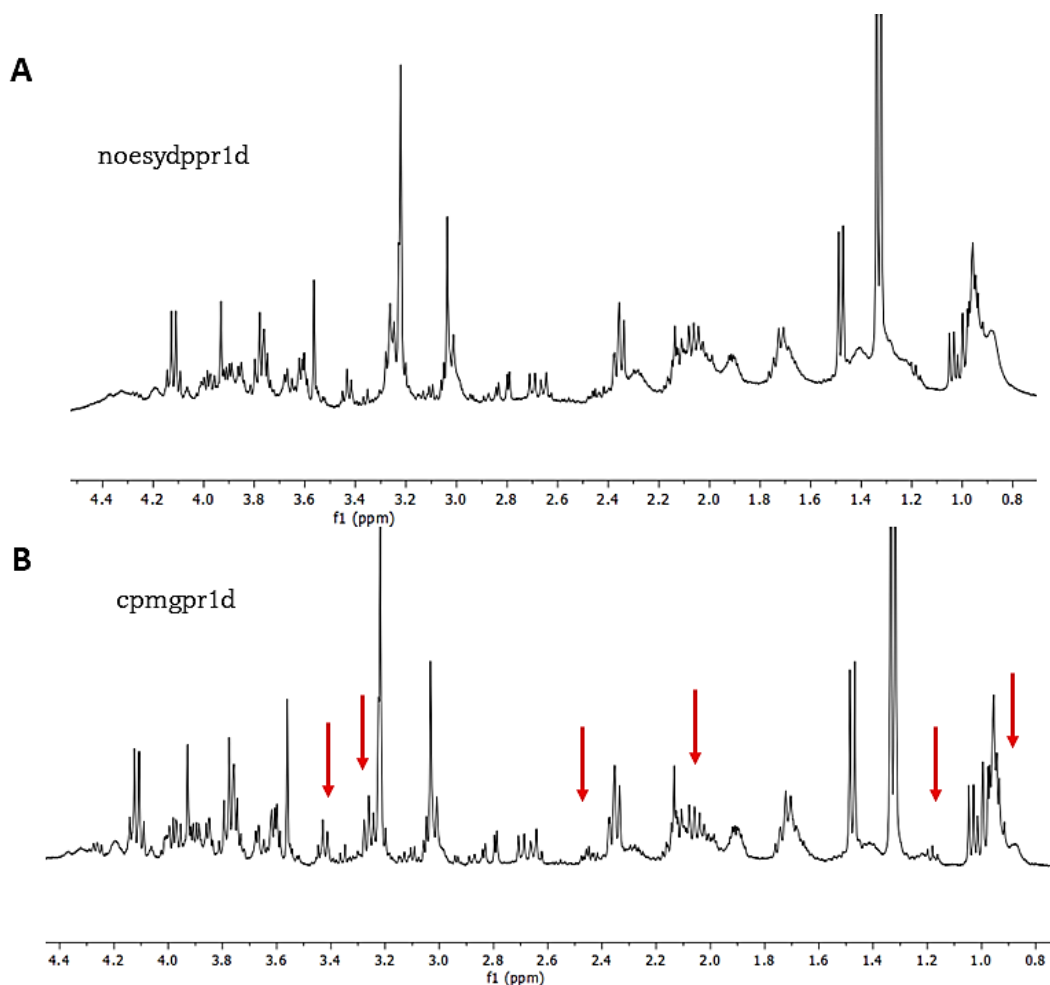
Figura 7. Esquema representativo da geração dos espectros de RMN.



Fonte: Adaptada de Silverstein et al., 2019 e Keeler et al., 2010.

Diferentes frequências de pulsos podem ser utilizadas na coleta de dados de RMN. No caso de amostras biológicas, a presença de água e/ou outros solventes em grande proporção pode ser observada nos espectros como um grande pico que pode atrapalhar na identificação de pequenos sinais de outros metabólitos. Para resolver esse problema, uma das sequências de pulsos que é comumente aplicada é a *noesygppld*, com pré-saturação do sinal de água para supressão desse sinal no espectro e facilitar a visualização de pequenos sinais (**Figura 8A**). Além disso, também é desejável suprimir o sinal de compostos de alta massa molecular como as macromoléculas (proteínas, lipídeos, etc.) para evitar que estes dificultem a visualização de sinais menores. Portanto, utiliza-se um experimento com filtro T_2 denominado CPMG (*Carr-Purcell-Meiboom-Gill Sequence* - *cpmgrp1d*), resultando na filtração e supressão dos sinais de macromoléculas (**Figura 8B**) (GUENNEC et al., 2017).

Figura 8. (A) Espectro de RMN ^1H (400 MHz, D_2O) de uma amostra de células Vero E6, adquirido utilizando a sequência de pulsos *noesyppr1d*. **(B)** Espectro de RMN ^1H (400 MHz, D_2O) de uma amostra de células Vero E6, adquirido utilizando a sequência de pulsos *cpmgrp1d*. As flechas em vermelho indicam os pequenos sinais que se tornam visíveis ao filtrar os sinais de macromoléculas com o experimento CPMG (Carr-Purcell-Meiboom-Gill Sequence). Ambos foram adquiridos em espectrômetro de RMN Bruker Avance III 400 MHz do Instituto de Química da UNICAMP.



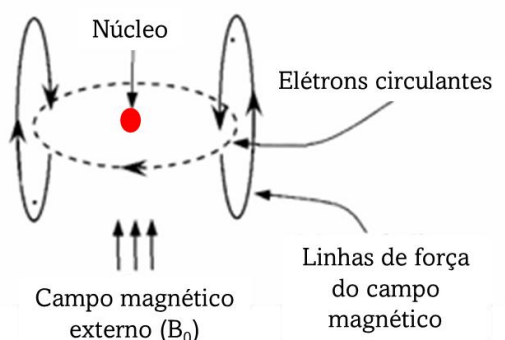
Fonte: Autoria própria, gerada a partir do software MestReNova.

1.6.1 Blindagem e deslocamento químico

Em um espectro de RMN pode-se observar a presença de sinais em frequências de absorção diferentes, mesmo que sejam de um único tipo de núcleo, como o de hidrogênio. O fenômeno que explica a disparidade nas frequências de absorção é denominado blindagem. Esse evento decorre das diferenças nos ambientes químicos onde esses núcleos estão inseridos. Em outras palavras, a absorção de energia varia conforme os núcleos e elétrons circundantes desse próton, e a maneira como interagem entre si. Se isso não ocorresse, todos os prótons de uma determinada molécula forneceriam sinais em uma mesma frequência quando submetidos a um campo externo. Porém, devido à densidade eletrônica que envolve os prótons em um ambiente químico molecular, cada núcleo experimentará o campo magnético externo de maneira diferente, pois, o movimento dos elétrons gera um campo magnético induzido (B_i) que é proporcional ao campo magnético externo (B_0) (**Figura 9**). Quanto maior a densidade eletrônica em volta dos núcleos vizinhos, mais blindados em relação a campo magnético aplicado eles estão. Na presença de átomos eletronegativos, como por exemplo o oxigênio, a nuvem

eletrônica ao redor dos núcleos vizinhos diminui, tornando-os mais desblindados e mais suscetíveis ao campo externo (B_0). A frequência de absorção é diretamente proporcional ao campo magnético aplicado, portanto, núcleos mais blindados absorvem energia em frequências menores, enquanto os núcleos desblindados absorvem energia em frequência mais altas (BOB; SILVA, 2001; SILVERSTEIN et al., 2019; SOLOMONS et al., 2018).

Figura 9. Esquema representativo do efeito de blindagem em um próton sob a influência de um campo magnético externo (B_0). A movimentação dos elétrons ao redor do próton, gera um campo magnético induzido (B_i) que blindo o mesmo da influência do campo externo (B_0).



Fonte: Adaptada de Silverstein et al., 2019.

Essas diferenças de frequências de absorção em relação a um determinado núcleo em uma molécula são chamadas de deslocamento químico (δ), e é dada em escala delta com valores expressos em ppm (partes por milhão) nos espectros de RMN. Os valores de deslocamento químico podem ser calculados medindo-se a distância entre o sinal observado e o sinal de uma substância de referência que é adicionada à amostra. Comumente utiliza-se o tetrametilsilano (TMS) por ser uma substância quimicamente inerte, volátil e solúvel em muitos solventes orgânicos, mas outros tipos de substâncias também podem ser adicionados. O pico do TMS no espectro é considerado como valor zero na escala em Hertz (Hz) ou ppm (δ), e, como os equipamentos utilizados nas análises de RMN possuem diferentes forças de campo magnético (200 a 600 MHz, por exemplo), a seguinte fórmula é utilizada para obter-se os valores de deslocamento químico, independente da frequência operacional do instrumento utilizado (SILVERSTEIN et al., 2019):

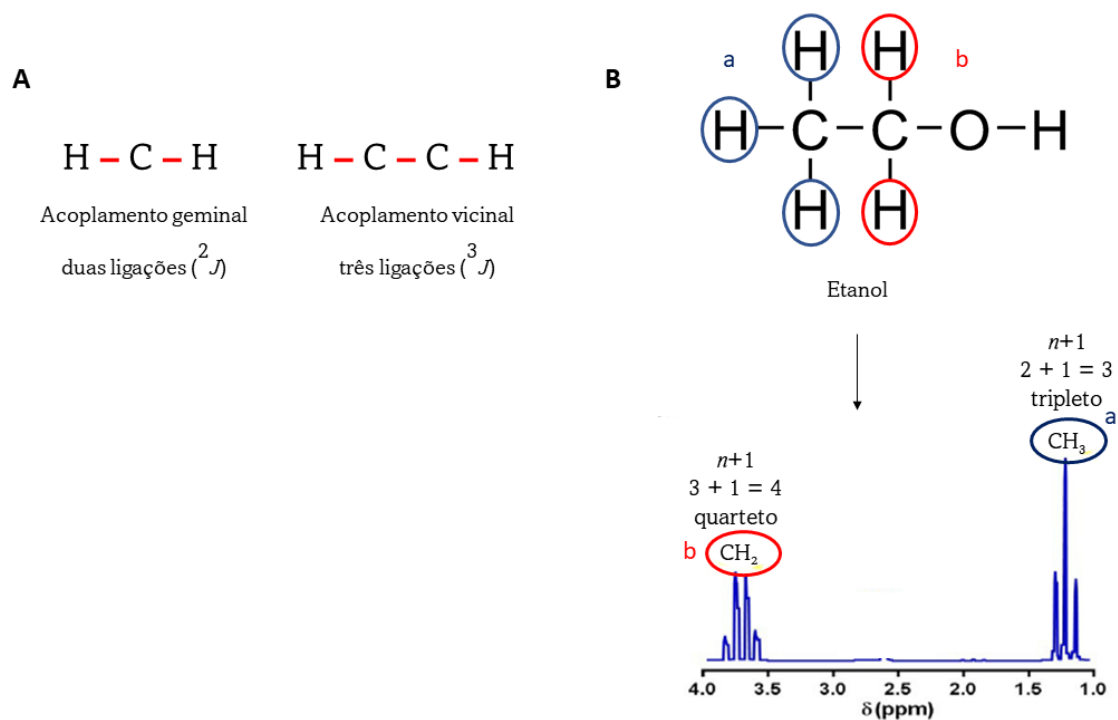
$$\delta \text{ (deslocamento químico em ppm)} = \frac{\text{Distância a partir da substância de referência (TMS)}}{\text{Frequência operacional do equipamento (MHz)}}$$

Por convenção, os deslocamentos químicos na escala de RMN aumentam da direita para a esquerda, sendo que, quanto mais blindados os núcleos, mais à direita os sinais se encontram, e quanto mais desblindados, os sinais no espectro aparecem mais à esquerda.

1.6.2 Desdobramento de sinal, Acoplamento e Constante de Acoplamento Escalar (J)

Os sinais de absorção do spin nuclear podem manifestar-se no espectro como uma ou mais frequências, em um fenômeno conhecido como desdobramento de sinal. Esse fenômeno ocorre devido ao acoplamento dos spins dos núcleos vizinhos, gerando campos magnéticos que exercem influência nos núcleos ao seu redor. Além disso, as diferentes condições químicas nos diversos ambientes moleculares também contribuem com o desdobramento dos sinais. Dois tipos de acoplamento spin-spin podem ser observados: o vicinal, que ocorre até três ligações de distância, e o geminal, que ocorre até duas ligações de distância (**Figura 10A**). Esse acoplamento de spins implica no desdobramento de sinais no espectro que originam picos únicos (singletos) ou picos de maior multiplicidade, como os dupletos, tripletos, quartetos, e assim sucessivamente, dando origem a multipletos. Para núcleos com spin $\frac{1}{2}$, essa multiplicidade do sinal é dada pela relação $n + 1$, em que n representa o número de hidrogênios vizinhos até três ligações de distância, fazendo com que os prótons apresentem $n + 1$ picos no espectro de ^1H , dependendo dos n prótons vizinhos equivalentes ao seu redor (**Figura 10B**) (SILVERSTEIN et al., 2019; SOLOMONS et al., 2018).

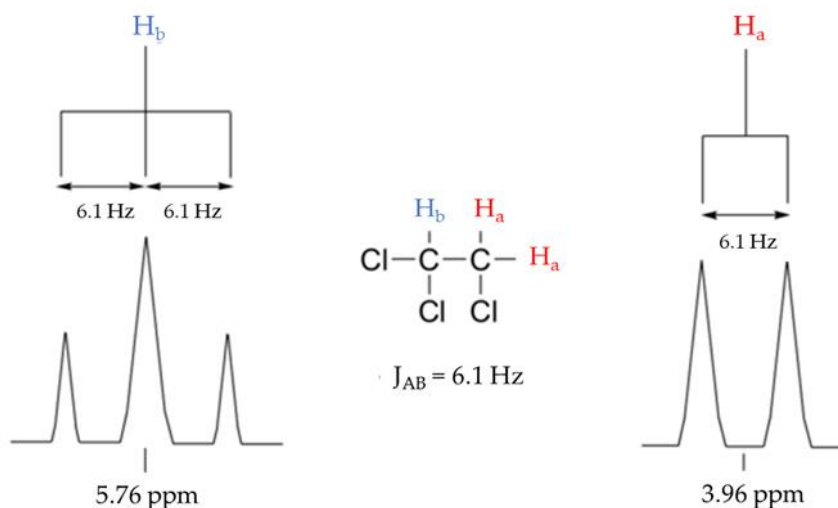
Figura 10. (A) Acoplamento vicinal e geminal. **(B)** Exemplo da multiplicidade de sinal com a molécula do etanol.



Fonte: Adaptada de Silverstein et al., 2019 e (ROCCHITTA; SERRA, 2013).

A distância entre os picos é denominada constante de acoplamento escalar (J), dada em unidades de frequência em Hertz (Hz), que indica o quanto um acoplamento é efetivo, e pode variar dependendo da molécula analisada (SILVERSTEIN et al., 2019). Núcleos e/ou prótons que acoplam entre si apresentam a mesma constante de acoplamento, por exemplo, se um próton A acopla com um próton B, a constante de acoplamento J_{AB} será igual ($J_{AB}=J_{BA}$) (**Figura 11**).

Figura 11. Exemplo de desdobramento de sinal e da constante de acoplamento (J). O sinal **a** observado é um dubleto por acoplar com um próton em **b**. O sinal de **b** é um tripleto por acoplar com dois prótons em **a**. A constante de acoplamento $J_{AB}=J_{BA}$ é a medida entre os picos em Hertz.



Fonte: Adaptada de Silverstein et al., 2019 e (BRUICE, 2005).

1.7 Espectroscopia de Ressonância Magnética Nuclear Bidimensional (2D)

Os espectros de RMN unidimensional (RMN 1D) muitas vezes apresentam informações complexas e uma alta superposição de sinais devido a semelhanças dos ambientes químicos dos núcleos em uma molécula, como, por exemplo, os hidrogênios, o que pode gerar deslocamentos químicos muito próximos nos espectros, dificultando sua interpretação e determinação de estruturas moleculares. Com o intuito de superar esse problema e facilitar as etapas de interpretação e identificação, utiliza-se técnicas de RMN bidimensionais (2D), que possibilitam a análise minuciosa de estruturas moleculares através da correlação de diferentes núcleos em uma molécula. O único desafio dessas técnicas consiste no tempo de coleta, que, dependendo do tipo de experimento e amostra, pode durar várias horas para coleta de dados de uma única amostra (BOB; SILVA, 2001; GIRAUDEAU; FRYDMAN, 2016).

Resumindo em uma forma simplista, enquanto na RMN 1D o espectro é projetado contendo as funções intensidade do sinal versus frequência e é obtido após a transformada de Fourier que depende somente de uma variável no tempo, a RMN 2D consiste em uma série de aquisições 1D projetadas em função de duas frequências (F_1 e F_2) e leva em consideração duas variáveis de tempo: o tempo de evolução (t_1), que consiste no período em que o sistema de

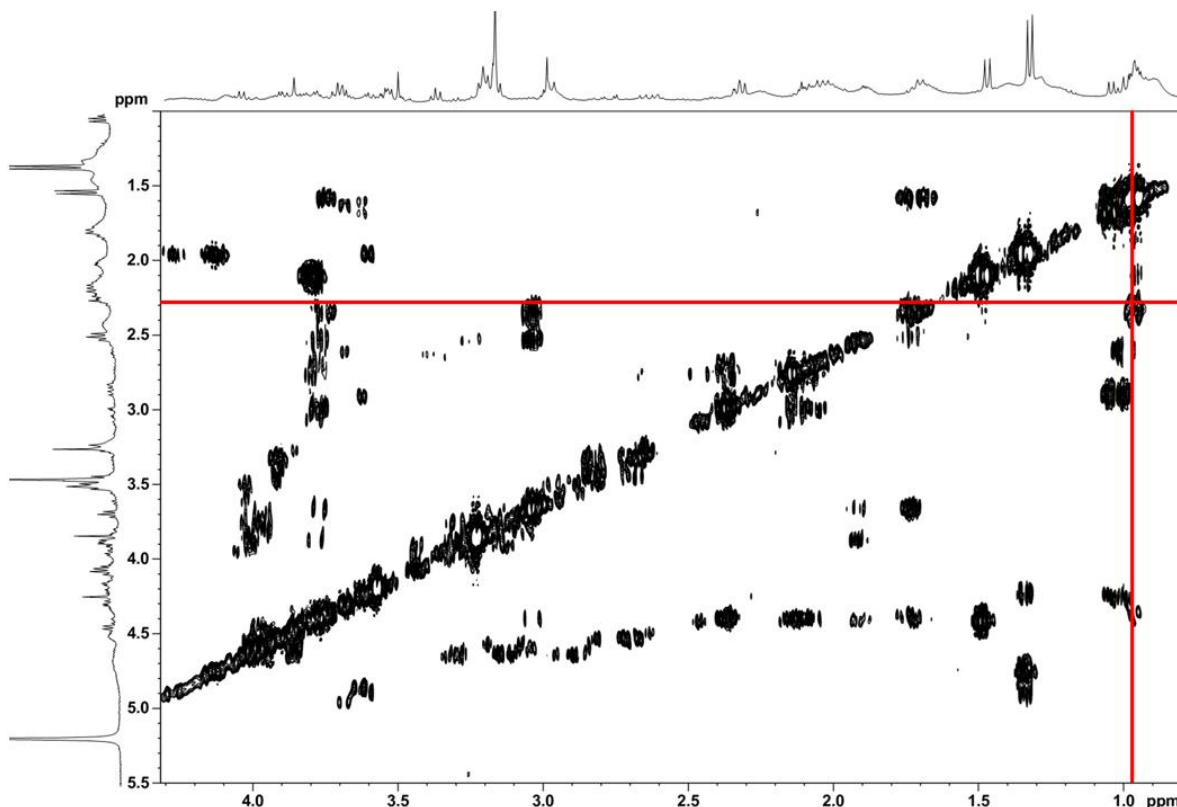
spins é excitado e sai do equilíbrio devido à aplicação dos pulsos de radiofrequência, e o tempo de detecção ou aquisição (t_2), que é o período em que se adquire o sinal do FID (SILVERSTEIN et al., 2019).

Para adquirir os espectros 2D, uma série de espectros é obtida com diferentes valores arbitrários de t_1 diferentes de zero em uma série de aquisições. Após todas as aquisições, obtém-se um FID de t_1 em função do tempo de aquisição (t_2), que quando submetido à transformada de Fourier resulta em um espectro bidimensional com sinais observados em duas dimensões denominadas F1 (Y) e F2 (X) (KRIWACKI; PITNER, 1989; SILVERSTEIN et al., 2019).

Os experimentos de correlação podem analisar interações homonucleares (H-H) ou heteronucleares (H-X) em uma variedade de experimentos. O foco da abordagem neste trabalho será dado aos experimentos TOCSY ^1H - ^1H (*T*otal *C*orrelation *S*pectroscop*Y*) e HSQC ^1H - ^{13}C (*H*eteronuclear *S*ingle *Q*uantum *C*orrelation *S*pectroscopy).

TOCSY ^1H - ^1H (*T*otal *C*orrelation *S*pectroscop*Y*) é um experimento que indica as correlações entre todos os prótons de um sistema de spin. Os espectros resultantes, tem os eixos F1(Y) e F2(X) representando os hidrogênios e a diagonal com as informações 1D, portanto, os picos cruzados no TOCSY indicam todos os spins que possuem acoplamento dentro do sistema, e não somente spins diretamente acoplados (até três ligações) (**Figura 12**) (EMWAS et al., 2019).

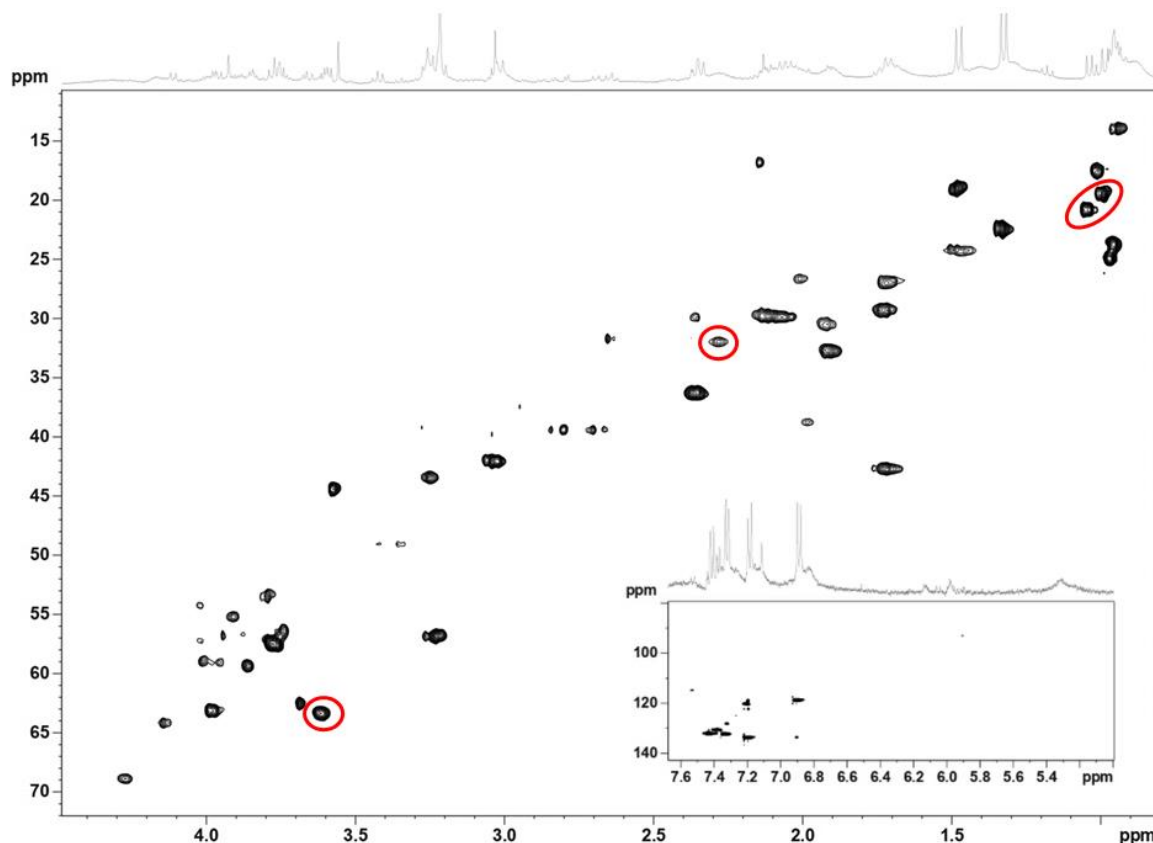
Figura 12. Espectro de correlação TOCSY ^1H - ^1H (400 MHz, D_2O) de uma amostra de células Vero E6. Espectro obtido no espectrômetro de ressonância magnética nuclear Bruker Avance III 400 MHz no IQ da UNICAMP. Representação das correlações de hidrogênio da valina em 0,97 e 2,28 ppm (deslocamentos químicos).



Fonte: Autoria própria obtida com o software MestreNova.

Para a análise de correlações entre diferentes núcleos (heteronucleares), utilizamos o experimento *HSQC* ^1H - ^{13}C (*Heteronuclear Single Quantum Correlation Spectroscopy*). Neste experimento, é possível visualizar as interações e correlações ^1H - ^{13}C que estão distantes por uma ligação química, onde os sinais do carbono aparecem ao longo do eixo F1(Y) e os dos prótons ao longo do eixo F2(X). O HSQC é um experimento muito útil para auxiliar na resolução das estruturas moleculares onde ocorre sobreposições de sinais de prótons e permite a identificação de metabólitos com grande eficácia (**Figura 13**) (EMWAS et al., 2019).

Figura 13. Espectro de correlação HSQC ^1H - ^{13}C (400 MHz, D_2O) de uma amostra de células Vero E6. Espectro obtido no espectrômetro de ressonância magnética nuclear Bruker Avance III 400 MHz no IQ da UNICAMP. Na imagem são representadas as correlações ^1H - ^{13}C atribuídas à valina.



Fonte: Autoria própria obtida com o software MestreNova.

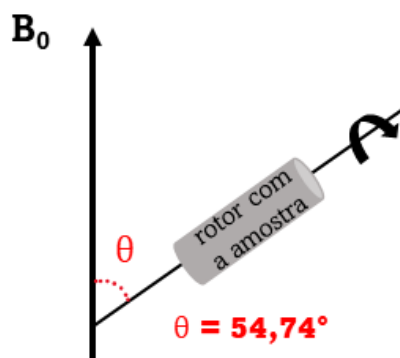
1.8 Ressonância Magnética Nuclear com sonda HR-MAS (*High Resolution Magic Angle Spinning NMR*)

Amostras em estado líquido apresentam homogeneidade e movimentação das moléculas de uma forma rápida e aleatória, enquanto amostras em estado sólido ou semi-sólido, como células e tecidos, apresentam heterogeneidade e distinção das moléculas em relação à susceptibilidade magnética, além do movimento molecular restrito por serem mais compactas, o que resulta em uma resolução espectral baixa e alargamento de sinais quando analisadas através de métodos de RMN convencionais (CHEN; SINGER, 2007). Para solucionar esses efeitos da heterogeneidade de amostras sólidas e semi-sólidas, Andrew e Lowe desenvolveram a técnica de giro no ângulo mágico (θ) (*Magic Angle Spinning - MAS*) de $54,74^\circ$ (ANDREW et al., 1959; LOWE, 1959), que posteriormente foi aprimorada na década de 90 para a técnica de

rotação no ângulo mágico em alta resolução (*High Resolution Magic Angle Spinning - HR-MAS*) (CHENG et al., 1996).

A técnica de alta rotação da amostra em um ângulo (θ) de $54,74^\circ$ em relação ao campo magnético (B_0) (**Figura 14**) simula o efeito da reorientação molecular para os spins e elimina os efeitos das interações e restrições moleculares em estado sólido, que causam baixa resolução e alargamento dos sinais. Isso permite que essas amostras se comportem como amostras em solução quando submetidas a um campo magnético externo, resultando em alta resolução espectral, assim como nas amostras de RMN em estado líquido, além de possibilitar o uso de protocolos de preparação de amostras relativamente simples, sem a necessidade de metodologias para a extração de metabólitos em fases polares e apolares, preservando também a estrutura do tecido, órgão ou células a serem estudados (KAEBISCH et al., 2018; VERMATHEN et al., 2017).

Figura 14. Esquema representando o ângulo de (θ) $54,74^\circ$ entre eixo de rotação da amostra e o campo magnético aplicado (B_0) para amostras sólidas e semissólidas.



Fonte: Adaptada de Andrew et al. 1959; Lowe, 1959.

1.9 Quimiometria

A quimiometria é uma área da química que consiste na utilização de métodos matemáticos e estatísticos para extrair o máximo de informações relevantes a partir da análise de dados químicos. Dados multivariados como os dados obtidos na metabolômica necessitam de tratamentos quimiométricos para a identificação de informações relevantes para objetivo do estudo (ERIKSSON et al., 2004).

Primeiramente, o conjunto de dados metabolômicos obtido necessita de uma etapa de pré-processamento, que consiste em ajustes que aprimoram ou eliminam informações relevantes ou não relevantes dos espectros. As etapas de pré-processamento incluem o ajuste de

fase dos espectros, que promove simetria dos picos, o ajuste de linha de base que auxilia na remoção de artefatos que são irrelevantes e podem gerar distorções nas análises, e o referenciamento em relação a um pico de referência (por exemplo, o do TMSP), que permite o alinhamento dos picos para a identificação dos respectivos metabólitos. A fim de reduzir a dimensionalidade dos dados e ruídos, assim como possíveis desalinhamentos para tornar as análises viáveis, utiliza-se um procedimento denominado “*binning*”, que divide o espectro em janelas (*bins*) de mesma largura em ppm, cujas áreas totais representam o espectro original. Por fim, é realizada uma etapa de normalização dos dados, para transformá-los em valores comparáveis, reduzindo os efeitos das discrepâncias de intensidades e concentrações relativas (EMWAS et al., 2018; WORLEY; POWERS, 2013).

Após o pré-processamento dos dados, estes são organizados na forma de matriz de dados, onde encontram-se as amostras nas colunas (os casos) e os *bins*, referentes aos deslocamentos químicos nos espectros em ppm, nas linhas (as variáveis), ou vice-versa. Para extrair as informações dessas matrizes de dados, são realizadas as análises estatísticas e quimiométricas que visam avaliar as diferenças e similaridades das amostras, assim como identificar as variáveis relevantes no conjunto de dados.

As análises estatísticas univariadas são realizadas com o intuito de obter-se uma análise exploratória dos dados que fornecem uma visão inicial de variáveis possivelmente significativas que serão validadas em conjunto com as análises multivariadas. Vários métodos estatísticos podem ser utilizados como *Fold change*, *teste t*, *ANOVA*, *Volcano plot*, entre outros.

As análises multivariadas consistem em métodos de reconhecimento de padrão que permitem identificar, avaliar e caracterizar as diferenças presentes entre os grupos e amostras em um conjunto de dados. Tais métodos podem ser não-supervisionados, como a Análise de Componentes Principais (*PCA – Principal Component Analysis*), que transforma as variáveis originais em um grupo de Componentes Principais (PC) que representam as direções de maior variância dos dados e permitem explorar os agrupamentos naturais intrínsecos das amostras e identificar *outliers* sem consultar a classe das mesmas (WORLEY; POWERS, 2013).

Já nos métodos supervisionados, existe a discriminação das classes às quais as amostras são categorizadas, e são construídos modelos que explicam as variações dos dados e também são capazes de prever a qual classe uma amostra pertence. Neste trabalho, foi utilizada a Análise Discriminante por Mínimos Quadrados Parciais (*PLS-DA – Partial Least Squares Discriminant Analysis*), juntamente com os escores das Variáveis Importantes na Projeção (*VIP – Variable Importance in Projection*) para identificar as variáveis discriminantes entre as classes (GHOSH et al., 2020).

Os modelos da análise multivariada não-supervisionada precisam ser validados para avaliar o caráter representativo dos dados, o número de variáveis necessárias para a construção do modelo, assim como a capacidade preditiva do modelo. Um exemplo de validação cruzada que é comumente utilizada é a Validação Cruzada Total (*LOOCV – Leave-One-Out-Cross-Validation*). Esse método consiste na retirada de uma amostra do conjunto de treinamento, e um novo modelo é construído sem a amostra que foi retirada, que é utilizada para validar o modelo. Subsequentes retiradas de amostras são realizadas para o teste do modelo, e valores do Coeficiente de Determinação (R^2), da Capacidade Preditiva (Q^2) e Acurácia do modelo são calculados como parâmetros de avaliação da previsibilidade e eficácia do mesmo. (TRYGG; LUNDSTEDT, 2007; WORLEY; POWERS, 2013).

2 Objetivos

2.1 Objetivo Geral

Identificar os metabólitos e vias metabólicas alteradas em células Vero E6 causadas pela infecção do arbovírus Chikungunya e pela adição da Wedelolactona em meio de cultura celular.

2.2 Objetivos Específicos

- Avaliar a citotoxicidade da Wedelolactona em cultura celular;
- Avaliar a ação antiviral da Wedelolactona sobre a infecção por Chikungunya em células Vero E6;
- Identificar os metabólitos e vias metabólicas alteradas pela infecção por Chikungunya em células Vero E6 utilizando a metabolômica por RMN com sonda de HR-MAS;
- Identificar os metabólitos e vias metabólicas alterados pela adição da Wedelolactona em células Vero E6 saudáveis e infectadas utilizando a metabolômica por RMN com sonda de HR-MAS.

3 Materiais e Métodos

Todos os experimentos e procedimentos passíveis de contaminação foram realizados em cabines biológicas de fluxo laminar, em condições de acordo com as normas de biossegurança NB-2. As soluções, reagentes, vidrarias e produtos foram esterilizados pelo processo de autoclavagem ou filtração. Os experimentos de cultura celular, análise da citotoxicidade, ensaios antivirais da Wedelolactona, infecção viral e preparação das amostras para análise por RMN foram realizados no Laboratório de Pesquisas em Virologia (LPV) da Faculdade de Medicina de São José do Rio Preto (FAMERP) sob permissão do Prof. Dr.

Maurício Lacerda Nogueira e supervisão da Profa. Dra. Carolina Colombelli Pacca, coorientadora deste projeto.

A coleta de dados de RMN, análises estatísticas e identificação dos metabólitos foram realizadas juntamente ao Instituto de Química da UNICAMP, no Laboratório Multiusuário de Ressonância Magnética Nuclear de Amostras Sólidas, sob supervisão e em colaboração com a Profa. Dra. Ljubica Tasic juntamente com o auxílio dos envolvidos no projeto.

3.1 Linhagens celulares e manutenção da cultura

A linhagem celular Vero clone E6 (ATCC C1008, células de rim de macaco verde africano, *Cercopithecus aethiops*) foi mantida em meio MEM (Meio Eagle's Minimum Essential - Gibco, Waltham, MA, USA) suplementado com 10% de Soro Fetal Bovino – SFB (Gibco, Waltham, MA, USA), 100 U/mL de Penicilina, 0,1 mg/mL de Estreptomicina 0,5 µg/mL de Anfotericina B (Gibco, Waltham, MA, USA), à temperatura de 37 °C e 5% de CO₂ em incubadora de atmosfera umidificada.

A manutenção por propagação ou repique celular ocorreu conforme a necessidade das células ou necessidade de realização dos experimentos. Para a manutenção por repique celular, a monocamada de células com aproximadamente 80% de confluência em frascos de cultura é lavada, gentilmente, com tampão fosfato-salino (PBS) e incubada com solução de tripsina-EDTA (0,25%) por um período de cinco a dez minutos a 37 °C e atmosfera de 5% de CO₂ para digestão e clivagem das proteínas de adesão das células e desprendimento da superfície de contato. Após esse período, novo meio MEM suplementado com 10% de SFB é adicionado, inativando a ação da tripsina, e as células são homogeneizadas e ressuspensas em novo meio para crescimento e proliferação em uma proporção de 1:10.

Para todos os experimentos realizados, é necessário a distribuição das células em placas e microplacas de *n* poços. Portanto, as células eram previamente contadas utilizando-se a câmara de Neubauer para posterior distribuição das quantidades necessárias de acordo com o tipo de placa e experimento utilizado.

3.2 Vírus e produção do estoque viral

O arbovírus Chikungunya, proveniente de uma linhagem asiática isolada de uma amostra clínica de um paciente virêmico (número de acesso BHI3762/H804917), foi cordialmente cedido pelo Prof. Dr. Maurício Lacerda Nogueira do Laboratório de Pesquisa em Virologia da Faculdade de Medicina de São José do Rio Preto (FAMERP), o qual dispõe de toda infraestrutura para a realização dos experimentos.

Para produção do estoque viral, os vírus foram propagados em células Vero E6, que são altamente permissíveis ao vírus, em frascos de cultura de 75 cm² (Corning Incorporated, EUA) com 80% de confluência e mantidos sob atmosfera de 5% de CO₂ a 37 °C por três dias, conforme a observação do efeito citopático (ECP) no tapete celular por microscopia de luz. Após esse período, as garrafas infectadas passaram por um período de congelamento a -80 °C por 24 horas e posteriormente foram descongeladas. A suspensão viral foi coletada e transferida para tubos tipo falcon e centrifugadas a 4.000 rpm, a 4 °C durante 10 minutos. Decorrido este período, o sobrenadante contendo as partículas virais foi aliquoteado em criotubos de 1,5 mL e congelados em freezer -80 °C.

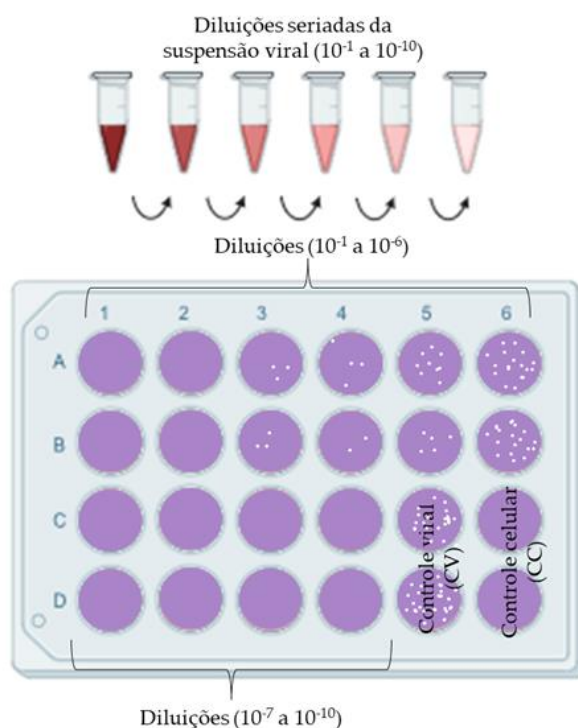
3.3 Titulação viral

A determinação do título viral para o CHIKV foi realizado através do ensaio de placas de lise. Para isso, microplacas de 24 poços foram preparadas com 1x10⁵ células/poço e mantidas em estufa umidificada com 5% CO₂ a 37 °C por 24 horas. Para o ensaio de placas, diluições seriadas de razão 10 (10⁻¹ a 10⁻¹⁰) da alíquota viral em meio MEM com 1% de SFB foram inoculadas em duplicatas na microplaca e incubadas por uma hora em estufa de 5% CO₂ a 37 °C para adsorção do vírus às células, com períodos de agitação gentil a cada 15 minutos. Decorrido este período, o inóculo viral foi removido, foi adicionado o meio *overlay* (meio MEM acrescido de CMC – carboximetilcelulose – a 2%, 1% SFB, 100 U/mL de Penicilina, 0,1 mg/mL de Estreptomicina 0,5 µg/mL de Anfotericina B) e as placas foram incubadas novamente a 37 °C e 5% CO₂ por três dias. Após esse período específico, o sobrenadante das placas foi desprezado para fixação das células vivas com solução de Formaldeído a 10%, à temperatura ambiente durante 20 minutos, e corados com solução de Cristal Violeta a 3% para visualização e contagem das placas de lise (**Figura 15**) e posterior cálculo do título de infectividade viral - expresso pelo número de unidades formadoras de placas (PFU/mL) - através da fórmula:

$$\text{Título viral} = P \times F \times 10^x \text{ (PFU/mL)}$$

Em que, **P** é a média do número de placas virais obtido na diluição em que se contou as placas; **F** é o fator de correção para expressar o título viral de PFU/mL (1000 µL / volume do inóculo), e **10^x** é diluição em que as placas foram contadas.

Figura 15. Esquema representativo da titulação realizada através do ensaio de placas de lise. As células Vero E6 são expostas a diferentes diluições seriadas de razão da suspensão viral para adsorção e infecção por uma hora. Decorrido este período, é adicionado novo meio acrescentado de CMC a 2% e as placas são mantidas em estufa de CO₂ por 72 horas, com observação recorrente em microscópio invertido do efeito citopático. As células vivas são fixadas com formaldeído e coradas com cristal violeta para a visualização e contagem das placas de lise provenientes de uma única partícula viral infectante.



Fonte: Autoria própria gerada na plataforma Biorender©.

O título obtido é então utilizado nos próximos experimentos de acordo com o MOI (multiplicidade de infecção) desejado. O MOI é a proporção do agente utilizado para seu alvo de infecção, e é calculado a partir da seguinte fórmula:

$$\text{MOI} = \text{título viral} / \text{número de células}$$

Para os experimentos realizados e para a preparação das amostras para análise metabolômica, foi utilizado o MOI de 0,1, visando um baixo nível de lise celular total, pois a morte celular é um processo indesejado nas análises metabolômicas, visto que objetivamos analisar o metabolismo das células vivas afetado durante a infecção viral.

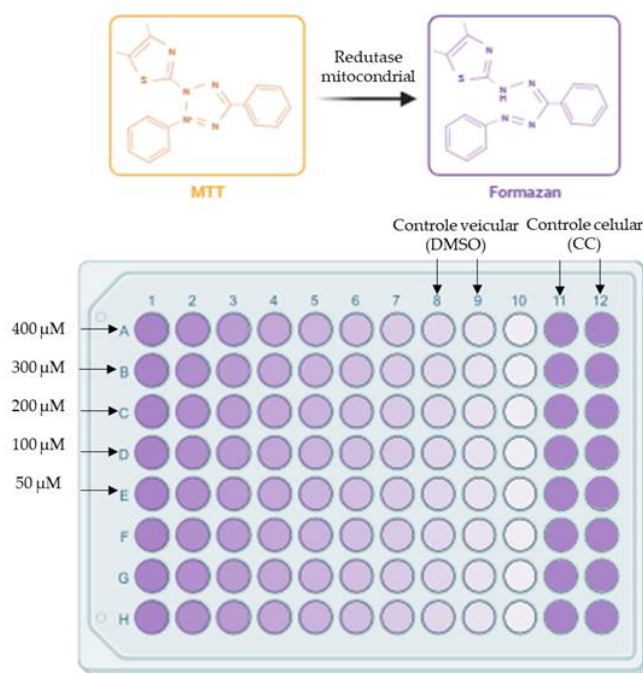
3.4 Wedelolactona e citotoxicidade

A Wedelolactona (“WDL”, 7-methoxy 5,11,12-trihydroxy-coumestan) (Sigma Aldrich, USA, nível de pureza $\geq 98\%$) foi adquirida para os experimentos realizados com o CHIKV. Para utilização nos experimentos, a WDL foi dissolvida em DMSO (Dimetil-sulfóxido, Merck, USA – 10 mg/mL) em uma concentração estoque de 40 mM e congelada em freezer a $-20\text{ }^{\circ}\text{C}$ até a utilização nos experimentos.

A citotoxicidade da WDL foi avaliada em células Vero E6 por meio do ensaio colorimétrico do MTT (brometo de 3- 21 [4,5-dimetil-tiazol-2-il]-2,5 difeniltertrazólio, Sigma-Aldrich, USA). O processo ocorre nas mitocôndrias de células metabolicamente ativas, onde as enzimas desidrogenases mitocondriais atuam na clivagem do anel de tetrazólio presente no sal de MTT. Esse sal, inicialmente de coloração amarela e solúvel em água, é modificado pelas enzimas, resultando na formação de cristais de formazan. Esses cristais exibem uma coloração púrpura/roxa, são insolúveis em água, e podem ser quantificados espectrofotometricamente (KUMAR et al., 2018; MOSMANN, 1983).

Para o ensaio, monocamadas de células Vero E6 aderidas em microplaca de 96 poços (3×10^4 células/poço) foram expostas a diferentes concentrações da molécula (50 μM , 100 μM , 200 μM , 300 μM e 400 μM) diluída em meio MEM acrescido de 2% SFB por 48 horas em estufa a $37\text{ }^{\circ}\text{C}$ e 5% de CO_2 . Para o ensaio, utilizou-se os seguintes controles: **(a)** controle celular, que são as células e meio de cultivo e **(b)** controle veicular, das células somente com o solvente utilizado (DMSO) a menos de 2% (**Figura 16**).

Figura 16. Esquema representativo do experimento de citotoxicidade da Wedelolactona por meio do ensaio colorimétrico do MTT. As células são tratadas com diferentes concentrações do composto (fileiras A a E, poços 1 a 5) por um período de 48 h. Também são feitos controles veiculares (DMSO – fileiras 9 e 10) e controles celulares (fileiras 11 e 12). Após o período de incubação, é adicionado novo meio MEM com o sal de MTT (1 mg/mL) e as células são mantidas em estufa de CO₂ por uma hora. Transcorrido esse período, adiciona-se DMSO em todos os poços para a solubilização dos cristais de formazan, e as placas são submetidas a leitura de absorbância a 550 nm em espectrofotômetro para o cálculo da viabilidade celular.



Fonte: Autoria própria gerada pela plataforma Biorender©.

Após o período de incubação decorrido, o sobrenadante foi cuidadosamente removido e foi adicionado novo meio de cultura MEM suplementado com 2% de soro fetal bovino e MTT (1 mg/mL), e as células foram novamente incubadas em estufa a 37 °C e 5% CO₂ por uma hora. Transcorrido esse período, os sobrenadantes foram gentilmente removidos e foi adicionado 100 µL de DMSO para a solubilização dos cristais formazan, que são indicativos de atividade celular devido à atividade da enzima redutase mitocondrial em células viáveis que realiza a clivagem o anel tetrazólio do MTT (KUMAR et al., 2018). A placa foi então mantida sob agitação por 10 minutos para posteriormente ser realizada a leitura da absorbância a 550 nm no espectrofotômetro Spectramax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

O ensaio foi realizado em triplicatas independentes, e a partir dos valores de absorbância, as porcentagens de viabilidade celular foram calculadas no Microsoft Excel® a partir da fórmula:

$$\frac{\text{Absorbância das células tratadas com a WDL ou controle veicular (b)} \times 100}{\text{Média das absorbâncias do controle celular (a)}}$$

3.5 Ensaio antiviral de tratamento pós infecção

Visando estabelecer a concentração da WDL que seria utilizada na preparação das amostras para análise metabolômica, assim como complementar os resultados encontrados nessas análises, também foram realizados ensaios antivirais com a molécula para avaliar sua capacidade de inibição viral *in vitro*. Visto que, na preparação das amostras para análise metabolômica a molécula é adicionada ao meio após a adsorção e infecção do vírus às células, foram realizados somente ensaios de tratamento pós infecção, que consistem em avaliar a atividade inibitória da molécula nas etapas após adsorção e entrada do vírus nas células, compreendendo o processo de replicação, montagem, maturação e liberação de novos vírions.

Nos ensaios, foram preparadas previamente microplacas de 48 poços (6×10^4 células/poço) incubadas em estufa de 5% CO₂ a 37 °C por 24 horas com meio de crescimento. Após esse período, o sobrenadante foi desprezado, e as células foram infectadas com 0.1 MOI do vírus e incubadas por 1h em estufa de ambiente umidificado de 5% CO₂ a 37 °C com períodos de agitação delicada a cada 15 minutos. O inóculo foi então removido, e as diluições da WDL em MEM 1% SFB em diferentes concentrações (30 µM, 20 µM, 15 µM, 10 µM e 5 µM) foram adicionadas aos poços, e as células foram novamente incubadas em estufa de 5% CO₂ a 37 °C por 24 horas, que corresponde ao pico de replicação dos alfavírus.

O ensaio de inibição foi avaliado pela redução de placas de lise formadas durante a infecção viral com a adição da molécula, e para isso, após o período de incubação decorrido, a titulação do conteúdo dos poços com as diferentes concentrações em diluição seriadas de razão 10 (10^{-1} a 10^{-8}) foi realizada. As placas de lise formadas, que representam um processo de infecção completo por uma partícula viral, foram contadas para determinar o título viral sob as diferentes concentrações da molécula e comparadas ao controle de infecção viral (onde não há adição da molécula). O ensaio foi realizado em triplicatas independentes e as porcentagens de inibição do título viral foram calculadas no Microsoft Excel® utilizando-se a fórmula:

$$100 - \frac{(\text{Título viral obtido com adição da WDL na concentração "x" (PFU/mL)} \times 100)}{\text{Título do controle viral (PFU/mL)}}$$

3.6 Análises estatísticas dos ensaios de citotoxicidade e antiviral

Os resultados obtidos nos ensaios de citotoxicidade e no ensaio antiviral pós infecção foram analisados utilizando-se o Microsoft Excel® para a montagem das tabelas de porcentagem de viabilidade celular e inibição da replicação viral. O cálculo dos valores de CC₅₀ (concentração necessária da molécula para reduzir a viabilidade celular em 50%) e IC₅₀ (a concentração da molécula capaz de inibir 50% da replicação viral) foram realizadas no GraphPad Prism (versão 8.0, GraphPad Software, La Jolla, CA, USA) utilizando regressão não-linear e quantificados por meio da análise de variância (ANOVA) com o teste post-hoc de Dunnett e $p \leq 0,05$ como nível de significância estatística..

3.7 Preparação de amostras para análise metabolômica por ¹H-RMN HR-MAS

Verificamos através da realização de experimentos pilotos que seriam necessárias no mínimo 6×10^6 células por amostra para a análise por ¹H-RMN HR-MAS. Portanto, as células foram cultivadas em placas individuais de cultivo celular de 100mm de diâmetro em meio MEM 10% SFB, mantidas sob atmosfera umidificada de 5% CO₂ a 37 °C por um período de 24 horas antes da realização dos tratamentos e infecção conforme as condições a serem avaliadas: **(i)** controle celular sem adição da WDL e sem infecção viral – **CC**; **(ii)** controle viral - células somente com infecção viral – **CV**; **(iii)** células somente com adição da WDL – **CW**, e **(iv)** células infectadas e com adição da WDL – **CWV**.

Para a preparação das amostras, o meio de cultura foi removido das placas, e as células do controle viral **(ii - CV)** e **(iv - CWV)** da infecção com adição da molécula WDL foram infectadas com 0.1 MOI do CHIKV. As células do controle celular **(i - CC)** e as células somente com a molécula **(iii - CW)** foram acrescentadas de meio MEM 1% SFB, e todas foram incubadas em estufa a 37 °C e 5% CO₂ por uma hora com períodos de agitação gentil a cada 15 minutos para adsorção do vírus às células infectadas.

Decorrido o período de incubação e infecção das células determinadas **(ii - CV)** e **(iv - CWV)**, o inóculo das amostras que foram infectadas, assim como o sobrenadante das amostras não infectadas foram desprezados, e meio MEM 1% SFB com adição da molécula de WDL na concentração estabelecida (30 µM para CHIKV) foi adicionado nas placas das condições **(iii - CW)** e **(iv - CWV)**, enquanto somente meio MEM 1% SFB foi adicionado nas condições de controle celular e viral, **(i - CC)** e **(ii - CV)**. As células foram novamente incubadas por um

período de 24 horas em estufa de 5% de CO₂, a 37 °C. Ao todo, seis amostras de cada condição foram preparadas, totalizando 24 amostras.

Para a análise dos metabólitos intracelulares, após o período de incubação decorrido, as células foram lavadas com PBS e depois tripsinizadas por um período curto de cerca de dois minutos. Foi adicionado meio MEM 10% SFB para inativação da tripsina, e a suspensão celular foi recolhida e centrifugada a 3.000 rpm e 4 °C por 5 minutos. O sobrenadante foi descartado e os *pellets* celulares foram cuidadosamente lavados novamente com PBS e submetidos a mais uma etapa de centrifugação para garantir a remoção de quaisquer componentes do meio e tripsina. Os sobrenadantes foram descartados delicadamente, e os *pellets* congelados em criotubos a -80 °C para as análises de metabolômica.

3.8 Coleta de dados de metabolômica por ¹H-RMN HR-MAS

Os espectros de RMN de ¹H foram coletados utilizando a técnica de Ressonância Magnética Nuclear de alta resolução com giro no ângulo mágico (RMN HR-MAS), utilizando um espectrômetro Bruker Avance III (Bruker Biospin, Germany) do IQ da UNICAMP, operante a 400 MHz para ¹H e 100 MHz para ¹³C, equipado com uma sonda de núcleo triplo de 4-mm d-HR-MAS 400 SB performando no ângulo mágico a uma frequência de spinning de 3,5 kHz e a 298 K (para a análise de semissólidos em rotor de rotação máxima de 15kHz).

Espectros unidimensionais de hidrogênio (¹H) foram coletados utilizando a sequência de pulso noesygppr1d para supressão do sinal de água, assim como a sequência cpmgpr1d com filtro *T*₂ para supressão de sinais de macromoléculas, possibilitando a análise das pequenas moléculas de interesse – os metabólitos. Ambos os experimentos foram coletados com 256 repetições e tempo de espera – *delay* – de 4 segundos.

Também foram coletados experimentos bidimensionais (RMN 2D) para o auxílio na identificação dos metabólitos presentes nas amostras, através da correlação de núcleos moleculares. Espectros de correlação homonucleares e heteronucleares como TOCSY ¹H-¹H (aplicado com a sequência de pulso mlevgpphw5, número de scans 110, tempo de mistura 60 milissegundos) e HSQC ¹H-¹³C (aplicado com a sequência de pulso hsqcetgpprsisp2.2, número de scans 384) foram coletados para cada condição analisada.

3.9 Processamento dos dados, quimiometria e identificação dos metabólitos

Os espectros coletados foram salvos e uma série de pré-processamentos foi realizada através dos softwares TopSpin 3.5 (Bruker Biospin, Germany) e MestReNova (versão 12.0.2-20910).

Em todos os espectros, a transformada de Fourier foi aplicada, que consiste em uma operação matemática que converte o FID obtido em função do tempo captado pelo equipamento para o domínio das frequências. Também foram realizados manualmente o ajuste de fase, correção de linha de base, normalização em relação à área total para reduzir o efeito das diferenças de concentração, referenciação (utilizou-se o sinal do lactato a δ 1.33, $J = 7.0$ Hz como referência) e *binning* de 0.005 ppm foi aplicado.

Após essa etapa de pré-processamento dos espectros, foram geradas matrizes na extensão .csv para as análises estatísticas. As matrizes são compostas pelas amostras nas colunas e os deslocamentos químicos em ppm nas linhas. Algumas regiões do espectro foram removidas manualmente das análises por serem regiões de ruído (até δ 0.5 ppm e depois de δ 8.7 ppm) e a região que compreende sinais de água (δ 4.55 – 5.10 ppm).

As análises estatísticas foram realizadas na plataforma online *MetaboAnalyst 5.0* (PANG et al., 2021). Para as análises, as matrizes foram carregadas no módulo “*Statistical Analysis - one factor*”, selecionando-se o tipo de dados como “*spectral bins*” e no formato de amostras nas colunas (não-pareadas). A normalização foi feita pela média dos dados (“*Normalization by median*”) que transforma os dados em uma distribuição mais próxima à normal gerando dados comparáveis para as análises estatísticas. O autoescalamento foi escolhido (que centra os valores na média e divide-os pelo desvio padrão de cada variável) para evitar que os sinais mais intensos tenham maior influência na construção dos modelos.

Para as análises multivariadas, foram realizadas análises dos componentes principais (PCA) para observar os agrupamentos naturais das amostras, detectar amostras anômalas e reduzir a dimensionalidade dos dados. Com o objetivo de classificar as amostras, foram construídos modelos supervisionados de PLS-DA (Análise Discriminante por Mínimos Quadrados Parciais) que foram validados pela Validação Cruzada Total (LOOCV – *Leave-One-Out-Cross-Validation*), e a Análise de Cluster Hierárquico (HCA) foi utilizada para visualizar os agrupamentos das amostras na forma de um mapa de calor (*heatmap*), que também demonstra a intensidade dos sinais no espectro na região determinada (em ppm) através das cores no mapa. Os *VIP scores* foram analisados para identificar as variáveis de interesse utilizadas na construção do modelo de PLS-DA, juntamente com a análise univariada utilizando o teste *t* para as condições comparadas ao controle celular (CCxCW; CCxCV; CCxCWV; CVxCWV).

A partir desse conjunto de dados coletados, as variáveis que apresentaram valores de *p* menor que 0,05 ($p < 0,05$) foram consideradas estatisticamente significativas e foram anotadas para o assinalamento dos metabólitos, juntamente com os *boxplots* produzidos nas análises

estatísticas para avaliar as diferenças nas concentrações relativas dos metabólitos discriminantes em cada condição.

A identificação dos metabólitos no espectro foi realizada a partir de dados publicados na literatura (KOSTIDIS et al., 2017), aliado ao conhecimento adquirido de química analítica para analisar as estruturas químicas de metabólitos, ao uso de bancos de dados (HMDB - *Human Metabolome Database*, e BMRB – *Biological Magnetic Resonance Bank*), e consulta nos espectros unidimensionais e bidimensionais de correlação homonucleares (^1H - ^1H TOCSY) e heteronucleares (^1H - ^{13}C HSQC).

Uma primeira varredura nos espectros homonucleares TOCSY foi realizada para visualização dos acoplamentos de ^1H em todo o sistema de spin. Verificamos posteriormente as correlações heteronucleares ^1H - ^{13}C no espectro HSQC. Após a identificação, os metabólitos foram destacados nos espectros unidimensionais com base em seus deslocamentos químicos. Esses deslocamentos foram então empregados para identificar os metabólitos correspondentes às variáveis significativas derivadas das análises estatísticas e quimiométricas no *MetaboAnalyst 5.0*.

A identificação das vias metabólicas relacionadas aos metabólitos encontrados foi realizada utilizando-se os bancos de dados como o HMDB (*Human Metabolome Database*) (WISHART et al., 2007) e KEGG (*Kyoto Encyclopedia of Genes and Genomes, KEGG Pathway maps*) (KANEHISA; GOTO, 2000), além da busca na literatura de informações dos metabólitos e suas respectivas vias e funções em mamíferos para compreender as alterações encontradas.

4 Resultados e Discussão (apresentado na forma de artigo científico)

4.1. Artigo científico publicado na revista internacional “*International Journal of Molecular Sciences*” (FI = 5.6)

HR-MAS NMR METABOLOMICS PROFILE OF VERO CELLS UNDER THE INFLUENCE OF VIRUS INFECTION AND NSP2 INHIBITOR: A CHIKUNGUNYA CASE STUDY

ABSTRACT

The arbovirus Chikungunya (CHIKV) is transmitted by *Aedes* mosquitoes in urban environments, and in humans, it triggers debilitating symptoms involving long-term

complications, including arthritis and Guillain-Barré syndrome. The development of antiviral therapies is relevant, as no efficacious vaccine or drug has yet been approved for clinical application. As a detailed map of molecules underlying the viral infection can be obtained from the metabolome, we validated the metabolic signatures of Vero E6 cells prior to infection (CC), following CHIKV infection (CV) and also upon the inclusion of the nsP2 protease inhibitor wedelolactone (CWV), a coumestan which inhibits viral replication processes. The metabolome groups evidenced significant changes in the levels of lactate, myo-inositol, phosphocholine, glucose, betaine and a few specific amino acids. This study forms a preliminary basis for identifying metabolites through HR-MAS NMR (High Resolution Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy) and proposing the affected metabolic pathways of cells following viral infection and upon incorporation of putative antiviral molecules.

Keywords: Arbovirus, Chikungunya, Metabolomics, HR-MAS ¹H-NMR, Vero cells, Wedelolactone.

INTRODUCTION

Chikungunya virus (CHIKV) is a reemerging arbovirus (Arthropode-Borne virus) from the *Togaviridae* family and *Alphavirus* genus mainly transmitted by mosquitoes of the *Aedes* genus (*Ae. aegypti* and *Ae. albopictus*), which along with other arboviruses such as Dengue, Zika and Yellow Fever virus are a public global health concern, as they are spread worldwide (BELLONE; FAILLOUX, 2020) and are responsible for significant global human morbidity, mortality and recurrent epidemics (YOUNG, 2018). The aforementioned viruses belong to neglected tropical diseases (NTD), which, although manageable, affect more and more of the world's population (FERANMI FOLAHAN, 2023). Low investment in the already fragile healthcare systems and a lack of potent vaccines or antiviral drugs make it difficult to control the spread of these infectious diseases (JONES et al., 2008; MAYER; TESH; VASILAKIS, 2017).

Acute symptoms of CHIKV infection include high fever, myalgia, arthralgia, skin rashes, nausea, headaches, intense asthenia and conjunctivitis. Eventually, CHIKV may also cause long-term debilitating conditions and complications, such as arthritis, meningoencephalitis, encephalopathy, hepatitis, myocarditis, and Guillain Barré syndrome (VAIRO et al., 2019; VAN DUIJL-RICHTER et al., 2015).

CHIKV is a positive sense RNA virus (12 kb) with two open reading frames that encode four nonstructural proteins (nsP1-nsP4) which are primarily responsible for viral replication,

transcription and host evasion, and five structural proteins (C – capsid, E3, E2, 6K/TF and E1 – envelope), which are encoded by subgenomic RNA in infected cells (BURT et al., 2017). Among the nonstructural proteins, nsP2 plays diverse roles in replication, propagation, and host gene expression shutdown, displaying proteolytic, NTPase and RNA helicase activities within its C- and N-terminal domains, making nsP2 a potential target for antiviral research (KHAN et al., 2021; STRAUSS, J.H.; STRAUSS, 1994).

Clinically approved vaccines or antiviral drugs are not yet available against CHIKV. Thus, disease prevention still relies solely on vector control and the treatment of symptoms by anti-inflammatory, analgesic, and antipyretic drugs. Globally, scientific efforts have focused on the discovery of novel antiviral drugs and repurposing existing drugs such as chloroquine (CHOPRA; SALUJA; VENUGOPALAN, 2014), ribavirin (BRIOLANT et al., 2004), favipiravir (PASTORINO et al., 2014), immunotherapy (WARTER et al., 2011), and other inhibitor molecules (DELANG et al., 2019), but further research is vitally important (DEEBA et al., 2016). In this context, Wedelolactone (WDL), a coumestan derived from *Eclipta prostrata* (*Asteraceae*), has demonstrated potential as an antioxidant (LI et al., 2020a), anti-inflammatory (KOBORI et al., 2004; MAO-MAO et al., 2019b; PAN et al., 2020; YUAN et al., 2013), hepatoprotective (QINGQIONG et al., 2018; XIA et al., 2013; ZHAO et al., 2015), and anti-cancer (BENES et al., 2011; CHEN et al., 2015; HSIEH et al., 2015; NEHYBOVA et al., 2015; SARVESWARAN et al., 2016; SARVESWARAN; GAUTAM; GHOSH, 2012) molecule, and also as an inhibitor of HIV-I (Human Immunodeficiency Virus type I) (TEWTRAKUL et al., 2007), hepatitis C virus RNA polymerase (MANVAR et al., 2012) and replication of HCMV (Human Cytomegalovirus) (SVRLANSKA et al., 2020). However, no studies have been reported on its activity toward CHIKV nsP2 nor CHIKV infection on cellular models.

Understanding how viruses hijack the host cellular machinery and how potential antiviral drugs affect cell metabolism when placed in the cellular environment is imperative. HR-MAS ¹H-NMR metabolomics permits the identification of metabolites with experimental reproducibility and relatively rapid sample preparation, which makes it well-suited for diverse solid/semi-solid sample types (EMWAS et al., 2019). The spectral data obtained represent unique chemical fingerprints that, combined with statistical methods, provide valuable information on metabolic alterations caused by different conditions, such as diseases, pathogens, and inhibitor molecules (LETERTRE; GIRAUDAU; TULLIO, 2021; VERMATHEN et al., 2017; WEYBRIGHT et al., 1998).

In the present study, we explored the inhibitory potential of wedelolactone against the nonstructural protein 2 of CHIKV as well as the metabolic disturbances in model mammalian

cultured cells (Vero E6—African green monkey kidney cells). Vero E6 cells are highly permissive and commonly used in viral propagation and viral assays, and while they lack interferon response genes, our primary objective was to explore the impact of the virus's presence and potential inhibitors on their metabolism. We explored the changes caused by both CHIKV and WDL using HR-MAS ¹H-NMR spectroscopy methods. We suggest that metabolomics studies of viruses and inhibitors may represent a rapid and efficient approach for unveiling viral and inhibitor mechanisms that serve as the basis for further investigation of metabolic pathways and drug development through omics integration.

MATERIALS AND METHODS

Cloning, expression and purification of CHIKV nsP2^{pro}

The codon-optimised cDNA encoding CHIKV nsP2^{pro} (GenBank Protein Accession number AAN05101.1, strain S27-African prototype) was cloned, expressed and purified as described previously (EBERLE et al., 2021, 2022).

Inhibition assay

The CHIKV nsP2 protease activity assay was performed as described previously (EBERLE et al., 2021, 2022), using DABCYL-Arg-Ala-Gly-Gly-Tyr-Ile-Phe-Ser-EDANS (BACHEM, Bubendorf, Switzerland) as substrate. The inhibition assay was performed in Corning 96-well plates (Merck, Darmstadt, Germany). An amount of 10 µM CHIKV nsP2^{pro} was incubated with 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25 and 50 µM WDL for 10 min at RT—the measurement started by adding the substrate with a final concentration of 5 µM. The fluorescence intensities were measured at 60 s intervals over 20 min at 37 °C using an Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). The excitation and emission wavelengths were 340 nm and 490 nm, respectively.

Activity and inhibition of the investigated protease was calculated using Equation (1):

$$\% \text{ protease activity} = (\text{Intensity of enzyme activity} - \text{intensity left after inhibition}) / \text{Intensity of enzyme activity} \quad 1)$$

The experiments were performed in triplicate and the results are shown as mean ± standard deviation (SD). Each experiment was performed with freshly purified protein. The half-maximal inhibitor concentration (IC₅₀) value of WDL was calculated using GraphPad

Prism 5 software (San Diego, CA, USA).

Cell, virus and reagents

Vero E6 cells (African green monkey kidney-derived cells - *Cercopithecus aethiops*, clone E6, ATCC C1008) were grown and maintained at 37 °C, and 5% CO₂ in a humidified incubator in Eagle's Minimum Essential Medium (MEM - Gibco, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS - Gibco, Waltham, MA, USA) and addition of 100 U/mL of penicillin (Hyclone Laboratories, United States), 0.1 mg/mL of streptomycin and 0.5 µg/mL of amphotericin B (Gibco, Waltham, MA, USA). CHIKV [BHI3762/H804917 access number (NUNES et al., 2015)] stocks were propagated and titrated in Vero cells using plaque-forming units assay (PFU). Wedelolactone ("WDL", 7-methoxy 5,11,12-trihydroxy-coumestan - Sigma Aldrich, MA, USA, purity ≥ 98%) was dissolved in DMSO (dimethyl sulfoxide – 10 mg/mL, Merck, NJ, USA) and used for the cell viability and antiviral assays, as well as in HR-MAS ¹H NMR sample preparation.

Cytotoxicity assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Aldrich, MA, USA) assay according to Mosmann (MOSMANN, 1983) with slight alterations. Vero E6 cells at a 10⁴ density were grown in 96-well plates and treated with different concentrations of WDL ranging from 50 to 400 µM – for 48 h at 37 °C with 5% CO₂, and DMSO (Dimethyl sulfoxide - Merck) was used as vehicle control. After this period, molecule-containing media was removed, and MTT solution at 1 mg/mL was added to each well, incubated for 1 h and later replaced with 100 µL of DMSO for formazan crystals solubilisation. Absorbance was measured at 550 nm by a Spectramax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The CC₅₀ (cytotoxic effects on 50% of cultured cells) was defined as the concentration that reduced the viability of cells to 50% when compared to controls. The MTT assay was performed in triplicate, and the results calculated in GraphPad Prism 8 software (San Diego, CA, USA) using a normalised dose-response curve fitting.

Virus infection and titration

A CHIKV stock was used to infect Vero E6 cells on a T-75 culture flask (ThermoFisher) at 80% confluency. To determine the viral titer, we seeded 10⁵ Vero E6 cells in each well of a 24-well plate 24 h prior to infection. Cells were then infected with 10-fold serial dilutions of CHIKV and incubated at 37 °C for 1 h with gentle agitation every 15 min. The virus inoculum

was removed, overlay media containing 4% v/v carboxymethylcellulose (CMC – Synth, Sao Paulo, Brazil) and 1% FBS were added to the wells, followed by incubation for two days at 37 °C and 5% CO₂ in a humidified incubator. Finally, cells were fixated with 10% formaldehyde and stained with 4% violet crystal, and viral plaques were counted to determine the virus titer, expressed as plaque-forming units, PFU/mL.

Antiviral in vitro activity assay

A post-infection treatment assay was performed to evaluate the in vitro antiviral activity of WDL. A 48-well tissue culture plate with 6×10^4 Vero E6 cells/well was prepared and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. After that, media were removed, and cells were infected with CHIKV at a MOI (multiplicity of infection) of 0.1 and incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO₂. The inoculum was removed, five different diluted concentrations of WDL in MEM 1% FBS were added (30, 20, 15, 10 and 5 µM) to the wells and cells were incubated for a 24 h period at 37 °C in a humidified atmosphere with 5% CO₂. Supernatants were collected, and viral titration was performed and revealed through the plaque formation assay to evaluate plaque reduction and antiviral activity. The assay was performed in triplicate, and data were analysed by a four-parameter dose-response curve fitting using GraphPad Prism 8 software (San Diego, CA, USA).

HR-MAS ¹H-NMR sample preparation

An amount of 6×10^6 Vero E6 cells were seeded in 100 mm culture dishes 24 h prior to infection and treatment. Six independent samples of each of the following treatment conditions were produced: healthy cell control (CC), cell-WDL (CW), cell-CHIKV (CV) and cell-WDL-CHIKV (CWV).

First, cells were infected with CHIKV at 0.1 MOI (as we identified high cell death rates with higher MOIs) and incubated for 1 h at 37 °C and 5% CO₂ with gentle agitation every 15 min. The virus inoculum was removed, and MEM 1% FBS was added to the CC and CV conditions. On the other hand, for the CW and CWV conditions, MEM supplemented with 1% FBS and the established diluted concentration of WDL (30 µM) was added. Cells were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. Culture media were removed, and cells were washed twice with cold phosphate-buffered saline (PBS) and harvested by trypsinisation, and a subsequent addition was made of cold MEM supplemented with 10% FBS for trypsin neutralisation and collection of cells. Centrifugation was performed, and the supernatant was removed and discarded. The cell pellets were then washed with cold PBS,

followed by another centrifugation step and complete removal of the supernatant for subsequent HR-MAS ^1H -NMR analysis.

HR-MAS ^1H -NMR and 2D analysis

All HR-MAS ^1H NMR spectra were acquired on a Bruker Avance spectrometer (Bruker Biospin, Germany) operating at 400 MHz for ^1H , equipped with a triple nuclei 4-mm g-HR-MAS 400 SB BL4 probe, and performing at a magic-angle spinning frequency of 3.5 kHz and 298 K. Cell pellets were resuspended in 12 μL D_2O (deuterated water) for the experiments. One-dimensional water-suppressed ^1H -NMR spectra were acquired with `noesygppr1d` (NOESY 1D) pulse sequence. T₂-edited spectra were obtained using `cpmgpr1d` (Carr-Purcell-Melboom-Gill, CPMG) pulse sequence, both with 256 transients and recycling delay of 4 s. Two-dimensional ^1H - ^1H T_{OT}al Correlation SpectroscopY (TOCSY) experiments were recorded using `mlevgpphw5` pulse sequence, $n_s = 110$, mixing time = 60 ms, and ^1H - ^{13}C Heteronuclear Single Quantum Coherence (HSQC, `hsqcetgpprsisp2.2` pulse sequence, $n_s = 384$) experiments were also performed to randomly selected samples.

Data pre-processing and analysis

The HR-MAS ^1H -NMR cell spectra obtained were manually processed for phase and baseline corrections using MestReNova 12.0 software. Lactate (3H, δ 1.33, $J = 7.0$ Hz, doublet) was used as a chemical shift reference. The spectra were binned (0.005 ppm) and exported as csv matrices. Spectra regions below δ 0.5 and water region (δ 4.55–5.10) and above δ 8.7 were excluded from the analysis. Normalisation to a constant sum (100) of the spectra's intensities was performed to reduce concentration differences. Assignment of the metabolites was based on chemical shifts, correlation assignments (TOCSY and HSQC) and databases, such as the Human Metabolome Database (WISHART et al., 2007) and Biological Magnetic Resonance Data Bank (ULRICH et al., 2008).

All statistical analyses were performed using the MetaboAnalyst online platform (PANG et al., 2021). Multivariate analyses were performed using the matrices with a total of 810 variables for the parts of the aliphatic regions of water suppression (`noesy1d`) and T₂-edited (CPMG) spectra and constructed for all conditions ($\text{CC} \times \text{CW} \times \text{CWV} \times \text{CV}$, with 24 spectra in total), as well as matrices containing each condition compared to the healthy control cells (CC), namely, $\text{CC} \times \text{CW}$, $\text{CC} \times \text{CWV}$, $\text{CC} \times \text{CV}$, and also compared to the viral control ($\text{CV} \times \text{CWV}$). Data were normalised by median and autoscaling.

Principal component analyses (PCA) were performed for all spectra (all conditions,

separately for data obtained by the noesygppr1d and cpmgpr1d pulse sequences) to explore intrinsic groupings within samples, reduce data dimensionality and identify outliers (REN et al., 2015). Partial least squares discriminant analysis (PLS-DA) models were generated to classify samples from the four conditions evaluated together and compared to the healthy control (CC) and identify the essential variables (BARKER; RAYENS, 2003; REN et al., 2015). Model validation was assessed by leave-one-out cross validation (LOOCV) to evaluate its accuracy and performance, and variable of importance in projection (VIP) scores were analysed to identify the most different chemical shifts among samples. The validated models' accuracy, specificity and sensitivity values were computed (BERRUETA; ALONSO-SALCES, 2007). Univariate analysis was performed to confirm the discriminant metabolites identified by multivariate analysis (PLS-DA) between two conditions (CC × CV; CC × CW; CC × CWV; CV × CWV) and for visualisation of the relative metabolite levels between the groups, measured as peak intensities, using a *t*-test with $p < 0.05$ as the threshold for statistically significant difference.

RESULTS

In vitro inhibition assay of the CHIKV ns2 protease (nsP2) by Wedelolactone

Experiments employing a wide range of WDL concentrations (ranging from 0 to 50 μM) were performed, and the titration of the molecule demonstrated 100% protease inhibition at 25 μM WDL. Using the titration data, the half-maximal inhibitory concentration (IC_{50}) of WDL toward nsP2 was determined to be $2.3 \pm 0.4 \mu\text{M}$ (Figure 1). This value is in the same range as previously reported flavonoids and small molecules targeting CHIKV nsP2: hesperetin (2.5 μM) and hesperidin (7.1 μM) (EBERLE et al., 2021) and 1,3-thiazolbenzamide derivatives

(between 8.3 and 13.1 μM) (IVANOVA, et al., 2021).

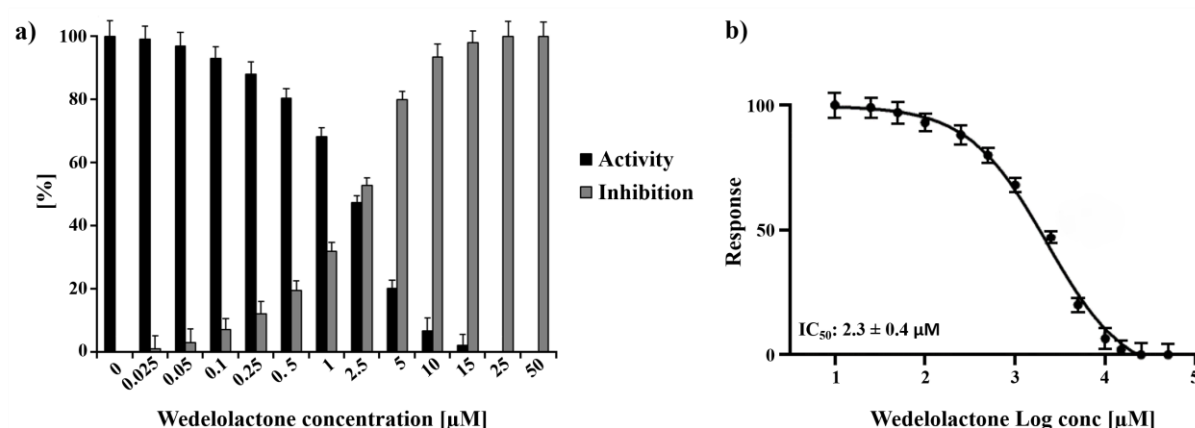


Figure 1. Normalised activity and inhibitory effects of WDL on nsP2 protease. (a) Inhibition of nsP2 by WDL with the concentrations ranging from 0 to 50 μM . (b) Dose response curve of WDL and nsP2 depicting the the half-maximal inhibitory concentration (IC_{50}) of $2.3 \pm 0.4 \mu\text{M}$. The experiments were performed as triplicates and the results are shown as mean $\pm$ standard deviation (SD).

Assessment of Wedelolactone cytotoxicity in Vero E6 cells

A cytotoxicity assay of WDL was performed on Vero E6 cells to obtain the CC_{50} value. Cells were treated with different concentrations of WDL and DMSO (negative control) for an incubation period of 48 h. Living cells were quantified through the MTT assay by assessing the production and solubilisation of formazan crystals (KUMAR; NAGARAJAN; UCHIL, 2018). The results yielded a CC_{50} value of $373.5 \pm 154.2 \mu\text{M}$, calculated using GraphPad Prism (8.0) from a normalised dose–response curve fitting (Figure 2). Cell viability was also calculated for

each concentration used and is available at Table S1.

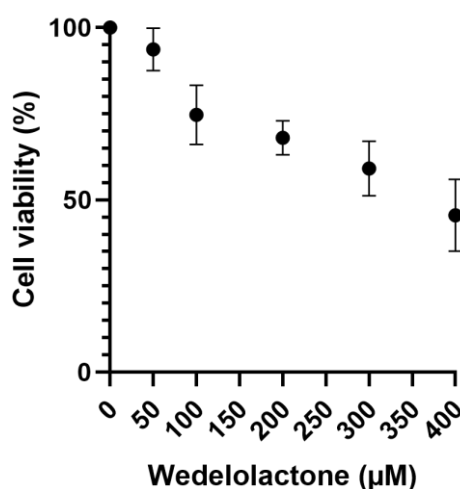


Figure 2. Vero E6 cell viability MTT assay using a range of WDL concentrations from 50 to 400 μM where live cells were quantified. Cell viability percentages are shown on the y axis, while WDL concentrations (μM) are on the x axis. Error bars represent standard deviations. Values are the mean $\pm$ standard error obtained from three independent experiments.

Effects of Wedelolactone on CHIKV-infected cells

To evaluate the *in vitro* inhibitory potential of WDL on CHIKV infection, we treated infected cells with different concentrations of WDL (5, 10, 15, 20 and 30 μM) based on our nsP2 inhibition assay results. This was performed to evaluate the antiviral effects of the molecule on postentry steps, including replication, virion assembly and release, as these were the focus of our metabolomics analyses conducted during the postinfection period. After a 24 h period postinfection and treatment, supernatants were collected, and viral titration was performed. We observed a slight decrease (ANOVA, Dunnett's test, $p < 0.0001$) in viral titres with concentrations of 20 μM and 30 μM , with 0.44 and 0.45 Log_{10} units decreases in viral titres, respectively, compared to the viral progeny titres in controls (6.92 Log_{10} units, corresponding to approximately 10^7 PFU/mL); see Figure 3. The IC_{50} for WDL in this assay

was calculated and determined as $22.9 \pm 6.7 \mu\text{M}$.

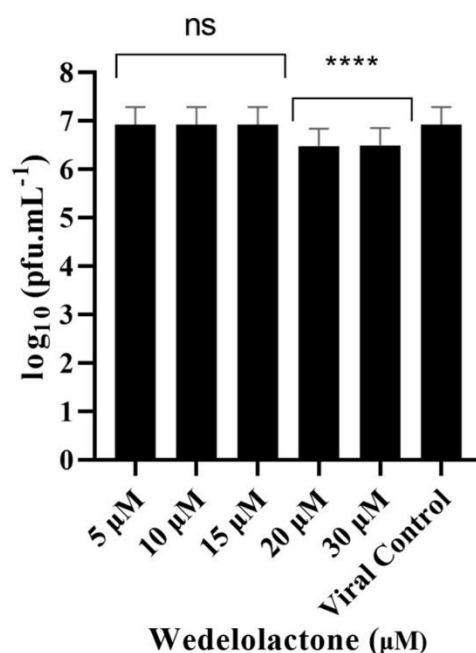


Figure 3. Dose-dependent inhibitory effects of WDL on CHIKV titre yields. Cells were infected with CHIKV at MOI of 0.1 and treated with different concentrations of WDL ranging from 5 μM to 30 μM . The reduction in the viral titre was determined by plaque-forming assay after collection and titration of the supernatants 24 h postinfection and treatment. PFU infectivity titration of CHIKV is shown on the left vertical axis. Error bars represent standard deviations. Values are the mean $\pm$ standard error obtained from three independent experiments. Asterisks indicate statistical significance between the control and each group as determined by two-way ANOVA and subsequent Dunnett's test (ns, not significant; ****, $p < 0.0001$).

HR-MAS NMR spectroscopy applied to the analysis of Vero E6 cells

Twenty-nine metabolites were assigned in the ^1H -NMR spectra of the Vero E6 cell control group (Figure 4, Table S2). The 2D-HSQC (Figure S2) and 2D-TOCSY experiments were used to identify and assign the metabolites accurately.

Representative NMR spectra of each group with identified metabolites are shown in Figure S1. The T_2 -edited (CPMG) ^1H NMR spectra highlighted the most relevant differences

in the aliphatic region (0.5–4.55 ppm).

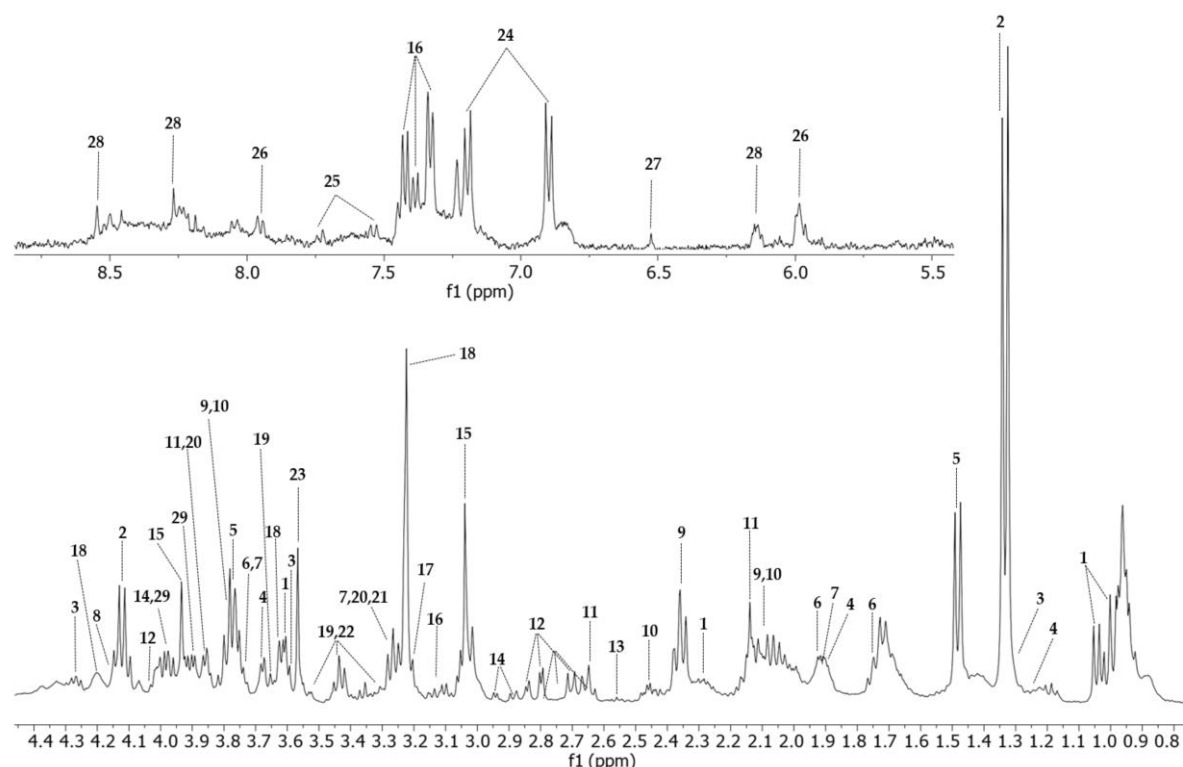


Figure 4. HR-MAS ^1H -NMR T_2 -edited (CPMG) spectra (400 MHz) of all four conditions. Vero E6 control sample is represented in green (CC), cells treated with WDL (CW) are in blue, cells infected and treated with the molecule (CWV) are represented in purple and CHIKV-infected cells are represented in red (CV). Identified metabolites are Val (valine); Lac (lactate); Iso (isoleucine); Pro (proline); Met (methionine); Asp (aspartate); Cre (creatine); Phe (phenylalanine); Pho (phosphocholine); Glu (glucose); Bet (betaine); Myo (myo-inositol). HR-MAS ^1H -NMR with T_2 filter spectrum of a Vero E6 cell sample acquired with a Bruker Avance III 400 MHz using a CPMG (*cpmgpr1d*) pulse sequence. The upper image of the spectrum comprises the region between 5.0 and 9.00 ppm, amplified 20 times, and the lower image shows the 0.80 to 4.55 ppm region. All peaks assigned to a particular metabolite are numbered. 1: valine; 2: lactate; 3: threonine; 4: isoleucine; 5: alanine; 6: lysine; 7: arginine; 8: proline; 9: glutamate; 10: glutamine; 11: methionine; 12: aspartate; 13: hypotaurine; 14: asparagine; 15: creatine; 16: phenylalanine; 17: choline; 18: phosphocholine; 19: glucose; 20: betaine; 21: trimethylamine-N-oxide; 22: myo-inositol; 23: glycine; 24: tyrosine; 25: tryptophan; 26: UDP-N-acetylglucosamine, UDP-glucose, UDP-glucuronate; 27: fumarate; 28: ATP; 29: serine.

Chemometric analysis

We first used an unsupervised discriminant model (PCA) to explore intrinsic groupings

and reduce dimensionality of datasets from the different conditions. The clustering tendency between the control samples and the other groups reflects the overall changes affected by infection or the inhibitor (Figure S3). The postscattering between the first two components of NMR data of CC $\times$ CW, PC1 (27.2%) and PC2 (18.7%), presented a net variance of 45.9% (Figure S3a). The other groups presented a net variance of 56.1% (CC $\times$ CV), 60.9% (CC $\times$ CWV) and 60.3% (CV $\times$ CWV) (Figure S3b–d), which indicates significant variability before and after the infection and following the addition of the WDL. PCA analysis was performed on the data matrices of all groups, indicating that the control cells and cells treated with the inhibitor (CC and CW) were similar among themselves, and as expected, similarities among the infected cells (CV and CWV) were also observed (Figure S4a).

To focus our attention on the influence of the virus and the inhibitor on the metabolomic profiles of the Vero E6 cells, we assessed the significance of the results using a supervised PLS-DA model. As presented in Figure 5a–d, the PLS-DA class discrimination model maximised the difference between the groups and obtained better separation effects than PCA, demonstrating substantial differences in cellular metabolism among the analysed conditions and presenting adequate cross-validation results of the model through values of accuracy, R^2 and Q^2 (Table 1).

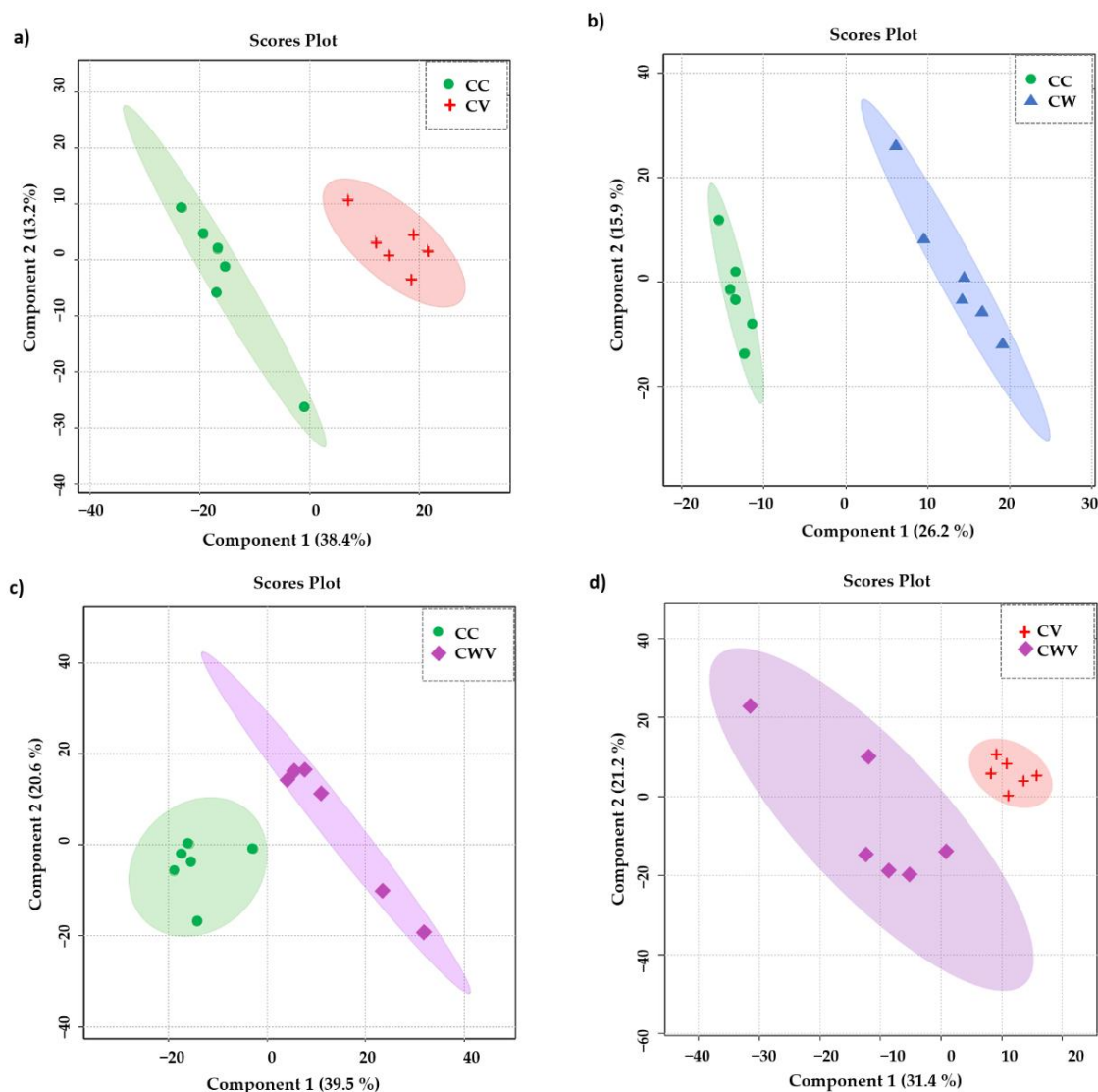


Figure 5. PLS-DA scores plot in 2D of the HR-MAS ^1H -NMR data showing the discrimination between the healthy cell control (CC, green circles) and the other conditions: **(a)** CC × CV (red crosses); **(b)** CC × CW (blue triangles); **(c)** CC × CWV (purple diamonds); **(d)** CV × CWV. The explained variance of the models with the two first latent variables is represented on the x and y axes.

Table 1. Accuracy, R^2 and Q^2 values of the best performance PLS-DA models calculated by LOOCV.

Classes	Accuracy	R^2	Q^2
CC × CV	0.91	0.99	0.78
CC × CW	1.0	0.99	0.90
CC × CWV	1.0	0.99	0.85
CV × CWV	0.91	0.99	0.72
CC × CV × CW × CWV	0.87	0.98	0.85

Metabolite discrimination

The most significant variables contributing to class discrimination in the PLS-DA models were identified through VIP graphs. Metabolites with VIP scores > 1.5 in the PLS-DA model (Figure S5) and $p < 0.05$ in the t -test were selected as differential metabolites. Box and whisker plots show relative concentrations for the most significantly altered metabolites ($p < 0.05$) in cell control (CC, green) and CV (red), CW (blue) and CWV (purple) (Figure 6). False discovery rate (FDR) estimates and the p -value threshold are presented in Tables S3–S6.

Six of the twenty-nine identified metabolites showed significant differences between CC × CV; they are proline (δ 4.14), phosphocholine (δ 4.22), myo-inositol (δ 3.52), lactate (δ 1.33), valine (δ 2.28) and phenylalanine (δ 3.11) (Figure 6a). The cells treated with WDL (Figure 6b) presented differences in four metabolite levels: glucose (δ 3.54), betaine (δ 3.89), isoleucine (δ 3.67) and aspartate (δ 2.68). The corresponding CWV samples showed similar tendencies with five significant metabolite differences between the compared groups (CC × CWV): methionine (δ 3.85) lactate (δ 1.33), proline (δ 4.14), betaine (δ 3.89) and aspartate (δ 2.68) (Figure 6c). Lastly, we compared the infected cells (CV) with the infected WDL-treated cells (CWV), and the most significant metabolites were methionine (δ 3.85), glucose (δ 3.54), aspartate (δ 2.68), myo-inositol (δ 3.52), creatine (δ 3.93), proline (δ 2.36) and lactate (δ 1.33) (Figure 6d).

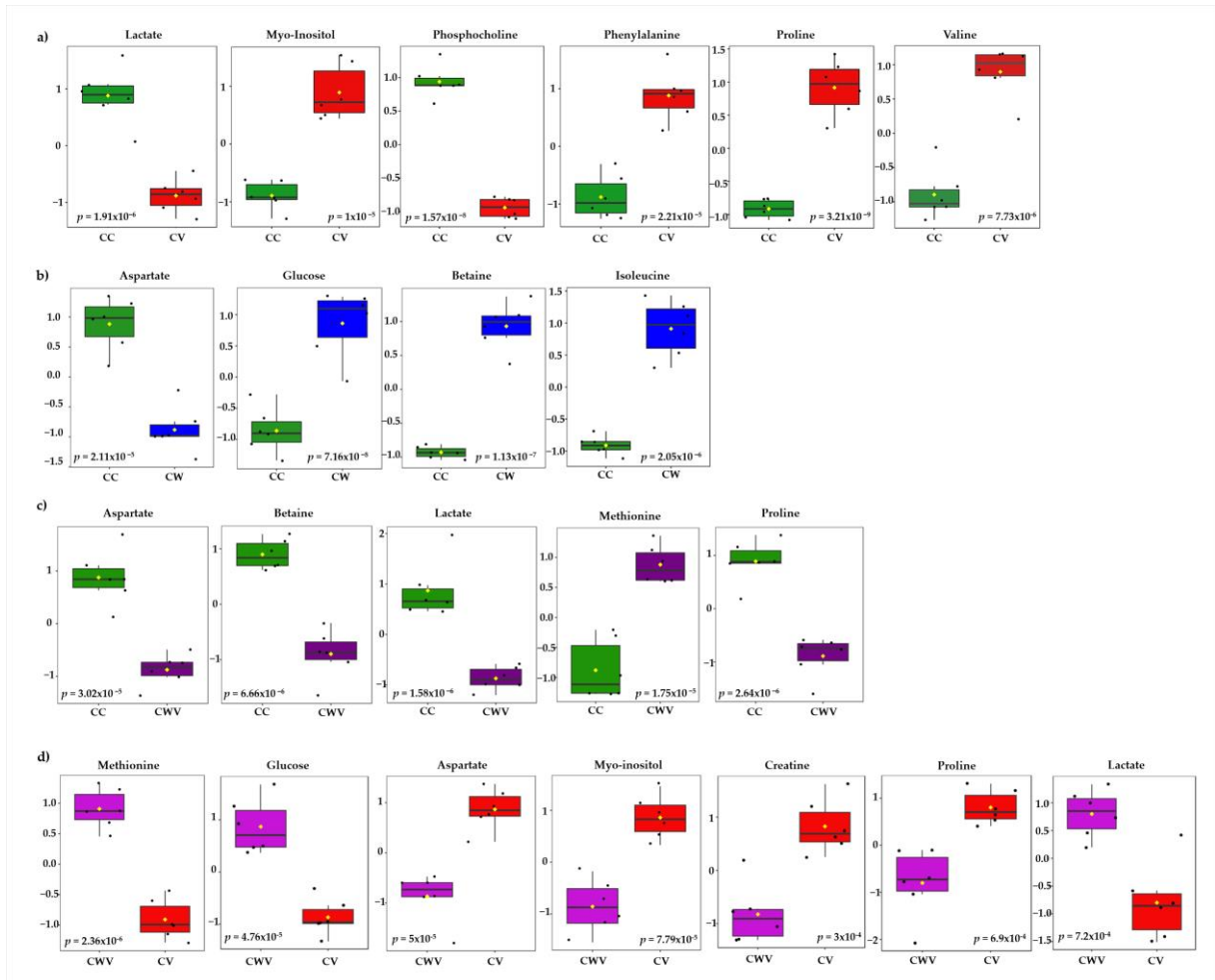


Figure 6. Box plots of discriminant metabolites identified in the univariate analysis (t -test, $p < 0.05$) of the (a) CC $\times$ CV, (b) CC $\times$ CW, (c) CC $\times$ CWV, (d) CV $\times$ CWV groups. Relative metabolite levels between the groups are shown (measured as peak intensities). The black dots represent metabolite levels in the samples, and the yellow diamond represents the average value for the group, as indicated on the y axis. Groups are CC, green; CV, red; CW, blue; CWV, purple.

Heatmap analysis of metabolites

Hierarchical clustering analyses (HCA) of the PLS-DA data were performed to explore the substantial changes in each metabolite among the groups. The heat maps visually present the relative increase or decrease in differential metabolite levels in Vero E6 cells in each

condition described in the previous analyses (Figure 7).

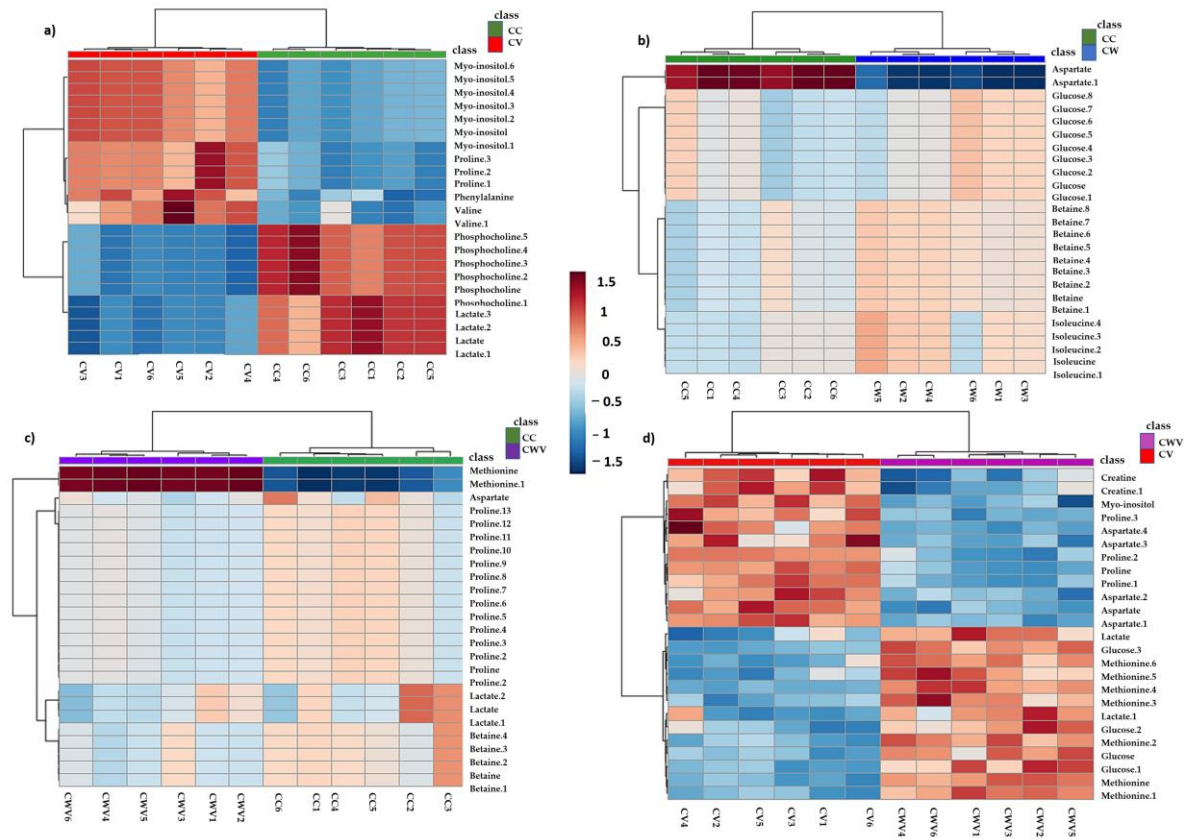


Figure 7. Hierarchical clustering through heat maps analysis using Euclidean distance. The lines represent the discriminant variables from the PLS-DA analysis for (a) CC $\times$ CV, (b) CC $\times$ CW, (c) CC $\times$ CWV and (d) CV $\times$ CWV. The columns represent the samples, and the colour bars on the right represent the relative concentrations of the discriminant metabolites (red: higher relative concentration; blue: lower relative concentration).

The heat map displayed significant differences and clustering of groups along with differences in metabolites' relative concentrations, demonstrating the overall metabolic variation in the different conditions. In CHIKV-infected cells, levels of myo-inositol and amino acids (proline, phenylalanine and valine) increased, while decreased levels of phosphocholine and lactate were observed (Figure 7a). Cell treatment with WDL decreased relative levels of aspartate, whereas levels of glucose, betaine and isoleucine increased (Figure 7b). Furthermore, the CHIKV-infected cells treated with WDL showed increased methionine levels, while aspartate, betaine, lactate and proline showed lower intensities (Figure 7c). When comparing the CV $\times$ CWV results, we observed a decrease in metabolites that were increased during viral infection (CV); they were myo-inositol and proline. Moreover, similar to the CW samples, an increase in glucose, methionine and lactate and decreased aspartate levels were observed.

Interestingly, here we also identified an alteration in creatine, which presented higher levels in CHIKV-infected cells and lower levels when the infected cells were treated with WDL (Figure 7d). Figure S4b shows the heat map with all conditions, and the metabolic alterations will be discussed further.

DISCUSSION

Our results showed that WDL possesses an inhibitory potential towards CHIKV nsP2 protease in vitro in the low μM concentration range (IC_{50} value of $2.3 \pm 0.4 \mu\text{M}$). Our value is similar to IC_{50} values reported previously for flavonoids targeting CHIKV nsP2 (HST: $2.5 \pm 0.4 \mu\text{M}$, HSD: $7.1 \pm 1.1 \mu\text{M}$) (EBERLE et al., 2021). In contrast, we observed a slight antiviral effect in CHIKV-infected Vero cell cultures. A major problem of the development and discovery of potent antiviral molecules is an efficient cellular delivery; molecules with a poor permeability possess a reduced bioavailability (ZHANG et al., 2019a). Additionally, the molecules can be stored in cellular compartments where they are not available for the virus target proteins. However, the treatment of CHIKV-infected Vero cells with WDL demonstrated metabolic alterations that we could observe through our metabolomic experiments.

As virus formation depends on the host cell's metabolic capacity to provide the required low-molecular-weight metabolites (nucleotides, amino acids and fatty acids (FAs)/lipids) and energy in favour of viral infection, the current study speculates about the metabolic changes of Vero E6 cells induced by CHIKV infection and the inclusion of an nsP2 inhibitor (WDL) exclusively through the HR-MAS ^1H NMR method.

In our findings, we observed a significant reduction in lactate production in infected cells (CV) when compared to control cells (Figure 6a). This stands in contrast to the findings in MAYV-infected cells, where lactate production exhibited an increase 8–10 h postinfection (EL-BACHA et al., 2004), suggesting a dynamic shift in glycolytic activity throughout the infection process. The sustained decrease in lactate production over a prolonged period (24 h postinfection) leads us to hypothesise that glucose may be metabolised into pyruvate, subsequently entering the TCA cycle. Alternatively, it is plausible that lactate is repurposed as a gluconeogenic agent, serving as a vital energy source (BROOKS, 2018). It is important to emphasise the role of the TCA cycle in synthesising amino acids, lipids, nucleotides (purine and pyrimidines) and nucleic acids (DEBERARDINIS; CHENG, 2010; MULUKUTLA et al., 2010; NEWSHOLME et al., 2003; POLCICOVA; BADUROVA; TOMASKOVA, 2020; SA; ANGE; RODR, 2021), and its significant contribution to ATP synthesis, which is crucial to meet the high-energy requirements during infection. Additionally, overall evidence of

glycolysis requirement for efficient replication of alphaviruses has been reported for Semliki, Sindbis and Mayaro viruses, where glycolysis inhibitors substantially diminish viral yields, indicating that glycolysis plays a pivotal role during infection (EL-BACHA et al., 2004; FINDLAY; ULAETO; FINDLAY, 2015).

In cultured cells, amino acids from culture media are assimilated and synthesised to form the necessary pools crucial for sustaining normal metabolism, biosynthetic activities and overall cellular homeostasis, and they also play a pivotal role during viral infection (BASTIN; CHOTTEAU; WOUWER, 2021; EAGLE, 1955; THAKER; CH; CHRISTOFK, 2019). Accordingly, our results showed that valine, phenylalanine and proline were increased during CHIKV infection. These findings demonstrate broad alterations in glycolysis and TCA cycle metabolism to actively support and facilitate the infection process (NELSON; COX, 2017) (Figure 6a and Figure 8).

We also observed elevated levels of myo-inositol in response to CHIKV infection (Figures 6a and 8). Myo-inositol serves as a major component of phospholipids and inositol phosphate derivatives, such as phosphatidylinositol (HOLUB, 1986). Beyond its primary role in sensing metabolic shifts and energy demands through inositol phosphate signalling pathways (TU-SEKINE; KIM, 2022), there is a presumed involvement in the assembly and maintenance of the integrity of replication complexes along with recruitment of host factors or viral components, as described by Zhang and collaborators (ZHANG et al., 2019b).

Viral infection also regulates cellular lipid metabolism (CHUKKAPALLI; HEATON; RANDALL, 2013). Accordingly, our results demonstrated perturbation of phospholipid metabolism with decreased phosphocholine levels during infection (Figures 6a and 8). Choline and phosphocholine are substrates for the synthesis of phosphatidylcholine (PC), which constitutes approximately half of the phospholipid species in most mammalian cells (VANCE, 2015) and is a biosynthetic precursor for lipid-signalling molecules and lipoproteins (LI; VANCE, 2008). PC synthesis is enhanced during infection of numerous viruses, including Dengue virus (PERERA et al., 2012), poliovirus (VANCE; TRIP; PADDON, 1980) and Brome mosaic virus (BMV), with a high accumulation of PC and choline metabolism enzymes for PC synthesis within the viral replication sites to sustain viral replication complexes (VRCs) and stimulate viral replication (ZHANG et al., 2016a).

The relevance of understanding the metabolic changes induced by a CHIKV nsP2 protease inhibitor may favour future studies on drug development. The inhibitor is introduced into an intricate environment and undergoes metabolisation by cells. In this condition (CW), alterations in central energy metabolism were observed upon WDL addition. The reduction in

aspartate, a critical building block for several biomolecules and a glucogenic amino acid within the TCA cycle (HOLEČEK, 2023), may reflect the promotion of gluconeogenesis upon the addition of WDL, a phenomenon further supported by the elevated glucose levels (Figures 6b and 8).

Metabolic changes upon adding WDL (CW) also indicates higher levels of betaine (Figures 6b and 8). Betaine, derived from choline oxidation, is an essential osmoregulatory metabolite and a cofactor of methylation during the methionine–homo-cysteine cycle (KIM et al., 2011; CRAIG, 2004). Interestingly, betaine acts as an antiviral and anti-inflammatory compound that may exert functions through epigenetic regulation and mitigation of oxidative and ER (endoplasmic reticulum) stress and cell apoptosis (ZHANG et al., 2016b). A notable decrease in betaine levels followed by elevated levels of the sulphur-containing amino acid methionine were observed in our CC × CWV analysis (Figure 6c). As previously mentioned, betaine serves as a methyl donor for methionine synthesis through the activity of the betaine homocysteine S-methyltransferase enzyme (HARRIS; FINHELSTEIN; KYLE, 1972). The subsequent steps in the methionine cycle generate a pivotal methylating agent, SAM (S-adenosylmethionine), a cofactor essential for various cellular processes (LAUINGER; KAISER, 2021) (Figure 8). Both methionine and SAM can be helpful in oxidative stress conditions, as induced by CHIKV infection (JOUBERT et al., 2012), and the betaine-methionine interaction exerts an antioxidant function by maintaining cellular SAM:SAH ratios through increased methionine and SAM synthesis (WANG et al., 2019; ZHANG et al., 2016b). Furthermore, methionine and SAM synthesis are inducers of anabolic pathways such as amino acid and nucleotide biosynthesis to support cells under nutritional stress conditions (i.e., viral infection), which might also be correlated to the presence of WDL in the cellular environment during infection (WALVEKAR et al., 2018).

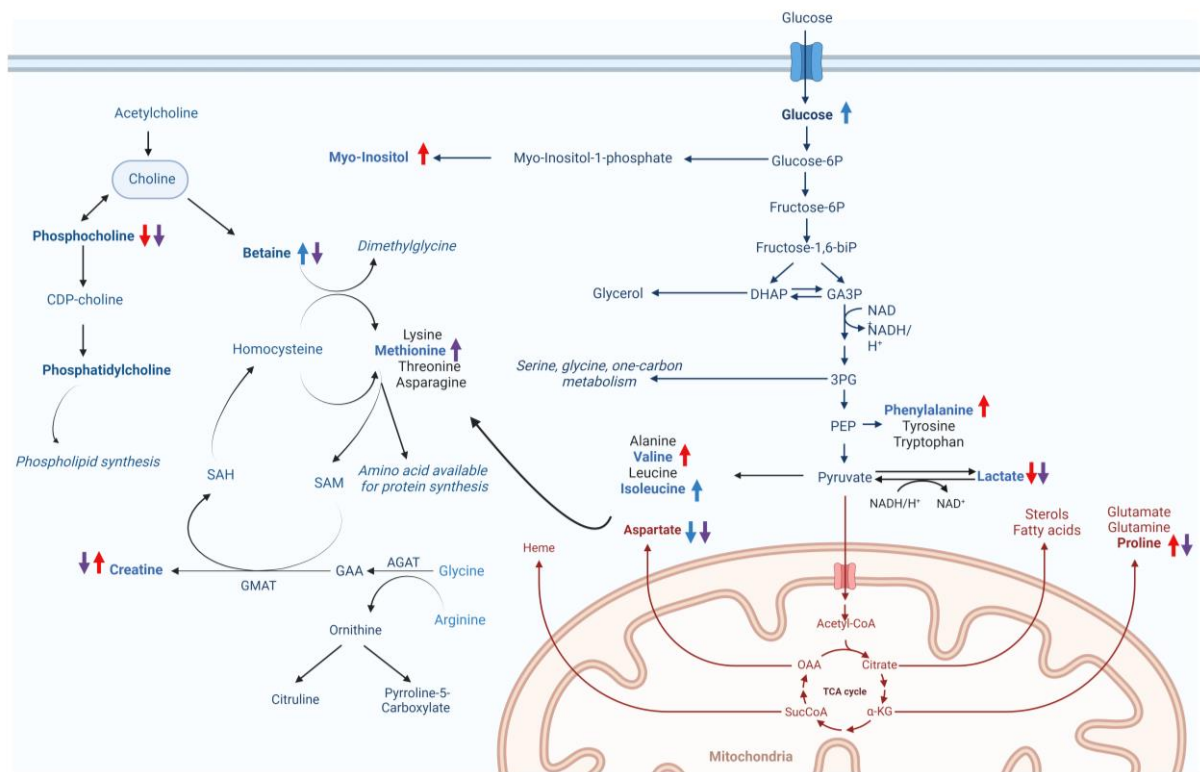


Figure 8. Hypothesised changes to metabolic pathways in Vero E6 cells caused by CHIKV infection and/or WDL treatment. The coloured arrows indicate increased (upwards) or decreased (downwards) metabolite levels for the different conditions analysed: CV (red), CW (blue) and CWV (purple).

WDL consistently appears in the literature as possessing antioxidant effects, demonstrated by its ability to diminish levels of reactive oxygen species (ROS) and mitigate oxidative stress damage (MAO-MAO et al., 2019a), promoting free radical scavenging (LI et al., 2020b) and alleviating oxidative stress and mitochondrial dysfunction (SHARMA et al., 2021). Due to the limitations of our study, further research is necessary to elucidate these effects and mechanisms and investigate whether WDL might play a role against oxidative stress and cell damage caused by CHIKV infection.

Lastly, overall metabolic alterations in the CV × CWV analysis represented similar changes induced by the molecule in the CC × CW conditions, possibly enhancing energy production even amidst viral infection. Nevertheless, we noted decreased levels of myo-inositol and proline, aligning with diminished viral production. This observation contrasts with the observed increase in these metabolites to support viral infection, as previously discussed in the CC × CV results. Interestingly, exclusively in this analysis, creatine was found to be

significantly decreased in CWV samples (Figure 6d). Creatine synthesis requires glycine, arginine and methionine (WYSS et al., 2000), and creatine and phosphocreatine are known to regulate mitochondrial ATP synthesis during high-energy-demanding periods to help maintain ATP efficiency in cells (BONILLA et al., 2021; BROSNAN; BROSNAN, 2007; HARA et al., 2009). Furthermore, it was elucidated that creatine kinase B (CKB), a pivotal ATP-generating enzyme responsible for modulating ATP levels within subcellular compartments, plays a crucial role in facilitating the efficient replication of the HCV genome and the subsequent propagation of the infectious virus (HARA et al., 2009). Specifically, the recruitment of CKB to the HCV replication complex (RC) compartment, characterised by high and fluctuating energy demands, is paramount for the optimised replication of the viral genome. This recruitment is facilitated through its interaction with NS4A, underscoring the significance of this molecular interplay in the intricate process of viral replication (HARA et al., 2009). Based on these findings, we speculate that the decreased levels observed in CWV when compared to the CV samples may be linked to diminished energetic demands, a consequence of the lower yields in viral progeny.

CONCLUSIONS

With recurrent epidemics worldwide and the risk of developing long-term complications in human hosts, the effects of arboviral infections by Chikungunya and other viruses are concerning. To date, no licensed antiviral drugs or vaccines are available. Although studies have provided numerous promising results in drug and vaccine development, research contributing to understanding viral mechanisms is crucial.

In this study, we aimed to explore the potential of HR-MAS ¹H NMR to understand the metabolic effects of viral infection and viral inhibition by WDL with in vitro and metabolomic assays. Our results demonstrate that WDL inhibits viral nsP2 protease with considerably low IC₅₀ values. At the same time, in cell culture, a much more complex environment, WDL slightly impairs the post-viral-entry steps in Vero E6 cells in a dose-dependent manner, where, perhaps, higher dosages of the molecule might have been more efficient in inhibiting viral replication in vitro, which were limitations in our study.

To date and to our knowledge, there are no HR-MAS NMR metabolomic studies regarding arboviruses of the *Togaviridae* family. HR-MAS NMR and chemometric analysis tools allowed us to trace a metabolic profile of Vero cells, along with metabolite disturbances triggered by Chikungunya virus infection, which mainly affected central energy, amino acid and phospholipid metabolism. We could also observe that WDL contributes to glucose synthesis

in Vero cells. During infection, it might support anabolic pathways, lowering oxidative stress and cell damage, although it is speculative and provides further insights into future studies regarding these allegations.

Acknowledgements

Special thanks for the support of the Institute of Biological Information Processing (IBI-7) Forschungszentrum Jülich, Germany, to FAPESP (Project Nr. 2020/03639-6; 2020/12875-5; 2023/09590-7; 2017/13341-1; 2018/15083-2; 2013/21719-3; 2018/24069-3; 2018/06510-4; 2020/08615-8) and CNPq (Project Nr. 309940/2019-2). Thanks for the partial support by the Centers for Research in Emerging Infectious Disease “The Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics (CREATE-NEO)” grant U01AI151807 awarded by the National Institutes of Health (NIH/USA) and INCT Viral Genomic Surveillance and One Health (grant 405786/2022-0).

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SUPPLEMENTARY MATERIAL

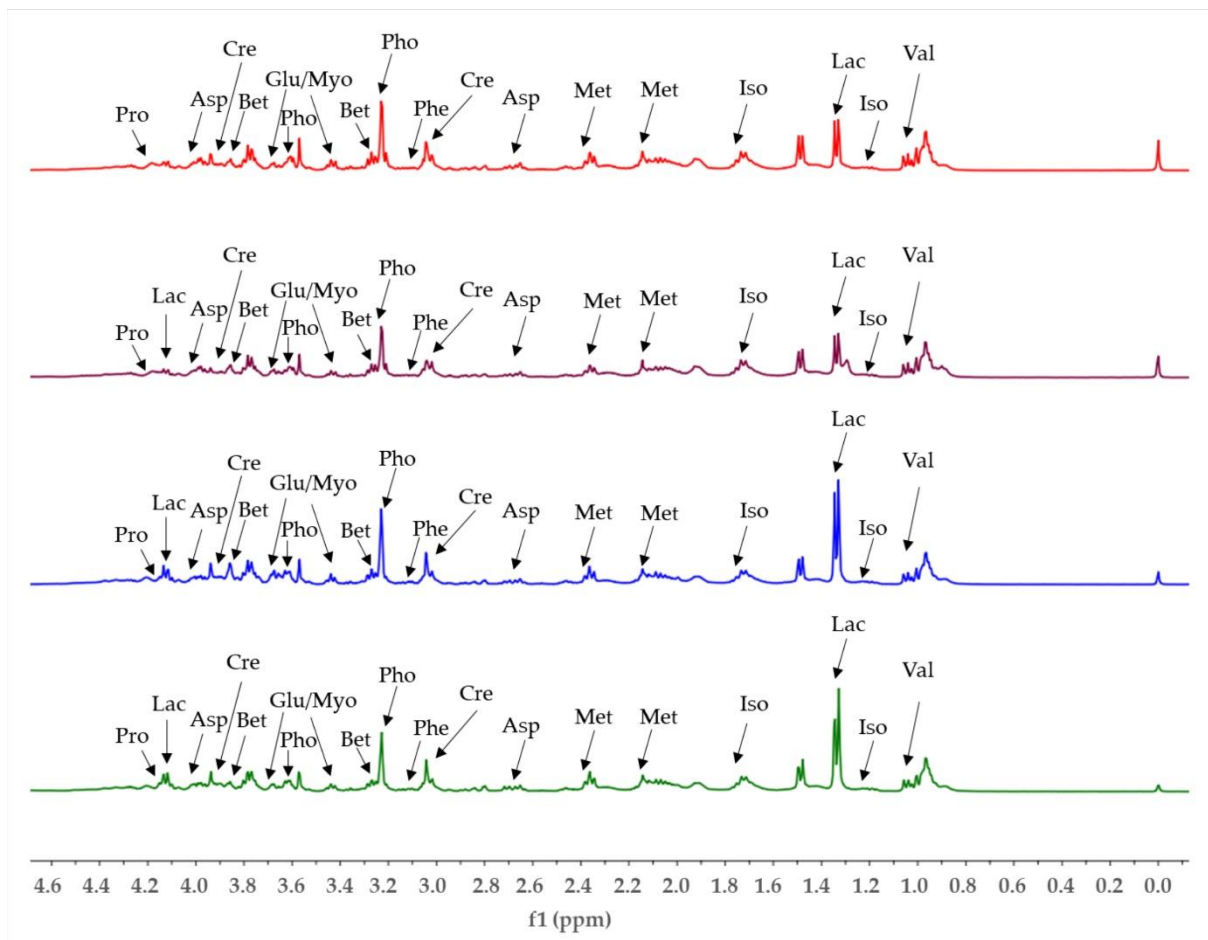


Figure S1. Figure S1. HR-MAS ^1H -NMR T_2 -edited (CPMG) spectra (400 MHz) of all four conditions demonstrating the most differential metabolites among them. Vero E6 control sample spectrum is represented in green (CC), cells treated with WDL (CW) are in blue, cells infected and treated with the molecule (CWV) are represented in purple, and CHIKV-infected cells are represented in red (CV). Identified metabolites are Val (Valine); Lac (Lactate); Iso (Isoleucine); Pro (Proline); Met (Methionine); Asp (Aspartate); Cre (Creatine); Phe (Phenylalanine); Pho

(Phosphocholine); Glu (Glucose); Bet (Betaine); Myo (Myo-inositol).

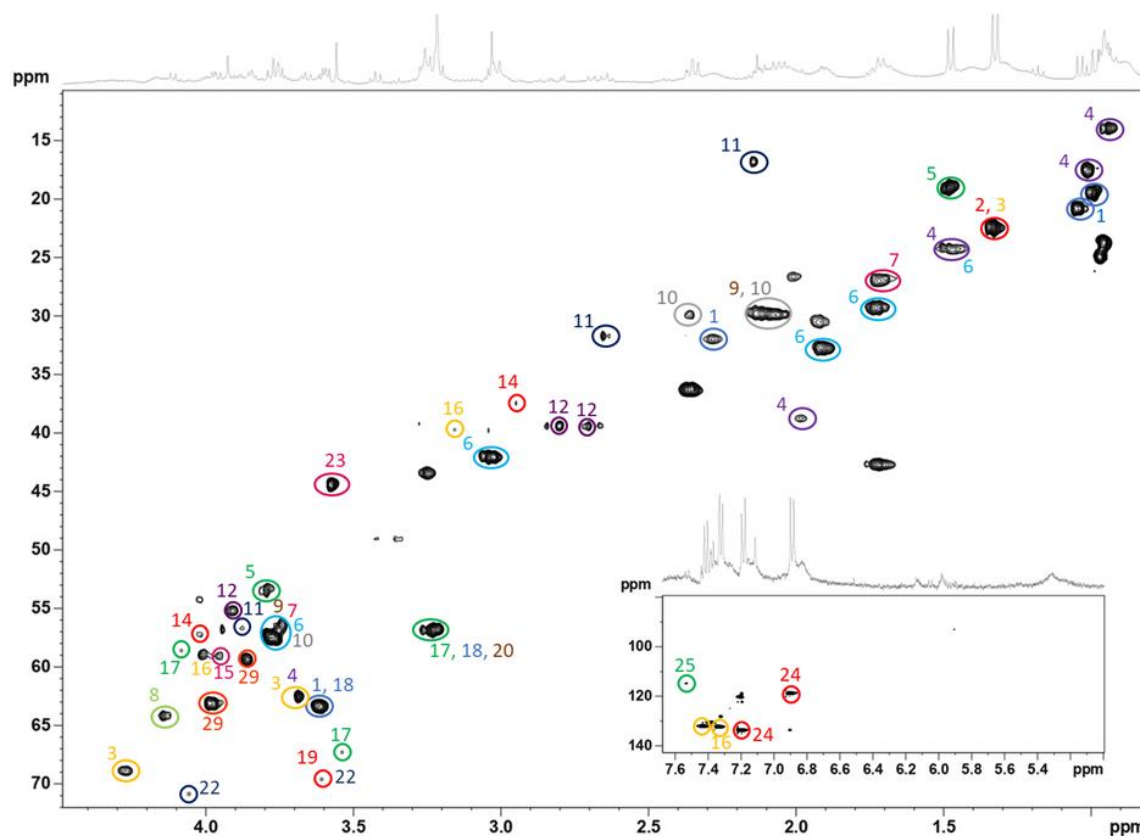


Figure S2. HR-MAS 2D-HSQC spectrum of Vero E6 cells samples acquired on a Bruker Avance III 400 MHz. The image of the spectrum comprises the region between 0 and 4.50 ppm, amplified 20 times, and the small image on the right corner shows the 5.20 to 7.60 ppm region for ^1H chemical shifts and from 10 to 70 ppm for ^{13}C . Identified metabolites are numbered

according to Figure S1 and Table S1.

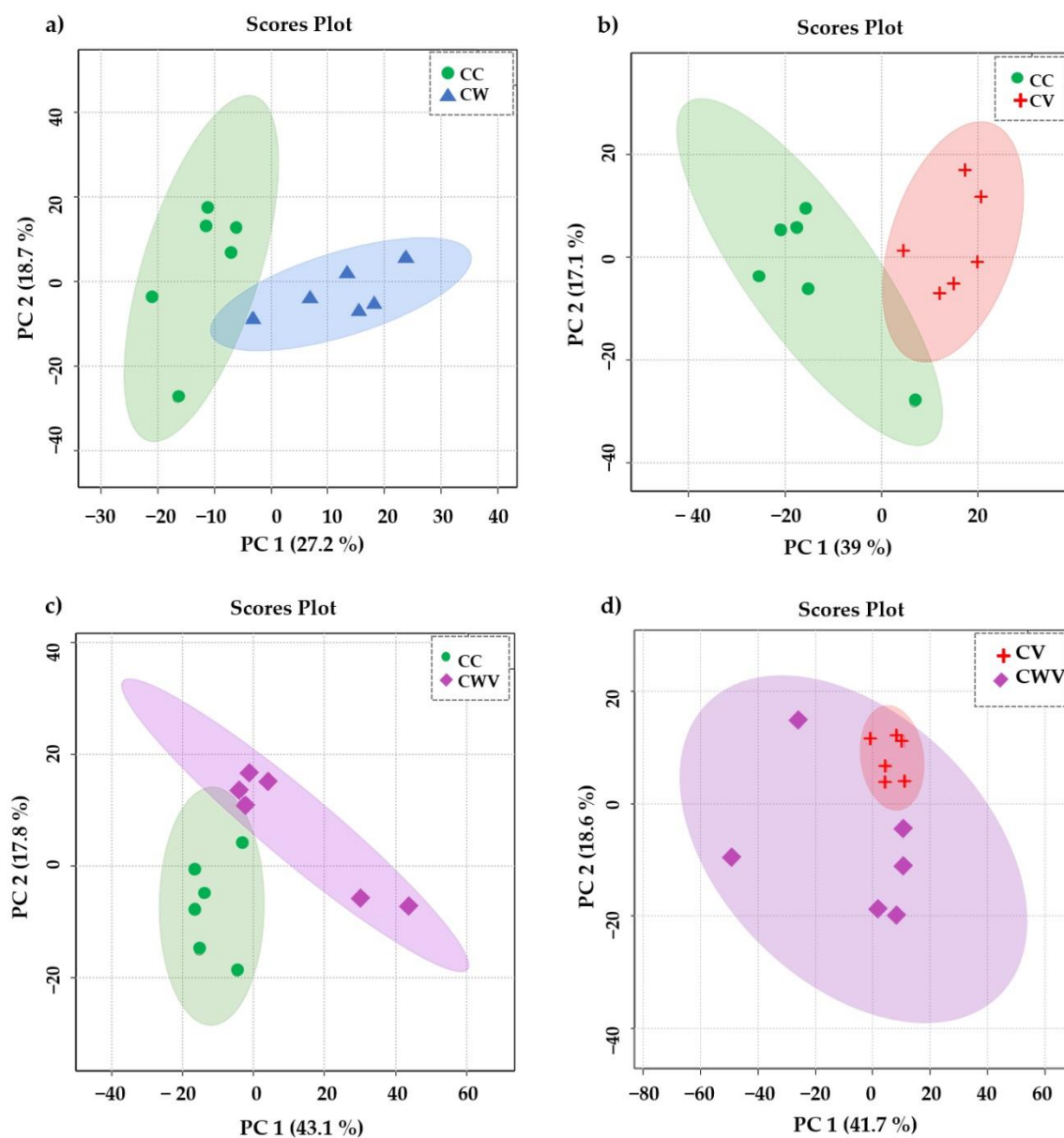


Figure S3. PCA scores plot in 2D of the HR-MAS ^1H -NMR data showing the intrinsic groupings of the conditions compared to the healthy cell control (CC), shown in green, and no outliers. **(a)** The cells treated with WDL (CW) in blue, **(b)** the cells infected with CHIKV in red (CV), and **(c)** the CHIKV-infected cells treated with WDL in purple (CWV). The explained

variance with the first two principal components is shown in the y and x axes.

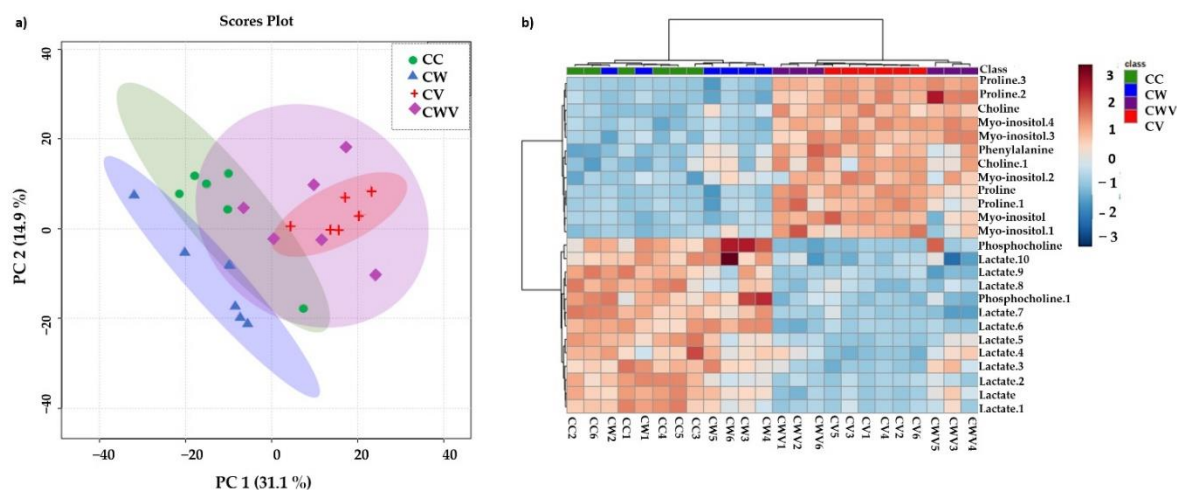


Figure S4. (a) PCA scores plot in 2D of the HR-MAS ^1H -NMR data showing the intrinsic groupings of all the conditions: the healthy cell control (CC), shown in green, the cells treated with WDL (CW) in blue, the cells infected with CHIKV in red (CV), and (c) the CHIKV-infected cells treated with WDL in purple (CWV). The explained variance with the first two principal components is shown in the y and x axes. (b) Hierarchical clustering through heat maps analysis using Euclidean distance. The lines represent the discriminant variables from the PLS-DA analysis for all conditions $\text{CC} \times \text{CV} \times \text{CW} \times \text{CWV}$. The columns represent the samples, and the colour bars on the right represent the relative concentrations of the discriminant

metabolites (red: higher relative concentration; blue: lower relative concentration).

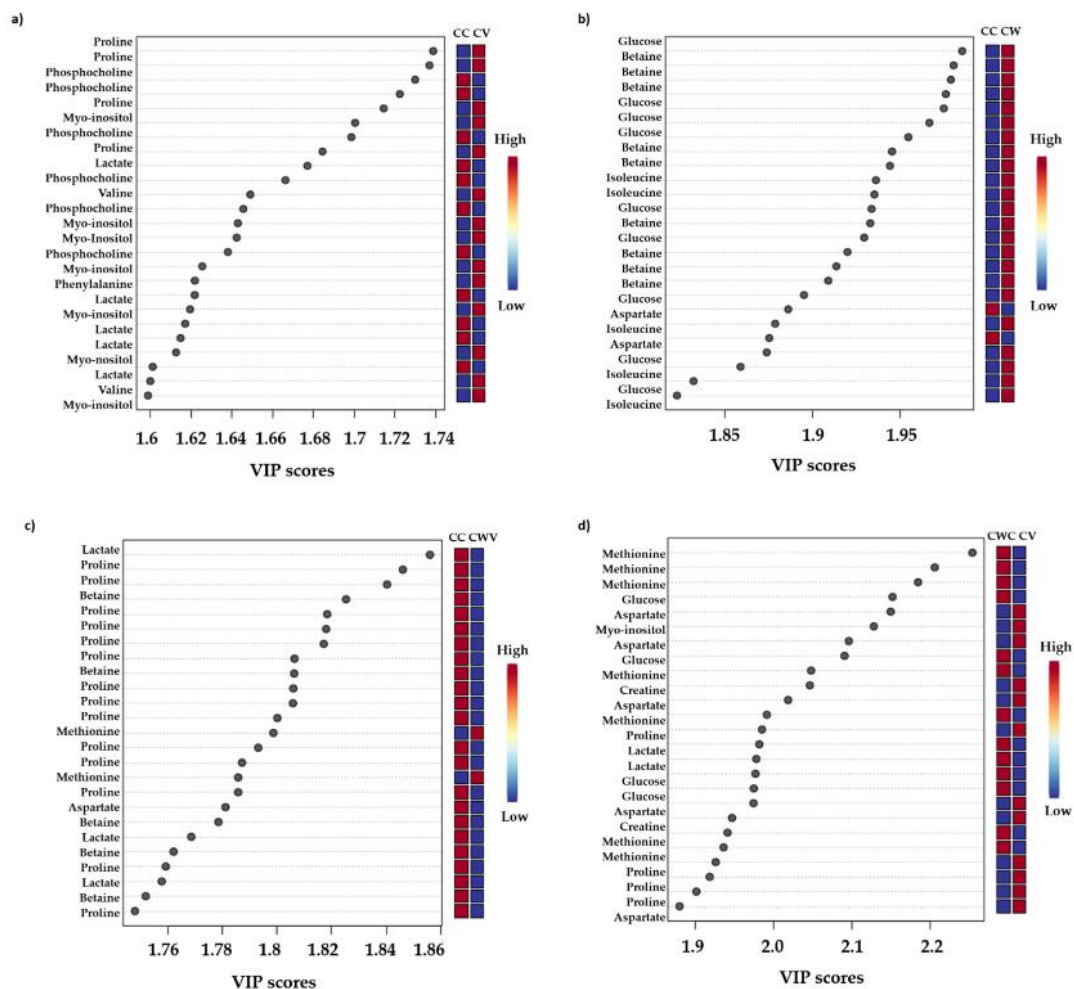


Figure S5. VIP plots of the most discriminant variables from the PLS-DA models, with scores > 1.5 . **(a)** CC $\times$ CV; **(b)** CC $\times$ CW; **(c)** CC $\times$ CWV; **(d)** CV $\times$ CWV .

Table S1. Cell toxicity of WDL in Vero E6 cell lineage.

Concentration	Cell Viability (%)*
400 μ M	45.5 $\pm$ 17.0
300 μ M	59.0 $\pm$ 13.0
200 μ M	68.0 $\pm$ 8.0
100 μ M	74.7 $\pm$ 14.0
50 μ M	93.6 $\pm$ 10.1

* Values with 95% confidence intervals (CI).

Table S2. Chemical shifts, spectral peaks multiplicities and coupling constants of some of the most important metabolites.

Metabolite	N°	¹ H Chemical shifts (ppm), spectral peaks multiplicities and coupling constant	¹³ C Chemical shifts (ppm), spectral peaks multiplicities and coupling constant
Valine	1	0.97 d (<i>J</i> = 8.0 Hz); 1.05 d (<i>J</i> = 7.0 Hz); 2.28 m; 3.61 d (<i>J</i> = 4.0 Hz)	17.1, 19.3, 20.5, 31.6, 63.2
Lactate	2	1.33 d (<i>J</i> = 7.0 Hz); 4.12 q (<i>J</i> = 7.0 Hz)	22.4
Threonine	3	1.33 d (<i>J</i> = 6.7 Hz); 3.60 (J = 6.7 Hz); 4.26 m	22.6, 63.2, 68.8
Isoleucine	4	0.93 t (<i>J</i> = 8.0 Hz); 0.99 d (<i>J</i> = 9.0 Hz); 1.24 m; 1.46 m; 1.97 m; 3.67 d (<i>J</i> = 4.0 Hz)	14.1, 17.1, 24.1, 38.6, 62.5
Alanine	5	1.48 d (<i>J</i> = 7.3 Hz); 3.79 q (<i>J</i> = 7.3 Hz)	18.8, 53.1
Lysine	6	1.43 m; 1.73 m; 1.91 m; 3.03 t; 3.75 t (<i>J</i> = 6.1 Hz)	24.1, 29.2, 32.6, 42.1, 57.4
Arginine	7	1.68 m; 1.90 m; 3.25 t (<i>J</i> = 6.9 Hz); 3.75 t (<i>J</i> = 6.1 Hz)	26.4, 30.4, 43.3, 57.2
Proline	8	2.10 m; 2.36 m 3.58m; 4.14 dd (<i>J</i> = 8.6, 6.4 Hz)	31.1, 64.2

Glutamate	9	2.12 m; 2.37 m; 3.77 dd ($J = 7.2$, 4.7 Hz)	30.2, 31.6, 56.5
Glutamine	10	2.14 m; 2.45 m; 3.77 t ($J = 6.2$ Hz)	29.4, 33.8, 57.2
Methionine	11	2.14 m; 2.17 s; 2.65 t ($J = 7.6$ Hz); 3.85 dd ($J=7.1$, 5.4 Hz)	16.6, 32.3, 31.5, 56.6
Aspartate	12	2.68 dd ($J = 17.5$, 8.9 Hz); 2.82 dd ($J = 17.5$, 3.7 Hz); 3.92 dd ($J = 8.8$, 3.8 Hz)	39.3, 55.1
Hypotaurine	13	2.55 t ($J = 6.8$ Hz); 3.23 t ($J = 6.8$ Hz)	-
Asparagine	14	2.88 m; 2.94 m; 3.99 dd ($J = 7.7$, 4.3 Hz)	37.4, 37.6, 54.1
Creatine	15	3.04 s; 3.93 s	59.1
Phenylalanine	16	3.11 dd ($J = 14.6$, 8.0 Hz); 3.28 dd ($J = 14.6$, 5.5 Hz); 4.00 dd ($J = 7.9$, 5.3 Hz); 7.33 m; 7.39 m; 7.42 m	39.1, 58.9, 132.2, 131.7, 131.4
Choline	17	3.21 s; 3.51 dd ($J = 5.8$, 4.2 Hz); 4.06 dd	56.5, 70.2, 58.1
Phosphocholine	18	3.23 s; 3.62 m; 4.22 m	56.7, 64.1
Glucose	19	3.31 m; 3.44 m; 3.54 m	74.1
Betaine	20	3.26 s; 3.89 s	56.8
Trimethylamine-N-oxide	21	3.27 s	-
Myo-inositol	22	3.27 t ($J = 11.8$ Hz); 3.52 dd ($J = 9.9$, 2.9); 3.61 t ($J = 9.7$ Hz); 4.05 t ($J = 2.9$ Hz)	77.2, 73.8, 75, 74.9
Glycine	23	3.57 s	44.3
Tyrosine	24	6.89 m; 7.19 m	118.8, 133.6

Tryptophan	25	7.53 m; 7.72 m	115.1, 121.3
UDP-N-acetylglucosamine	26	5.97 d; 7.95 d	144.2
UDP-glucose			
UDP-glucuronate			
Fumarate	27	6.53 s	-
ATP	28	6.15 d; 8.26 s; 8.56 s	-
Serine	29	3.86 dd; 3.97 m	59.2, 63.3

5. Conclusão

Através dos resultados obtidos na análise metabolômica utilizando ¹H-RMN HR-MAS, foi possível construir um perfil metabólico que revelou a presença de 29 metabólitos identificados nas células Vero E6. Dentre esses metabólitos, incluem-se Valina, Lactato, Treonina, Isoleucina, Alanina, Lisina, Arginina, Prolina, Glutamato, Glutamina, Metionina, Aspartato, Hipotaurina, Asparagina, Creatina, Fenilalanina, Colina, Fosfocolina, Glicose, Betaína, N-óxido de trimetilamina, Mio-inositol, Glicina, Tirosina, Triptofano, UDP-N-Acetilglucosamina, UDP-glicose, UDP-glucuronato, Fumarato, ATP e Serina. Também conseguimos avaliar diversas condições em comparação com células saudáveis, o que permitiu a identificação de 12 metabólitos afetados pela presença do vírus CHIKV e/ou da WDL, a saber: aspartato, betaína, creatina, fenilalanina, fosfocolina, glicose, isoleucina, lactato, mio-inositol, prolina e valina. Esses achados revelam, de maneira geral, alterações do metabolismo energético celular, metabolismo de aminoácidos e fosfolípidios, indicando adaptações para atender às demandas virais durante a replicação. Além disso, destaca-se a contribuição da WDL para o metabolismo energético celular, sugerindo um possível papel na redução do estresse oxidativo induzido pelo vírus. Embora esses resultados forneçam *insights* abrangentes sobre as alterações metabólicas associadas à infecção e o impacto da molécula no metabolismo celular, as limitações presentes no estudo e também na literatura impediram uma conclusão definitiva sobre o real potencial inibitório exercido pela WDL em um ambiente celular, as quais podem ser melhor abordadas por meio da integração de diversas técnicas ômicas para uma compreensão mais detalhada e precisa desses resultados.

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Anexo I

Artigo Publicado na revista *Virus Research*, Fator de Impacto 5.

Peinado, R. D. S., Eberle, R. J., Pacca, C. C., Arni, R. K., & Coronado, M. A. (2022). Review of -omics studies on mosquito-borne viruses of the Flavivirus genus. *Virus Research*, v. 307, 198610. <https://doi.org/10.3390/v14102194>

Anexo II

Artigo Publicado na revista *Viruses*, Fator de Impacto 4,7.

Peinado, R. D. S., Eberle, R. J., Arni, R. K., & Coronado, M. A. (2022). A Review of Omics Studies on Arboviruses: Alphavirus, Orthobunyavirus and Phlebovirus. *Viruses*, 14(10), 2194. <https://doi.org/10.3390/v14102194>

Anexo III

Artigo publicado na revista *International Journal of Molecular Sciences*, Fator de Impacto 5,6. Peinado, R. D. S., Martins, L. G., Pacca, C. C., Saivish, M. V., Borsatto, K. C., Nogueira, M. L., Tasic, L., Arni, R. K., Eberle, R. J., Coronado, M. A. (2024). HR-MAS NMR Metabolomics Profile of Vero Cells under the Influence of Virus Infection and nsP2 Inhibitor: A Chikungunya Case Study. *International Journal of Molecular Sciences*, 25(3), 1414. <https://doi.org/10.3390/ijms25031414>



Review of -omics studies on mosquito-borne viruses of the Flavivirus genus

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ARTICLE INFO

Keywords:

Arbovirus
Flavivirus
Genomics
Transcriptomics
Proteomics
Metabolomics

ABSTRACT

Arboviruses are transmitted by arthropods (arthropod-borne virus) which can be mosquitoes or other hematophagous arthropods, in which their life cycle occurs before transmission to other hosts. Arboviruses such as Dengue, Zika, Saint Louis Encephalitis, West Nile, Yellow Fever, Japanese Encephalitis, Rocio and Murray Valley Encephalitis viruses are some of the arboviruses transmitted biologically among vertebrate hosts by blood-taking vectors, mainly *Aedes* and *Culex* sp., and are associated with neurological, viscerotropic, and hemorrhagic reemerging diseases, posing as significant health and socioeconomic concern, as they become more and more adaptive to new environments, to arthropods vectors and human hosts. One of the main families that include mosquito-borne viruses is *Flaviviridae*, and here, we review the case of the *Flavivirus* genus, which comprises the viruses cited above, using a variety of research approaches published in literature, including genomics, transcriptomics, proteomics, metabolomics, etc., to better understand their structures as well as virus-host interactions, which are essential for development of future antiviral therapies.

1. Introduction

Flaviviridae is a family of viruses that mainly infects mammals and birds, and is divided into four genera: *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus*. Arthropod-borne viruses make up a large portion of the *Flavivirus* genus with a broad geographic range. The *Flavivirus* genus comprises about 70 viruses (Kuno et al., 1998), of which 39 are arboviruses species that cause numerous diseases. Dengue (DENV), Zika (ZIKV), Saint Louis Encephalitis (SLEV), West Nile (WNV), Japanese Encephalitis (JEV), Rocio (ROCV), Yellow fever (YFV) and Murray Valley Encephalitis (MVEV) viruses are important pathogens that infect humans (Lindenbach et al., 2001). Table 1 summarizes the main information of those viruses.

The transmission of arthropod-borne flaviviruses includes distinct cycles that involve the mosquito/insect vector and a mammalian or avian host. The transmissions are divided into horizontal transmissions - which happen between mosquitoes and vertebrate hosts, causing diseases in vertebrates, and vertical transmissions - which allow the spread

of the virus uniquely in mosquitoes (Rosen 1988). Urban cycles, Fig. 1 (b), are responsible for the transmissions of DENV, YFV, and ZIKV by the *Aedes* spp., with enzootic cycles, Fig. 1(a), in Africa, Asia and in the Americas. JEV and WNV maintained an enzootic cycle in affected regions between *Culex* spp. mosquitoes and pigs, Fig. 1(c), or aquatic birds (JEV) and birds (WNV) as increasing hosts, with humans, Fig. 1(d) (JEV, WNV) and horses, Fig. 1(c), (WNV) as dead-end hosts (Huang et al., 2014).

The *Flavivirus* viral particle has a diameter between 40 and 60 nm which is composed of an icosahedral protein capsid (C) surrounded by a lipidic envelope in which the membrane proteins (M) are inserted together with protein spicules (Lindenbach et al., 2001). Their genome is a single positive RNA strand, non-segmented, of about 11 kb with one open reading frame (ORF) and untranslated regions (UTRs) at both ends (Gubler et al., 2011). At the 5' end (~100 nucleotides (nt) long (Markoff 2003), a type I cap (m7G - 7-methylguanosine triphosphate) is present, followed by the conserved AG dinucleotide sequence. The 3' UTR is between 400 and 800 nt long (Ramos-Lorente et al., 2021), which

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<https://doi.org/10.1016/j.virusres.2021.198610>

Received 16 July 2021; Received in revised form 18 September 2021; Accepted 11 October 2021

Available online 27 October 2021

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varies among flaviviruses. This region is notable for the existence of structural duplications, which increases functional diversity and provides, for example, adaptation to different hosts (Chambers et al., 1990; Engelman et al., 2014; Ramos Lorente et al. 2021). The ORF encodes a polyprotein processed in three structural proteins (proteins C (capsid protein), precursor membrane protein (pre-M), and envelope protein (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), which are involved in regulating virus protein expression, replication, virulence, and pathogenicity. The whole genome structure is shown in Fig. 2 (Chambers et al., 1990; Ramos-Lorente et al., 2021).

Flaviviruses are internalized through clathrin-coated pits by different types of cell receptors, varying between different virus species, into a pre-lysosomal endocytic compartment with low pH, inducing virus fusion with the host cell membrane and releasing the nucleocapsid into the cell (Smit et al., 2011; Pinu et al., 2019). The viral polyprotein is cleaved by viral and host proteases in the N-terminal region releasing the structural and the non-structural proteins (Lopes et al., 2014; Byk and Gamarnik 2016). The viral capsid, composed mainly of the C protein, is icosahedral shaped and surrounded by a lipidic envelope with membrane proteins (M) and glycoproteins spicules (E proteins). It has a binding role to the cell membrane and assembly of the viral RNA into a nucleocapsid for the formation of the mature viral particles (Lopes et al.,

2014). The E glycoprotein, which has three structural domains – EI, EII, and EIII – is the main protein in the viral particle and has a highly hydrophobic loop that starts the infection and fusion to the membrane (domains EI and EII) and has putative receptor-binding sites in domain EIII promoting cell receptor binding and immune recognition (Slon Campos et al. 2018). As for the prM protein, it works as a chaperone for the E protein during viral assembly, and it is matured by host enzyme furin in the trans-Golgi network. The small envelope protein M may play a role in virus release and present cytotoxic effects by activating a mitochondrial apoptotic pathway (Trent et al., 1987; Tian et al., 2016).

Significant insight into the architecture of flavivirus-induced membrane structures associated with functional replication complexes within mammalian cells has been provided by combined immunoelectron microscopy (IEM) and electron tomography (ET) studies (Welsch et al., 2009). The study shows a complex set of DENV-modified structures, including double-membrane vesicles (Ve), membrane packets (Vp), convoluted membranes (CM), and tubular structures (T) covered by a single continuous ER membrane (Welsch et al., 2009). The replication complex where RNA synthesis takes place is formed by Ve, which are composed of viral RNA, replication proteins and probably cellular proteins. Negative RNA strands are produced to serve as templates for the synthesis of new positive RNA strands. At the end of the process, new

Table 1

Summary of the Flaviviruses discussed in this review: their main geographic areas, main vectors and hosts, genotypes/serotypes/lineages, their first isolation and main symptoms are described.

Flavivirus	Main geographic areas	Main Vectors	Hosts	Genotypes/lineages/serotypes	First isolated in/ from	Main symptoms
Yellow Fever Virus	Africa, America, Europe	<i>Haemagogus</i> and/or <i>Aedes</i> mosquitoes	Non-human primates (monkeys) and humans	I and II (from West Africa), III – IV (East, Central Africa and Angola), South America I (1A – 1E) and II	1927 from a Ghanaian patient	Fever, headache, jaundice, muscle pain, nausea, vomiting and fatigue
Zika virus	America, Africa, Southeast Asia	<i>Aedes aegypti</i> and <i>Aedes albopictus</i>	Humans, non-human primates, goats, horses, bats and even sheep (Vorou Rengina zikvtabela)	Two African lineages and one Asian lineage	1947 from a rhesus monkey blood sample in Zika forest, Uganda	Fever, skin rashes, arthralgia, conjunctivitis and headache, microcephaly
Dengue virus	Asia, Europe, America	<i>Aedes aegypti</i> / <i>albopictus</i>	Humans and non-human primates	DENV-1, DENV-2, DENV-3 and DENV-4	1943 from patient blood samples in Japan	Fever, headache, myalgia, retro-orbital pain, arthralgia, skin rashes, gastrointestinal discomfort (severe Dengue may cause hemorrhagic manifestations)
Saint Louis Encephalitis Virus	America	<i>Culex</i> sp.	Birds, humans, horses and hematophagous arthropods	I and II (US and Canada), III (Southern America), IV (Colombia and Panama), V (South America), VI (Panama), VII (Argentina), VIII (Amazon region in Brazil)	1933 from human encephalitis outbreak in Saint Louis, US	Viremia, fever and headaches (mild cases); high fever, spastic paralysis, convulsions, disorientation, tremors, meningoencephalitis (severe neurological cases)
Japanese Encephalitis Virus	Asia, Western Pacific countries, northern Australia	<i>Culex</i> sp.	Water birds, pigs, equids and humans, arthropods	GI – GV in Indonesia and Malaysia; GI and GII in Australia and New Guinea; GI, GII and GIII in Thailand, Cambodia and Vietnam; GI and GIII in Japan, Korea and China; GIII in India, Sri Lanka and Nepal	1924 from human brain tissue in Japan	High fever, chills, headache, nausea, fatigue, vomiting, paralysis, encephalitis, brain edema, coma (severe encephalitis cases)
West Nile Virus	Africa, Europe, Middle East, America, West Asia	<i>Culex</i> sp.	Birds, arthropods, humans and other animals	9 lineages Human outbreaks: Lineage 1: 1a – Europe, Africa, Americas; 1b – Oceania Lineage 2 – Africa, Hungary, Italy and Greece	1937, blood sample in the West Nile district in Uganda	Febrile illness, rashes, myalgia, headache, disorientation, meningitis, encephalitis, acute paralysis
Rocio Virus	South America (Brazil)	<i>Psorophora ferox</i> , <i>Aedes</i> and <i>Culex</i> mosquitoes	Birds, horses, humans, arthropods	Ilheu Virus subgroup (ILHV), close to the JEV serogroup	1975, from the cerebellum and spinal cord in Iguaçu, São Paulo, Brazil	Fever, headache, meningeal irritation, malaise, motor abnormalities, encephalitis
Murray Valley Encephalitis Virus	New Guinea, Australia and South Pacific Islands	<i>Culex annulirostris</i> and <i>Aedes</i> mosquitoes	Humans, water birds, horses, cattle, dogs, foxes, marsupials, arthropods	G1 – Australia and Papua New Guinea; G2 Western Australia; G3 and 4 also in Papua New Guinea	1951 from the human brain in Australia	High fever, headache, nausea, vomit, diarrhea, macular rashes, lethargy, irritability, confusion, seizures, tremor, paralysis, encephalitis

viral particles (virions) are assembled near the endoplasmic reticulum region, and the nucleocapsid is involved by an envelope, as shown in Fig. 3. These virion particles are trafficked towards the cell membrane through vesicles that will merge with the membrane to release the virions to infect other cells (Lindenbach et al., 2001).

The nonstructural proteins (NS1-NS5) associate to form the replicative complex and participate against host innate response. NS1 can be used as a diagnosis marker during the acute stage of the disease, where the increase of secreted NS1 in patient bloodstream by infected cells elicits humoral response and the antigen can be detected by ELISA (enzyme-linked immunosorbent assay) and rapid immunochromographic assays up to 9 days after the onset of the disease (Peeling et al., 2010). The NS1 glycoprotein, with a mass between 46 and 55 kDa, is initially translated as a monomer, but it binds to the membrane on the cell surface as a dimer. However, in blood circulation, it is found as a hexamer forming a hydrophobic central cavity carrying lipid molecules (Rastogi et al., 2016; Norazharuddin and Lai 2018). The dimer form probably interacts with NS4A and NS4B in the early stages of the infection, and the hexamer has been hypothesized to be linked to dengue physiopathology, transporting lipids in plasma from tissues to the liver, for its lipid content, i.e. triglycerides, phospholipids, cholesterol, is similar to high-density lipoproteins (HDL) (Gutsche et al., 2011; Norazharuddin and Lai 2018). NS1 has also been related to the activation of macrophages via Toll-like receptors, which causes vascular leakage by disruption of endothelial cells and is linked to dengue hemorrhagic fever (Modhiran et al., 2015; Norazharuddin and Lai 2018). Also, disruption of the endothelial glycocalyx layer (EGL) on human pulmonary endothelial cells is caused by NS1-induced expression of sialidases and heparanase that degrade sialic acid and heparan sulfate proteoglycans leading to endothelial barrier dysfunction during severe dengue disease (Puerta-Guardo et al., 2016). The C-terminal region of NS1 presents an octapeptide sequence that is conserved among flaviviruses and is essential for cleavage of the polyprotein. Cleavage is performed mainly by host (signalases, furin) and viral proteases (NS3 serine protease), except for the NS1/NS2A junction cleavage, which is performed by still unknown proteases in the ER lumen (Rastogi et al., 2016).

The N-terminal NS3 protein (~70 kDa) domain comprises a helicase and a protease domain. The protease domain is responsible for polyprotein processing along with NS2B as its cofactor with a catalytic role due to the serine protease catalytic triad composed of Histidine 51, Aspartic Acid 75, and Serine 135 residues (Ganesh et al., 2005; Norazharuddin and Lai 2018). It cleaves sites between two cationic regions in between domains, such as the NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4B-NS5 sites, upstream C-prM, NS4A-NS4B junctions, and within NS2A and NS2 itself (Niyomrattanakit et al., 2004). On the C-terminal region, NS3 has a nucleoside triphosphatase (NTPase), a 5' RNA triphosphatase (RTP), and helicase functions that are important for viral

replication (Junaid et al., 2013). NS3 with NS2B as its cofactor acts, therefore, as a serine protease, helicase, nucleoside triphosphatase, and RNA triphosphatase, being involved in the RNA replication along with NS5, which has a methyltransferase, guanylyltransferase, and RNA-dependent RNA polymerase activity (Thamizhmani and Vijayachari 2014; Afroz et al., 2016).

NS4A and NS4B are transmembrane proteins that function as scaffolds for the formation of the replication complex. NS4A also induces endoplasmic reticulum (ER) re-arrangement, localization, and stabilization of the replication complex; it is also responsible for autophagy induction during the viral infection protecting from cell death, which is important for the replication process (Miller et al., 2007; Zou et al., 2015). The NS4B protein presents conserved and unique interactors in host cells that are important for virus-host adaptation. ATP synthase, voltage-dependent anion-selective channel proteins, calcium-binding mitochondrial proteins, and even cytochrome C oxidase complex components are some of these interactors (Scaturro et al., 2019), as it has been confirmed that flaviviruses mainly target ATP production, calcium homeostasis, apoptosis, and the mitochondrial respiratory chain (Chatel-Chaix et al., 2016). Interaction with mitochondrial mechanisms contributes to perturbation of the innate immune response since mitochondria are key for antiviral response regulation. As well as mitochondrial protein, NS4B also probably binds to cellular proteins that are related to protein stability and quality control, as demonstrated in SK-N-BE2 (neuroblastoma) and HEK293 (kidney) cells (Heaton and Randall 2010a; Metz et al., 2015; Scaturro et al., 2018; Shah et al., 2018; Liu and Cherry 2019), and other cellular proteins involved with neurodegenerative disorders, retinal degeneration, defects in neuronal differentiation and axonal dysfunction (Scaturro et al., 2018).

With about 100 kDa, the NS5 protein is the most conserved protein among flaviviruses, and it possesses an RNA-dependent RNA polymerase (RdRp) at its C-terminal region that is essential for viral replication, which presents a Gly-Asp-Asp sequence (residues 667–669) that is conserved among flaviviruses and is critical for the RNA-dependent RNA polymerases (Lee et al., 1990). At the N-terminal region, there is a methyltransferase (MTase) and guanylyltransferase (GTase) domain that is responsible for the capping function of the NS5 protein for mRNA capping and processing. It is known to have a major role in viral replication probably also suppressing host cell antiviral response, which makes it a potential target for antiviral therapeutics (Zhou et al., 2007; Davidson 2009). NS5 interactions with members of the PAF1C (RNA polymerase-associated Factor 1 complex) complex have been demonstrated and, it has been deduced, that such interactions reduce the expression of interferon-stimulated genes and weakens immune responses (Shah et al., 2018). NS5 is also able to target the human signal transducer and activator of transcription factor 2 (STAT2) in order to inhibit type I IFN (type I interferons) immunity defense (Grant et al.,

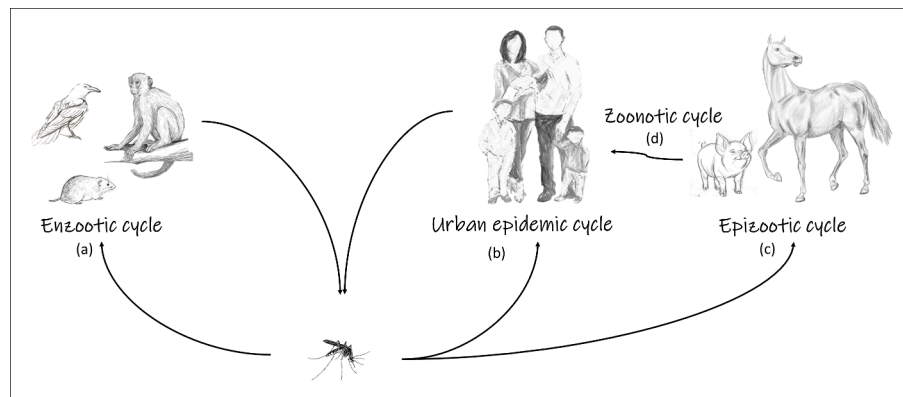


Fig. 1. Illustration of *Flaviviruses* transmission through an (a) enzootic cycle, (b) urban epidemic cycle, (c) epizootic cycle and, (d) zoonotic cycle, involving vectors, hosts and accidental hosts.

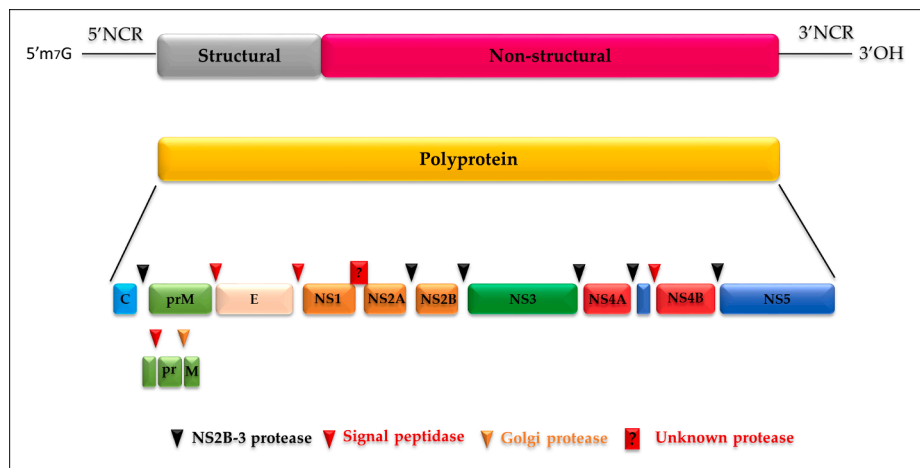


Fig. 2. Flavivirus genome. *Flavivirus* genome organization and polyprotein processing. In the upper scheme is the viral genome with the structural and nonstructural protein-coding regions and the 5'- and 3'-NCRs (non-coding regions). In the below scheme is the genome with the indicated proteins, which is generated by proteolytic processing by cellular and viral proteases (arrows). The question mark denotes a cleavage performed by an unknown protease (Rastogi et al., 2016). Figure based on: Fenner and White's Medical Virology (5th Edition), Chapter 36 – Flaviviruses, pages 493–518 (Burrell et al., 2017).

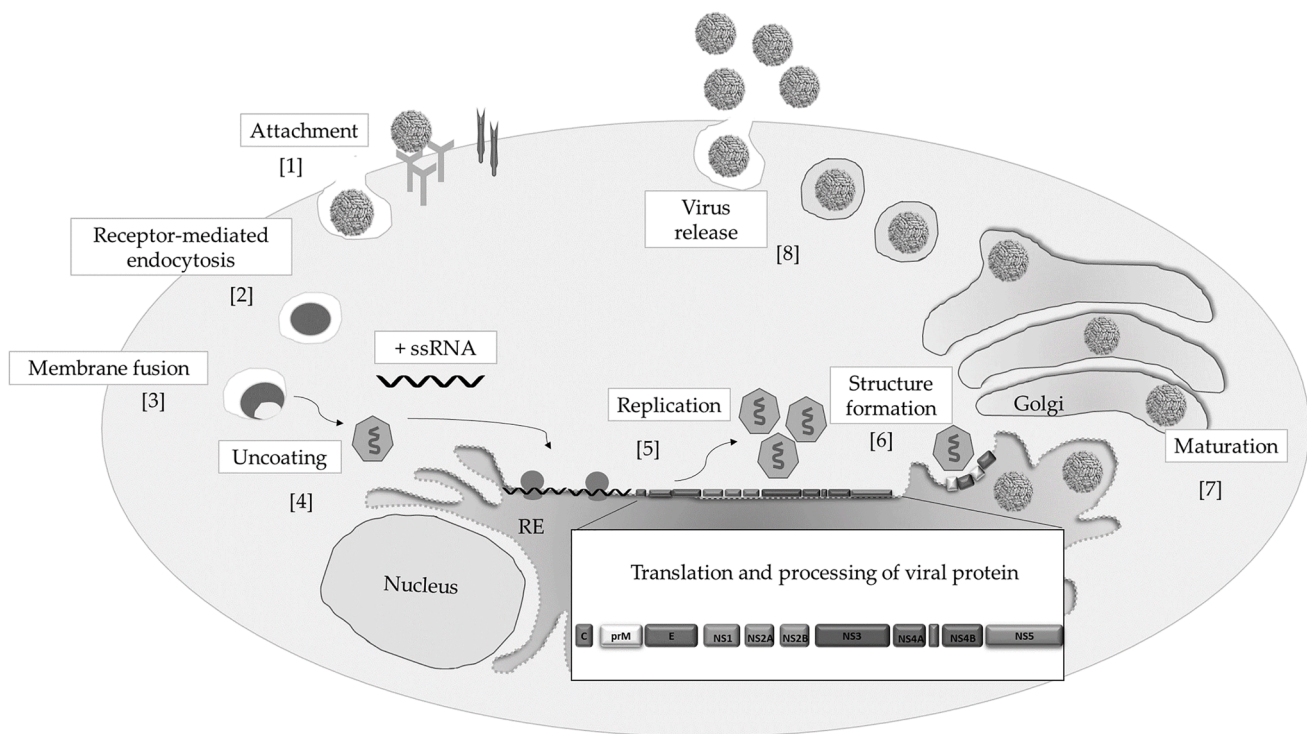


Fig. 3. Flavivirus replication illustration from virus binding, internalization, replication, generation and release of new virion particles. Based on: (Campos et al. 2018).

2016), as well as STAT1 (Signal transducer and activator of transcription 2) phosphorylation inhibition impeding proper IFN signaling (Hertzog et al., 2018). In this context, it was elucidated that NS5 can block IFN production by inhibiting TBK1 (TANK-binding kinase 1), which is responsible for phosphorylation of the transcription activator IFN regulatory factor 3 (IRF3). By doing so, TBK1 activation and IRF3 phosphorylation are compromised, allowing ZIKV evasion of innate immune response (Lin et al., 2019). NS5 has also been identified to interact with alternative splicing modulation which eventually leads to a different abundance of antiviral factors (De Maio et al. 2016).

As outbreaks increase throughout the world with clinical effects associated with neurological, viscerotropic and hemorrhagic diseases along with the viruses ability to adapt to new environments, arthropods

and human hosts cause a major health and socioeconomic concern (Slon Campos et al. 2018). Therefore, it has become extremely important to assess different techniques to study and develop new antiviral strategies and treatments. In this context, utilizing multiple omics data, i.e. genomics, transcriptomics, proteomics, and metabolomics, provides a wider and clearer perspective of biological systems and, their integration generates a better understanding of cells, organisms, and communities. Technological advancements in techniques such as DNA sequencing (Mardis 2008), transcriptome measurements via RNA-seq (Wang et al., 2009), SWATH-MS-based proteomics (Ludwig et al., 2018), Nuclear Magnetic Resonance (NMR) Spectrometry based Metabolomics (Emwas et al., 2019), and Mass Spectrometry based metabolomics (Dettmer and Aronov 2007) allows us to integrate data

from these different areas. This, however, is still a challenging task, as the specific analytical tools for each omics must be well-suited for comparisons among the data obtained (Pinu et al., 2019).

Here, we present a review of some of the available omics studies applied to arthropod-borne viruses whose vectors are mainly mosquitoes, comprised within the *Flavivirus* genus, including the following arboviruses: Dengue, Zika, Saint Louis Encephalitis, West Nile, Yellow Fever, Japanese Encephalitis, Rocio, and Murray Valley Encephalitis.

2. Mosquito-borne Flaviviruses

2.1. Yellow fever virus

Yellow fever virus (YFV) has been a recurrent and frightful disease since the 18th century in both the New and Old World. Reemerging large-scale epidemics happened during important historical events in America, Africa, and Europe, causing great devastation and death, and one of the hypothesis is that the virus originated in Africa was brought to the Americas and Europe through *Aedes aegypti* mosquitoes in sailing vessels during slave trades among these mainlands (Bryant et al., 2007). The virus is maintained through a cycle in nature that involves non-human primates and *Haemagogus* and/or *Aedes* mosquitoes, while humans sporadically are affected when bitten by the infected mosquitoes. *Aedes aegypti* mosquitoes can serve as viremic hosts for human transmission and cause epidemics (urban yellow fever) (Monath and Vasconcelos 2015).

Symptoms of the YFV can vary from mild febrile illness with or without rash/jaundice to severe hepatorenal failure and death (Clements and Harbach 2017). The first phase called the infection phase, which are around 4 days post-infection (dpi), is characterized by fever (up to 41 °C), myalgia, headache, nausea, and vomiting. After that phase, a remission period may happen (from 2 to 48 h) with a general improvement of symptoms and health. Most patients recover completely in the remission phase and the disease does not progress to the last and severe intoxication phase, which is characterized by fever, abdominal pain, nausea and vomiting, oliguria, jaundice, hepatic dysfunction, inflammation and renal failure, which can lead to fatal cases. YFV can even cause encephalopathy cases, although very rare (Waggoner et al., 2018).

In the 1900s, the vector *A. aegypti* was identified and since then, efforts to control mosquito dissemination were made and resulted in a decline of diseased numbers. In 1927, the virus was first isolated from a Ghanaian patient whose strain was named after as the Asibi strain. Later, in the 1930s, two live attenuated YFV vaccines were produced, which helped decline disease cases more intensively (Gardner and Ryman 2010; Monath and Vasconcelos 2015). However, some outbreaks occurred even after vaccination availability in Africa in the 1960s and late 1980s, and some events in Brazil, Argentina and Paraguay (2007–2009), Uganda in 2010, and Sudan and Ethiopia in 2012–2013. These outbreaks may be linked to YFV new lineages by genetic mutations background of absence of natural or acquired artificial immunity, although the underlying reasons for amplification remain unclear (Monath and Vasconcelos 2015).

Five YFV genotypes were originally documented: two of them originated in West Africa (I and II) and the other three originated in East and Central Africa (East, Central Africa, and Angola). When the virus was disseminated from Africa to the Americas in the 18th century together with *A. aegypti*, two new genotypes were generated derived from the Western African ancestor: South America I and II (SA-I and SA-II). SA-I was the most disseminated genotype in Brazilian epidemics, where until the 90's, outbreaks were restricted to the Amazon forest area and the savanna/cerrado in the Central/West region of the country. Within two decades, the epidemics spread throughout other areas and biomes in the country, and five other sub-lineages were generated from genotype I: 1A - 1E. A while later, genotype I was subdivided into two major lineages: the old lineage, which includes Old Para, 1A, 1B, and 1C, and the

modern lineage, with sub-lineages Trinidad and Tobago, 1D and 1E. Recent outbreaks in Brazil, in 2017, were genetically analyzed from howler monkeys in Espírito Santo state and it was concluded that the strain was from sub-lineage 1E, which presented new polymorphisms and amino acid substitutions in important domains of the NS3 and NS5 proteins (Gómez et al., 2018; Nunes et al., 2012; Figueiredo et al., 2018; Pan American Health Organization 2017).

Single-stranded RNA molecules that act as post transcriptional gene regulators - the so-called miRNAs or microRNAs - are able to silence certain expression products in host cells that could disturb viral replication and even regulate complete genetic pathways during pathological or physiological processes (Holanda et al., 2017). The protein DGCR8 which acts as a cofactor for Drosha mRNA in the production of miRNAs was found not expressed during YFV infection, probably due to a viral regulatory process, as YFV infection also reduces Drosha mRNA expression along the infection course due to viral replication and cytokine production (Best et al., 2005; Liu et al., 2005). Some miRNAs are also known to regulate the inflammatory response, for example, miR-29, miR-155, and miR-221 (Ender and Meister 2010), while others regulate TLR (Toll-like receptors) recognition of pathogens, such as miR-9, miR-125b, and miR-147 (Monath and Vasconcelos 2015), and they all can be negatively or positively regulated by their biogenesis components such as Dicer and Drosha, and YFV can act on this regulating process. Thus, the infection possibly has a direct role in the regulation and modulation of certain miRNAs for viral replication and host immune response, although other experiments are still required for further investigations on the function of each of the regulated proteins and how YFV affects such regulation (Holanda et al., 2017).

A study on Rhesus macaques infected with YFV showed that even low inoculum doses resulted in severe injuries to the liver and kidneys only a few days post-infection. Transcriptomic analysis of the infection showed that cytokine genes expression such as interleukins IL-8, IL-1 β , and IL-12 were downregulated, whereas, shortly before euthanasia, an increase of IFN γ , IL-15, IL-6, and MCP-1 plasma levels was observed, probably since at this final stage of the disease, injured organs like the liver produce these cytokines and also that high levels of inflammatory factors contribute to organ damage (Muñoz-Jordán et al., 2003; Amarilla et al., 2019).

Lymphopenia was also observed at 3 dpi which is probably linked to dysregulation of genes related to zinc binding and apoptosis processes (Terry et al., 2012; Bardina et al., 2015). The two YFV strains: the wild type YFV-DakH1279 and the attenuated vaccine strain YFV-17D, induced different host responses. An amount of 46 differentially expressed genes (DEGs) was revealed after infection with the YFV-17D strain, while 765 DEGs were found during strain YFV-DakH1279 infection. These differences are not only supported by transcriptomic and gene expression differences but also by differences in virulence and viral replication between the two strains. At 3 dpi with YFV-17D, the innate immune response presents very robust effects with upregulation of innate immune genes. Three common DEGs were found after infection with the two strains, one of them is gene RSAD2 (Radical S-Adenosyl Methionine Domain Containing 2), which encodes an antiviral protein called viperin that can be IFN dependent or not and is induced during several other viral infections (Mattijssen and Pruijn 2012; Engelmann et al., 2014). DEGs related to the YFV-DakH1279 strain are mostly related to zinc ion binding, which is strongly linked to immune response, as zinc deficiency induces B cell apoptosis, hence the lymphopenia presented in the study results. Therefore, it was concluded that the wild type strain induces severe lymphopenia and organ failure in adult Rhesus monkeys that are preceded by several indicators that may become diagnostic of disease severity. Host transcriptional alterations revealed inducement of apoptotic pathways, immune response dysregulation, lymphocyte inhibition, zinc-binding disruption leading to high apoptotic levels (Ibs and Rink 2003; Engelmann et al., 2014).

The attenuated 17D vaccination in humans elicits an elaborated regulation of innate immune cytokines, such as elevated plasma

interferon (IFN)- γ levels that can be observed 15 days post-vaccination (Da Costa Neves et al. 2009), along with others, for instance, interleukin (IL)- 1β , IL-12, tumor necrosis factor (TNF- α) and IL-10, which can also be decreased in other cases analyzed (Gaucher et al., 2008; Luiza-Silva et al., 2011; Silva et al., 2011). Although antiviral cytokines response may vary among vaccine recipients there is a general increase or decrease of IFN- α and (IFN)- γ transcripts only a few days after vaccine administration (Querec et al., 2009). It is also observed that 17D vaccination interacts directly with innate immune dendritic cells (DC), which induces the production of several cytokines mediated by TLRs and promotes downstream T cell responses, as both CD4+ and CD8+ T cells respond to the 17D attenuated strain. Early in virus exposure, CD8+ T cells are activated (3 days post immunization) (Akondy et al., 2015) and CD4+ T cells peak in 7–14 days post-immunization (James et al., 2013). Only a little percentage of the CD8+ cell clones produced to turn into memory populations and decrease over time but are still detectable after 25 years (Watson and Klimstra 2017). In patients with severe disease symptoms and visceral damage, there is a high infiltration of T cells such as CD4+, CD8+, B cells, macrophages, and NK (natural killer) cells with a high level of expressed inflammatory factors, which are probably either ineffective to control viral replication in the liver or strongly cytolytic contributing to damage and increased severity of the disease itself (Quaresma et al., 2007; Watson and Klimstra 2017).

A. aegypti mosquito transcriptomics analysis during infection with YFV, DENV and WNV were able to identify around 20 up-regulated genes and other 15 down-regulated genes on day 1 that were quite similar among the three flaviviruses and a possible *Flavivirus* “signature” of infection. YFV had the most characteristic up-regulated genes, with a total of 54 on day one of infection, 19 on day two, and 86 on day seven. Up-regulated genes included a putative juvenile hormone-inducible protein, peroxisomal membrane protein, and some other down-regulated genes related to a pupal protein and a matrix metalloproteinase. YFV also had seven up-regulated genes and 3 down-regulated during the three different days post-infection (D1, D2, and D7). Most of the up-regulated genes identified and analyzed were related to DNA-dependent transcription regulation and protein amino acid phosphorylation. Zinc binding, nucleic acid binding, nucleotide binding, and transcription binding genes were also abundant (Colpitts et al., 2011). Down-regulated genes were mainly related to chitin metabolism, transport, proteolysis, cuticle constituents, and serine-type endopeptidase activity and most of them were found to be extracellular, while the up-regulated were mostly intracellular. It was also observed that up and downregulation of gene expression during the different days of infection varied according to the tissue area where flavivirus disseminated in mosquitoes: midgut (MG) and through the body to the salivary gland (SG). Although there were similarities among the infections, this study suggested that specific alterations in *A. aegypti* transcriptome are present depending on the flavivirus and also on the days post-infection (Colpitts et al., 2011).

Blood transcriptomic and metabolomics after YF17D vaccine inoculation were analyzed and it was observed that individuals with endoplasmic reticulum (ER) stress and low tricarboxylic acid cycle (TCA) activity were more susceptible to YF symptoms with maladaptive ER stress; triggering mitochondria induced reactive oxygen species, proinflammatory responses, and cell death. Methionine was found abundant and reacted with ROS (reactive oxygen species) generating methionine sulfoxide, which was also increased at day 1 post-infection together with superoxide dismutase (SOD) that catalyzes ROS dismutation. As ROS production requires oxygen, high lactate levels were observed in symptomatic patients and low levels of glycerol, acylcarnitines, and acetylcarnitine which agrees with the fact the oxidative stress attenuates lipid production and increases fatty acid oxidation (Douglas et al., 2016; Chan et al., 2019). The gene that encodes a thioredoxin-interacting protein (TXNIP) which regulates cellular redox signaling (Oslowski et al., 2012) was significantly increased in symptomatic subjects and correlated with IFN signaling genes such as IFITM2 (Interferon Induced

Transmembrane Protein 2) and IRF5 (Interferon regulatory factor 5). In total, 2055 genes were more abundant and 1963 were less abundant in symptomatic subjects compared to the asymptomatic ones, and the most positively enriched pathways found were related to ER stress response, sumoylation (SUMO is a small ubiquitin-related modifier), and cell cycle. It was concluded that individuals with reduced ER stress and higher TCA cycle activity cope better with the infection without disturbing homeostasis and remain asymptomatic. Genetic, dietary, environmental and even microbiome factors influence metabolic pathways and immune response to pathogens although further experiments should be performed to better comprehend these possibilities (Rutkowski and Kaufman 2007; Chan et al., 2019).

2.2. Zika virus

Zika virus (ZIKV) was first identified from a rhesus monkey blood sample in 1947 in the Zika forest of Uganda (Dick 1952). Infection in humans was first described in Nigeria in 1954 and the first reported outbreak was in 2007 on the Western Pacific island of Yap in the Federated States of Micronesia (Duffy et al., 2009). This outbreak was later followed by an epidemic in 2013 and 2014 in French Polynesia where the number of 30,000 symptomatic infections were estimated and followed by other outbreaks where infections were associated with several neurological complications, including the Guillain-Barré syndrome that causes paralysis (Gardner and Ryman 2010; Clements and Harbach 2017; Waggoner et al., 2018). After this epidemic several other outbreaks took place throughout the Asian continent. In 2015 ZIKV was first spotted in the Americas and, by the beginning of 2016 autochthonous cases had already been registered in more than 20 countries and territories from North, South and Central Americas, and the Caribbean (Musso and Gubler 2016).

ZIKV is mainly transmitted by *Aedes aegypti* and *Aedes albopictus* (Diptera, Culicidae) mosquito vectors of the *Aedes* genus. The infection can be asymptomatic, but it is usually common to have mild fever, rashes, arthralgia, and conjunctivitis. Although these are the most common and prevalent symptoms, there have been several cases where the infection caused fetal loss in pregnant women, microcephaly of newborns, or even newborn brain/eye abnormalities (European Centre for Disease Prevention and Control 2015; Pan American Health Organization 2015; Johansson et al., 2016; Mlakar et al., 2016; Schuler-Faccini et al., 2016; Chimelli et al., 2017). Besides the horizontal transmission (from the bite of infected *Aedes* mosquitoes) and the vertical (from pregnant mother to child) it has also been reported that Zika virus can be transmitted in blood contact, as in blood transfusions, organ transplantation, sexually, in research laboratory exposure and even urine or saliva (Centers for Disease Control and Prevention 2019).

The Zika virus genome was sequenced in 2007 from the African ZIKV strain (MR 766), and comprises the three structural and seven non-structural *Flavivirus* proteins. The ZIKV E protein compared to the Dengue virus structure, which is glycosylated at Asn67 and Asn153 (important for viral release), is glycosylated at Asn154. Such variations among flaviviruses are important determinants for antibody specificity and recognition (Wang et al., 2017). Regarding the nonstructural proteins, which are mostly responsible for the replicative complex and participating against host innate immunity, the ZIKV NS1 has a divergent electrostatic potential on its loop surface when compared to other flaviviruses and such region is related to binding host factors and antibodies (Brown et al., 2016).

Analysis of ZIKV transcriptome through RNA-seq and effects on host cells has shown that the lncRNAs (long non-coding RNAs), can be involved in innumerable cellular processes such as RNA replication, gene expression, cell cycle, amino acid transport, act as regulatory factors during ZIKV infection to evade host immunity, and even induce cell apoptosis by decreasing apoptotic regulators (Rinn and Chang 2012). It was suggested that changes on the host transcriptome are directly connected to the cellular activation responses or pathways (Tang et al.,

2016; Ramaiah et al., 2016). ZIKV infected human umbilical vein endothelial cells transcriptome profiling revealed activation of pro-inflammatory cytokines (IL-1 β), which indicates caspase-1 activation that can lead to severe inflammation and cell death, and chemokines such as CCL2 (C–C Motif Chemokine Ligand 2), CCL5 (C–C Motif Chemokine Ligand 5), GM-CSF (Granulocyte Macrophage Colony-Stimulating Factor), G-CSF (Granulocyte Colony-Stimulating Factor), CXCL1 (C-X-C Motif Chemokine Ligand 1), and CXCL12 (C-X-C Motif Chemokine Ligand 12) possibly because ZIKV infection promotes chemotaxis of neutrophils and leukocytes across the endothelial barrier in the placenta. Also, cytochrome c release caused by caspase-8 leading to endothelial cell apoptosis was observed, and matrix metalloproteinases (MMPs) expression was affected by infection with ZIKV, showing upregulation of MMP10 and MMP13, which is commonly associated with dysregulation of the extracellular matrix leading to inflammation and tissue destruction (Khaiboullina et al., 2019).

A study on subgenomic ZIKV RNA (sRNAs, 300 – 500 bases non-coding RNA) showed that ZIKV 3'UTR can interrupt the cellular 5'–3' exoribonuclease XRN1 as it tries to degrade viral transcripts inside the infected cell, resulting in numerous copies of sRNAs and disturbing gene expression regulation to decrease cell response to viral infection. These sRNAs have also been shown to dysregulate post-transcriptional processes by sequestering proteins involved in such events, splicing and nucleic acid editing, generating aberrant mRNA products. Besides, sRNAs are significantly related to increase of short-lived cellular mRNAs and their stability along with sequestration of RNA decay factors diminishing transcripts decapping and deadenylation (Michalski et al., 2019).

Analysis of microglial cells during ZIKV infection has revealed a high number of pro-inflammatory mediators such as IL-6, TNF- α , IL-1 β , and IFNs, which demonstrates neuroinflammation of microglia, along with nitric oxide (NO) produced by these neuronal cells under the influence of inflammatory cytokines, which leads to degeneration, demyelination and neuronal death (Douglas et al., 2016; Chan et al., 2019). In the same study, metabolomics data generated via Liquid Chromatography High-Resolution Mass Spectrometry (LC–HMRS) revealed modulation of lipid production during infection. An increase in expression of lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), phosphatidylcholine (PC), ceramide, and sphingomyelin was observed, in contrast to a decrease in phosphatidylserine levels (PSer) (Diop et al., 2018). The high levels of lipid forms have been related to activation and release of inflammation modulators, and therefore, the activation of neuroinflammation pathways that leads to cell deterioration (Ghoshal et al., 2007; Brown and Vilalta 2015; Freeman et al., 2017). The study also showed an increase in carboxylic acids, i.e. dodecanedioic and undecanedioic acids, which act as important substrates in the central nervous system, and a decline in amino acids L-valine and 3 methyl-L-phenylalanine levels (Alexander et al., 1998; Diop et al., 2018). Plasmalogens levels, ether-linked glycerophospholipids synthesized by peroxisomes and major constituents of neural membranes, were detected as increased during viral infection and it has been suggested that viruses can utilize such compounds for envelope assemblage (Queiroz et al., 2019). Phosphatidylethanolamine (PE), which is important for binding host cell receptors and mediation of virus entry, was also found increased in ZIKV patients (Perera-Lecoin et al., 2013). These alterations suggest that viruses create strategies for a better replication environment, success in entry and particle building, hijacking host cell lipids and inducing inflammatory response (Cao–Lormeau 2014; Musso and Gubler 2016; European Centre for Disease Prevention and Control 2016). Through Direct Infusion Mass Spectrometry (DIMS) for analysis of human serum metabolites during ZIKV infection, it was detected that PI phosphates (PIPs) and angiotensin levels were increased, probably since these molecules contribute to mTOR signaling to prevent autophagy. However, the NS4A and NS4B proteins from ZIKV can inhibit mTOR pathway signals to induce cell autophagy and escape this defensive mechanism (European Centre for

Disease Prevention and Control 2015; Pan American Health Organization 2015).

A recent study, which combined two complementary methods (yeast two-hybrid screens – Y2H – and tandem affinity purification coupled to mass spectrometry – TAP-MS), showed a total of 189 protein-protein interactions between virus and host, where twenty-nine human proteins were shown to interact with more than one ZIKV protein, evidencing that different ZIKV NS proteins have common human proteome targets (Golubeva et al., 2020). Targets such as components of CCT (chaperonin containing TCP1 or TriC-TCP-1 ring complex) were identified, as they play a role in trafficking telomerase and small Cajal body RNAs (CB), which are critical for neuron biology (Lafarga et al., 2017). NS5 demonstrated to increase the number of CBs per cell and reduce their volume, which could lead to CB fragmentation (Coyaud et al., 2018). NS1 was found to interact with CBs and CCT complex components, which evidences it may play a role in CB stability and neural disorders (Golubeva et al., 2020). NS5 was also shown to be modulated by PIAS1, a modulator of STAT1 activated by type I interferon signaling (IFN-I) (Grant et al., 2016). PIAS1 reduces NS5 protein half-life and levels, although it is not known yet how it may affect ZIKV replication in host cells. In the same study (Golubeva et al., 2020), NS3 was suggested to interact and interfere in centriole duplication, which could be important for the microcephaly phenotype caused by ZIKV, as centrosomal abnormalities result in impaired mitosis. NS1 and NS2A have also been identified to target host factors related to mitotic phenotypes (Barbelanne and Tsang 2014; Golubeva et al., 2020). Therefore, valuable results were presented for comprehension of the central nervous system perturbations caused by ZIKV and identification of potential infection interaction targets. Also, related to the virus-host protein-protein interaction, other studies were able to identify interaction among virus and host proteins, such as the interaction of ZIKV capsid protein with nucleolar proteins involved in cell growth regulation and maintenance, along with other RNA-binding proteins. These interactions work either in favor or against replication, as several knock-down experiments proved a significant reduction in viral load due to gene depletion or significant increases due to certain proteins being depleted (Brown et al., 2016; Musso and Gubler 2016; Wang et al., 2017; Lin et al., 2018; Centers for Disease Control and Prevention 2019). Nonsense-mediated decay (NMD) pathways and exon-junction complex (EJC) were also found to be targets of ZIKV capsid along with DENV and WNV (Lindenbach and Rice 1999; Lin et al., 2018). NMD pathways were shown to be responsible for viral restriction, as its depletion resulted in an increase of 50% in viral replication and moderate antiviral activity was found for EJC complex (Fontaine et al., 2018). Furthermore, ZIKV capsid has also been reported as the only one specifically associated with factors related to neuronal development and disorders (Scaturro et al., 2018; Scaturro et al., 2019).

Proteome expression affected by ZIKV infection has also been studied. The infection with a Brazilian ZIKV isolate on human neurospheres revealed that 259 proteins were upregulated and 199 downregulated, including proteins related to the arrest of cell cycle progression, neuronal differentiation, and viral replication (Garcez et al., 2017). Among the upregulated, proteins related to activation of DNA repair machinery and identification of hyperphosphorylation events in DNA damage signaling pathways, including induction of double-strand DNA breakage and activation of DNA damage response were identified (Hammack et al., 2019). Downregulation of neuronal factors affecting neuronal differentiation and neurogenesis, and upregulation of antiviral response pathways such as type-I interferon response genes were also identified (Scaturro et al., 2018). Also, phosphoproteomic analysis of ZIKV infected cells revealed proteins that were modulated related to cellular functions such as cellular assembly and organization, cell-cycle regulation, neurological development, and diseases with phosphorylation and dephosphorylation of substrates being involved (Scaturro et al., 2018; Scaturro et al., 2019).

2.3. Dengue virus

Dengue virus is a very known pathogen that includes four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4 (Halstead 1988), which are spread in Asia, Africa, and in the Americas and share around 65% of their genome, but the severity of the disease can vary among the serotypes (Guzman et al., 2010). A predominance of DENV-2 being more related to shock and hemorrhagic manifestations has been observed, followed by DENV-3, while DENV-1 presented milder clinical manifestations and DENV-4 as well (Balmaseda et al., 2006). Despite these results, it must be considered that the sequence of infection with different serotypes may influence the severity of disease manifestations, and that infection by one serotype does not guarantee long-term immunity to the others (Rothman 2011). Historically, there are reports in the literature of its occurrence and epidemics from as early as 1779 in Asia, Africa, and North America, however, records from a Chinese encyclopedia of diseases, symptoms and remedies date the occurrence of infections from 265 A.D., where it was called “water poison” by the Chinese people who believed it was related to flying insects and water (Ooi and Gubler 2011). Nowadays, DENV affects more than 100 countries in the Asian, European, and American continents and it is estimated that over two billion people live in dengue-endemic areas, and data suggests that at least 400 million infections occur every year (Gubler 2002; Guzman et al., 2010; Wilder-Smith et al., 2010; Ferreira 2012; Hasan et al., 2016).

Dengue fever is characterized by flu-like symptoms such as fever episodes, strong headache, myalgia, retro-orbital pain, arthralgia, body rashes, and gastrointestinal discomfort. The infection can be asymptomatic, mild (grades I and II), or more severe cases such as dengue hemorrhagic fever, where the symptoms are similar to dengue fever and include hemorrhagic manifestations, coagulopathy, vascular fragility, permeability and thrombocytopenia, which are considered grade III and IV (Martina et al., 2009). The main vector for DENV transmission is the Culicidae mosquito from the *Aedes* genus, more commonly *Aedes aegypti*, and transmission rates increase every year due to globalization, easy mobility, and distribution of people, besides better adaptation of the vectors to urban spaces (Patramool et al., 2011).

Analysis of *A. aegypti* transcriptome with DENV revealed an expression profile of 20 upregulated and 15 downregulated genes on day one of the inoculations in the mosquitoes. Within the upregulated were the expression of the juvenile hormone-inducible protein gene that regulates expression of many other genes, along with the core histone H3 gene, probably related to the fact that the virus targets host chromatin and histones to disturb gene expression and other nucleic mechanisms. A gene that expresses a mosquito allergen protein that binds human IgE was found 20-fold downregulated on the first day of mosquito inoculation along with others such as pupal cuticle protein (PC), matrix metalloproteinase (MMP), an elongase protein, and an aquaporin protein gene (Colpitts et al., 2011). A total of 405 genes were found to be expressed differently due to viral inoculation, and they were categorized into groups belonging to biological process (BP), molecular function (MF), and cellular component (CC). Among the upregulated genes related to BP, around 3% were related to DNA-dependent transcription regulation and 2% to phosphorylation of protein amino acids. Related to MF, were transcripts responsible for zinc ion binding, nucleotide-binding, and transcription factor binding. The down-regulated genes belonging to the BP category were transcripts involved in chitin metabolism, transport, and proteolysis, and the ones within the MF group were associated with structural constituents of cuticle and serine-type endopeptidase activity. After 10 days of inoculation, most of the upregulated genes were intracellular, while most of the downregulated ones were extracellular. The most significant BPs upregulated were involved in transcription and phosphate metabolism, and the MFs were related to ion binding. Downregulated genes within the MF group had major involvement in peptidase activity, chitin-binding, and ion binding (Colpitts et al., 2011). In general,

metabolic and oxidoreductive processes are greatly affected by DENV infection in *Aedes*, such as genes involved in reducing oxidation and zinc ion binding, along with chitin metabolism and other metabolic processes (Xi et al., 2008).

Transcriptome meta-analysis of DENV infection microarray data sets was able to identify several perturbations in gene expression during infection (Afroz et al., 2016), such as in type-I interferon response gene transcripts OAS2 (2'–5'-Oligoadenylate Synthetase 2, an oligoadenylate synthetase isoform), which promotes ATP oligomerization and later activation of RNase L to degrade viral RNA and replication (Thamizhmani and Vijayachari 2014); and USP18, responsible for inhibition I-Interferon signaling during infection (Santin et al., 2012). Other genes, such as ATOX1 (antioxidant protein 1), related to oxidative stress, were also found upregulated, along with downregulation of genes involved with extracellular matrix components PATJ (Pals1 Associated Tight Junction protein) and CRTAP (Cartilage associated protein), which may be involved in a mechanism of DENV infection restriction by altering permeability and penetration of the virus in target cells, however, it is a mechanism yet to be more studied and comprehended (Afroz et al., 2016).

Through RNA sequencing (RNAseq) of a DENV1 strain isolated from a patient and an attenuated strain derived from the patient, it was possible to analyze over 18,000 modifications in the host transcriptome and over 41,000 in response to the attenuated strain. Regulation of different isoforms is the most relevant effect on infection with both types of strains, and the study suggested regulation of several RNA binding proteins, interfering in host splicing events, modulation of protein translation, pathways related to cellular growth and proliferation, and viral infection. Pathways related to inflammation, induction of apoptosis, and cell stress were mostly triggered by the attenuated DENV strain. The study also suggested that responses associated with cell signaling and metabolism were mostly related to the wild viral strain, while pathways related to transcriptional activation, cell cycle modification, and post-translation modulation were mostly linked to the attenuated virus strain. In general, the pathways implicated in DENV virus infection were related to splicing post-transcriptional modification, lipid metabolism changes, oxidative phosphorylation, autophagy, apoptosis, endoplasmic reticulum stress and virus recognition, among others (Sessions et al., 2013).

Characterization of metabolites from Dengue fever (DF) and Dengue hemorrhagic fever/ severe dengue (DHF) patients' blood sample was performed through Liquid-Chromatography Mass Spectrometry (LC-MS) where it was observed an alteration in metabolites such as bile acid components, acylcarnitine, phosphatidylcholine (PC), lysophosphatidylcholine (LysoPC), amino acids, uric acid from purine catabolism and pyrimidines (Cui et al., 2018). The bile acids metabolites levels, which are end products of cholesterol metabolism, were significantly higher in DHF patients due to liver dysfunction, as hepatocytes could be a viral target for DENV replication which can be observed by tissue damage in infected patients; while acylcarnitines, phenylalanine, and most lipids were in relatively lower levels when compared to DF patients. When analyzing the metabolic pathways, it was found that bile acid biosynthesis, fatty acid β -oxidation, phenylalanine, tryptophan, purine, and pyrimidine metabolism, and phospholipid catabolism were the main pathways disturbed (Cui et al., 2013).

^1H Nuclear Magnetic Resonance Metabolomics of plasma samples from patients in Recife, Brazil compared five groups: patients with DF during primary infection (DFp), patients with DF secondary infection (DFs), patients with dengue hemorrhagic fever or severe dengue primary infection (DHFp), patients with DHF during secondary infection (DHF s) and patients with ND-related disease (non-dengue). The results obtained through multivariate analysis and identification of metabolites showed a general alteration of lipid metabolism among the groups in the study, with VLDL (very low density lipoprotein) and LDL (low density lipoprotein) concentration and lipoprotein signals being responsible biomarkers for discrimination of the DF and DHF (both primary and

secondary infections) from ND-related disease. DHF patient samples showed a great decrease in these lipoproteins concentrations, even more than DF samples, and these alterations are possibly linked to the fact that DENV infection affects lipid metabolism and therefore, causes liver dysfunction, as it is the main organ related to lipid metabolism pathways (El-Bacha et al., 2016; Samsa et al., 2009; Heaton and Randall 2010b; Soto-Acosta et al., 2013). Also, other metabolites, such as N-Acetyl glycoproteins, glutamine, acetate, citrate, choline, valine, tyrosine, and histidine were altered and contributed to the differentiation among the groups of samples (El-Bacha et al., 2016).

NMR spectrometry metabolomics analysis of urine samples to search for metabolite biomarkers of DENV infection showed that some metabolites such as acetoacetic acid, 4-hydroxyphenylpyruvic acid, fructose, S-sulfocysteine, acetoacetic acid, betaine, and valerylglycine were in high concentrations when compared to healthy urine samples (Fontaine et al., 2018; Li et al., 2019). Some metabolites showed a drastic reduction in concentration such as creatinine, N-acetylglutamic acid, citrate, succinic acid, creatine phosphate, and 3-hydroxy-3-methylglutarate. Acetoacetic acid and 4-hydroxyphenylpyruvic acid are related to amino acid metabolism - phenylalanine - showing that DENV infection affects this metabolic pathway increasing these compounds (Birungi et al., 2010). In addition, it was found an increase of S-sulfocysteine in infected samples, a metabolite that is produced by the association of cysteine with endogenous sulfite, and, in high levels, it could cause neurological damage which has been linked to DENV-3 infection (Solomon et al., 2000). Valerylglycine, which was found increased in infected patients, is mostly related to disorders caused by mitochondrial fatty acid β -oxidation and could be related to the febrile condition that patients have. Depletion of N-acetylglutamic acid, which is important in the urea cycle allowing conversion of ammonia to urea, may result in liver failure and hepatic encephalopathy due to accumulation of these nitrogenous metabolites, and therefore, this metabolite possibly indicates acute liver failure in infected patients (Giri et al., 2008). Also, creatinine and creatine phosphate are indicators of glomerular filtration and their depletion may be a biomarker for renal dysfunction in DENV infected patients (Shahfiza et al., 2017).

Serum analysis of Mexican and Nicaraguan patients through hydrophilic interaction liquid chromatography (HILIC-MS) provided the identification of disturbed metabolites such as lysophosphatidylcholines (lysoPCs) and long-chain polyunsaturated fatty acids (α -linoleic acid - ALA), docosahexaenoic acid (DHA), and arachidonic acid (AA) for DF and DHF, which is similar to results found in an investigation of affected metabolism during ZIKV infection (Voge et al., 2016). DENV-2 serotype infection in a humice model (humanized mice) serum metabolomics analyzed via mass spectrometry at different periods, identified in total 48 metabolites including fatty acids, amino acids, purines, pyrimidines, acylcarnitines, acylglycines, phospholipids, sphingolipids, free fatty acids, and bile acids. All of these showed a different pattern depending on the number of days post-infection (0, 3, 7, 14, or 28 dpi) (Cui et al., 2017). It was concluded that the main pathways disturbed were related to fatty acid β -oxidation, phospholipids catabolism, bile acid biosynthesis, purine and pyrimidine metabolism, tryptophan, lysine, and phenylalanine metabolism, which are similar results to previous metabolomics studies and show commonalities between human and humice responses to DENV infection (Heaton and Randall 2010a; Grant et al., 2016; Lafarga et al., 2017; Liu and Cherry 2019).

Overlapping metabolomics results were shown with DENV and Influenza virus (IVI) infection, as both diseases overlap geographically in tropical and subtropical regions. Common metabolites involved in fatty acid biosynthesis and β -oxidation, amino acid metabolism, phospholipid catabolism, sphingolipid metabolism, and steroid hormone pathways were observed. Some differences were also spotted, for example, DENV seems to attenuate metabolites such as serotonin, bile acids, biliverdin, phospholipids, and acylcarnitines, and appear to have a more intense effect on fatty acids β -oxidation and lipid metabolism, while IVI significantly elevated hypoxanthine, inosine and xanthine

levels from the purine metabolism pathway (Cui et al., 2017a,b). Overall, IVI metabolic changes were significantly less dramatic than DENV alterations, which might suggest that IVI has milder effects on hosts when compared to DENV, as IVI affects respiratory areas more intensively, and DENV has a systemic effect with cellular targets that include dendritic skin cells, liver cells, macrophages, and lymph nodes, spleen and blood monocytes (Hertzog et al., 2018; Khaiboullina et al., 2019; Lin et al., 2019).

2.4. Saint Louis Encephalitis virus

Saint Louis Encephalitis virus (SLEV) was first reported in the USA during a human encephalitis outbreak in Saint Louis in 1933 (Heinen et al. 2015). In Brazil, it was first isolated from mosquitoes at the Amazon basin in the 1960s and later in the Amazon region and in Sao Paulo from both animals and arthropods (Vasconcelos et al., 1998), but human isolations were only described by 2005 from patients with febrile illness without neurologic symptoms and recent isolations in Sao Paulo from patients with incorrect Dengue diagnosis (Mondini et al., 2007). The virus strains show different levels of severity, viremia, and neurological damage in its wide geographical and temporal diversity as it is well dispersed through the Americas. SLEV belongs to the Japanese encephalitis virus complex, and it is maintained and disseminated through *Culex* spp. or even other mosquitoes as vectors of the disease, while humans and other animals are accidental final hosts and birds are amplifiers (Hoyos-López et al., 2015). It is divided into 8 genotypes according to phylogenetic analysis (Rodrigues et al., 2010), and genotypes I and II are mainly found in the USA and Canada, while genotype V is located in South America. Genotype III is distributed in Southern America, IV in Colombia and Panama, VI only in Panama, VII in Argentina, and VIII is restricted to the Amazon region in Brazil (Balmaseda et al., 2006; Guzman et al., 2010). The genetic diversity level of SLEV isolates is not very high (only 10.1% of nucleotide divergence has been found in isolates and 5.5% of nucleotide divergence within lineages) even though mutation rates are considerably fast similar to other viruses of the Japanese Encephalitis complex (May et al., 2008). In most cases, SLEV infection can be asymptomatic or mistaken by another viral infection or disease. However, when symptoms occur, it is usually characterized by viremia, fever, and headaches that are neutralized by the host defense, if not, it can evolve to a secondary viremia affecting the central nervous system into a meningoencephalitis case which can be lethal or can leave severe neurological sequelae in survivors (Carballo et al., 2016).

Homology analysis of the SLEV structural and non-structural proteins revealed a range of 64 - 73% of amino acid sequence homology among the encephalitis group including SLEV/Murray Valley and West Nile virus, while the comparison to other *Flavivirus* outside the encephalitis complex showed around 42% of sequence identity. Sequence comparison with other *Flavivirus* revealed that the envelope and NS1 protein is highly conserved, while the capsid protein. NS2A and NS2B, for example, are more distinct among the family, although the overall structure is well conserved in general (Trent et al., 1987). Through phylogenetic analysis containing 27 SLEV strains, a relative homology profile between them was observed (Diaz et al., 2015).

There has not been any study relating metabolome analysis during SLEV infection in patients or other animal hosts, possibly since metabolomics is a recent and emerging “omic” and is applied as a complement to systems biology to better understand how these pathogens affect their hosts and how possible antiviral molecules can be applied and their effects on both host and virus.

2.5. Japanese Encephalitis virus

The Japanese Encephalitis virus (JEV) is responsible for the Japanese encephalitis disease, which is an inflammatory brain disease that can cause irreversible damage and that is very common in Asia, Western

Pacific countries, and northern Australia (Yun and Lee 2014). It is suggested that JEV derived from an ancestral virus in the Malay Archipelago several thousands of years ago into different genotypes and spread through Asia, where it occurred mainly as encephalitis outbreaks. In 1871, the first case of Japanese Encephalitis was registered in Japan and other outbreaks followed in subsequent years. In 1924, the virus was isolated from human brain tissue and in 1938 it was demonstrated that pigs and birds are reservoir hosts (Erlanger et al., 2009).

There are two well defined epidemiological patterns to JEV: epidemic and endemic. In temperate countries such as China, Japan, South Korea, and Nepal, outbreaks usually occur when temperature and rain levels increase during the summer; meanwhile, in tropical and subtropical countries like Malaysia, Indonesia, Sri Lanka, and Vietnam, outbreaks occur all year round and peaks during the rainy season. JEV control is mainly via vaccination which is relatively effective, although poor and rural communities are still challenging areas for vaccine delivery (Martina et al., 2009; Wilder-Smith et al., 2010). JEV is transmitted by the *Culex tritaeniorhynchus* mosquito, which breeds in irrigated rice fields and infects mainly water birds, though the virus eventually spreads to pigs, equids, and finally humans (Fig. 4). Other mosquitoes from the *Culex* and *Anopheles* genus have also been reported as JEV vectors such as *C. pipiens*, *C. bitaeniorhynchus*, *C. modestus* and *A. sinensis*, although *C. tritaeniorhynchus* remains as the main vector. The symptoms of JEV infection are commonly mistaken by other types of encephalitis. The virus can cross the blood-brain barrier and manifest as high fever, spasms, and, in acute and severe cases it can cause irreversible brain damage that leads to mental disorders and even severe dystonia (Fang et al., 2019).

The JEV is the prototype of the Japanese encephalitis serogroup comprised of other *Flavivirus* such as West Nile virus, Saint Louis Encephalitis virus, Murray Valley virus, and other four lesser-known viruses, and genetically it is very close to other arthropod-borne viruses like Zika, Dengue, Yellow Fever virus and also the tick-borne

encephalitis virus (TBEV) (Wang and Liang 2015). The NS1 protein for the JE serogroup (NS1') is elongated and has 52 extra amino acids to its C-terminus due to a 1- ribosomal frameshifting at codons 8 - 9 of NS2A. The nucleotide sequence of the partial or complete viral genome divides JEV into five main genotypes GI - GV also based on their geographic patterns and distribution. The Indonesia and Malaysia regions have all five genotypes identified and isolated; the Australia and New Guinea regions have GI and GII; Thailand, Cambodia, and Vietnam isolated GI, GII, and GIII; Japan, Korea, and China have GI and GIII, while India, Sri Lanka, and Nepal have only GIII. The GIV and GV genotypes are considered the oldest and most divergent ones that still circulate in the Indonesia-Malaysia region where it was probably originated, but GV was also isolated in China in 2008, which demonstrates their current spread to other regions as well (Yun and Lee 2014).

A study with cDNA microarray for analysis of host global transcriptomic responses in mice have shown differences in infected and control groups within 5 days post-infection (dpi), showing that around 833 genes were upregulated while 479 were downregulated after 5 dpi, which correlates with viral replication, inflammation and immune response caused by the infection. Expression of several cell surface receptors in mice brain was also observed along with correlation to immune, inflammatory, and defense response, cell adhesion, endocytosis, and chemokine signaling. Most of these receptors were lectin receptors, involved in immune response such as Clec7a (C-Type Lectin Domain Containing 7A), Clec5a (C-Type Lectin Domain Containing 5A), Clec4e (C-Type Lectin Domain Family 4 Member E), and Masp2 (Mannose-binding protein-associated serine protease 2), and receptors related to inflammatory response were mostly Tlr2, Tlr7 (Toll-like receptors), Edg3 (Sphingosine-1-phosphate receptor 3), among others. Cell adhesion family receptors such as L-selection (Sell), intercellular adhesion molecule-1 (Icam1), vascular cell adhesion molecule-1 (Vcam1), and others were also upregulated 5 dpi, and Gpr84 (G protein coupled receptor 84), related to signal transduction was increased in expression as the disease progressed, along with an increase in defense response-related receptors, cytokine and chemokine signaling receptors. In general, genes related to activation of the immune response, signaling cascades, cell adhesion, apoptosis, inflammatory response, protein processing, chemotaxis, and others were shown to be similar to other neuroinvasive viruses like West Nile virus (WNV) (Gupta and Rao 2011).

There is evidence of JEV regulation of host microRNAome in human microglial cells at different time intervals of 6, 24, and 48 h post-infection, with enhanced expression patterns for miR-3646, miR-4470, miR-572, and miR-3687, which suggests their association with JEV pathogenesis. Regulatory RNA, which competes with mRNAs for providing binding sites to corresponding miRNAs, is a new class called competing endogenous RNA (ceRNA) and have been identified in this study as 8 mRNAs that can act as ceRNA and interact with miRNAs to regulate innate immune pathways, along with several other complex host microRNAs that are intimately associated with JEV infection whether suppressed or overexpressed. Also, miR-34c-5p was found to be suppressed in JEV infection, and when overexpressed, it modulates proinflammatory cytokine production induced by JEV infection probably by downregulating the Notch signaling pathway activation, that mediates microglia cells maturation and activation to maintain normal brain function and regulates immune response (Grandbarbe et al., 2007; Hamilton Outtz et al. 2010; Kumari et al., 2016). Microarray analysis for mice brain during JEV infection provided an insight into the transcriptomic landscape with changes in expression and regulation of mRNAs and lncRNAs (long non-coding RNAs, important regulators of gene expression). A total of 618 lncRNAs and 1007 mRNAs were found differentially expressed, and within these results, 116 lncRNAs and 795 mRNAs were upregulated, while 402 lncRNAs and 212 mRNAs were downregulated. Analysis of the pathways related to such changes in expression revealed that the most significant up-regulated lncRNAs were involved with innate immune and inflammatory response, for example,

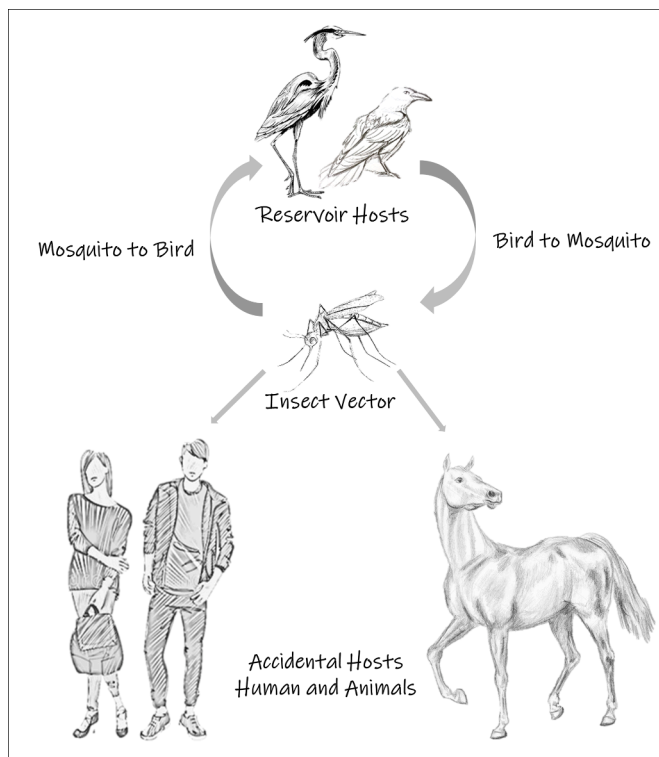


Fig. 4. West Nile and Japanese Encephalitis virus biological cycle. Illustration of the reservoir hosts, which are usually birds, the insect vector, mainly of the *Culex* sp., and the accidental hosts such as humans in urban cycles and horses in rural cycles for both WNV and JEV.

some lncRNAs that could trigger the MKK4/JNK pathway (Mitogen-Activated Protein Kinase Kinase 4 - MKK4; c-Jun NH2-terminal kinases - JNK) related to the regulation of neuroinflammation caused by JEV infection and other inflammatory pathways were detected. The apoptosis process, acetylation, nucleotide-binding, and defense response to the infection pathways were also identified. Pathways such as Toll-like receptor signaling pathway, the Janus kinase-signal transducer and activator of transcription Jak-STAT signaling pathway, Mitogen-activated protein kinase MAPK signaling pathway, chemokines, and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) - PI3K-Akt - signaling pathway were the most significantly upregulated, and the downregulated pathways were involved in ion transportation, cell adhesion, synaptic transmission, and signal transduction, such as calcium signaling, retrograde endocannabinoid and neuroactive ligand-receptor signaling (Li et al., 2017).

Quantitative proteomics analysis of JEV infected HeLa cells showed a variety of regulated proteins related to immune response reactions, to the ubiquitin-proteasome system, intracellular membrane system, and lipid metabolism (fatty acid and cholesterol). Also, protein functional studies indicated interferon-induced transmembrane protein 3 (IFITM3), Ran-binding protein 2 (RANBP2), sterile alpha motif domain-containing protein 9 (SAMD9) and vesicle-associated membrane protein 8 (VAMP8) as antiviral factors during JEV infection that were changed in cells and tissues at the mRNA level, and whose knock-down can increase viral load in cells (Zhang et al., 2013).

2.6. West Nile virus

West Nile virus (WNV) also belongs to the Japanese Encephalitis virus serogroup. It was first isolated in 1937 in the West Nile district in Uganda from the blood of a woman suffering from febrile illness, and later through the decades, several cases of WN fever were documented in Israel, Egypt, France, and South Africa until the 1990s where major outbreaks occurred and expanded global awareness. Since 1999, when it first emerged in New York and spread throughout the Western Hemisphere it has caused thousands of fatalities in humans and birds (Smithburn et al. 1999; Asnis et al., 2001; Murgue et al., 2001; Nash et al., 2001; Murray et al., 2010). Nine lineages of WNV have been proposed, but only lineages 1 and 2 have been credited for human outbreaks (Fall et al., 2017). Lineage 1 is composed of two main clades: 1a, which comprises strains from Europe, Africa, and the Americas; and 1b, restricted to Oceania and also called Kunjin virus; meanwhile, lineage 2 was reported in Africa until 2004 and isolated from birds and humans in Hungary, Italy and Greece (Lanciotti et al., 1999; Hall et al., 2001; Murray et al., 2010; Rizzoli et al., 2015). It was considered less pathogenic than lineage 1, however, it caused severe encephalitis outbreaks in humans and birds in South Africa, so it was concluded that both lineages 1 and 2 present different levels of pathogenicity of strains. Other less spread lineages are lineage 3, also known as Rabensburg virus, isolated in the Czech Republic (Hubálek et al., 1998; Hubálek and Halouzka, 1999), lineage 4 (Russia) (Lvov et al., 2004), lineage 5 (India) (Lanciotti et al., 2002), a putative lineage 6 described in Spain (Vázquez et al., 2010), lineage 7 which is very rare was isolated from ticks and rodents (Charrel et al., 2003), lineage 8 isolated in Senegal in 1992 from *Culex perfuscus* (Gould et al., 1997), and finally, lineage 9, also considered as sublineage 4 isolated in Austria from *Uranotaenia unguiculata* mosquitoes (Pachler et al., 2014).

Culex mosquitoes are the main vectors for WNV transmission with different species such as *C. tarsalis*, *C. quinquefasciatus*, *C. stigmatosoma*, *C. thriambus*, *C. pipiens* and *C. nigripalpus* that can feed on both mammalian and avian hosts, and there are also some reports of transmission by *Aedes* mosquitoes, although they are not the primary vectors in nature. WNV maintains a cycle between mosquitoes and animal hosts with birds as their main reservoir (Fig. 4), which usually become ill with some symptoms of the disease and may die, but others can survive as carriers without any symptoms. Equine infection has also been reported

and these animals usually develop acute febrile illness that may lead to death by neurological complications. Humans, on the other hand, are considered the dead-end hosts and may present a wide range of symptoms, from asymptomatic or mild febrile illness, rashes, and myalgias to neurological diseases such as meningitis, encephalitis, or acute flaccid paralysis that may cause death (Flores and Weiblen 2009; Colpitts et al., 2012; Fall et al., 2017).

Proteomic analysis of WNV infection shows a variety of proteins expressed in neurons that range from 150 kDa to 15 kDa at a pH 5 - 6. Among them are Ras-GTPase and SH3-domain-binding proteins, which have been shown to be very important for activation of the Ras signal transduction pathway and their role in cell cycle regulation and apoptosis process. Another protein found to be increased in WNV infected neurons was Restin, which shows homology with endostatin and has a role in the inhibition of cell proliferation and induction of cell apoptosis along with the β -amyloid peptide that suggests activation of apoptotic pathways leading to cell death. Likewise, the Ret finger protein (RFP) was increased during infection, and it is known to be able to activate two cell death pathways: via caspase-independent of mitochondrial events and the pathway via a stress-activated MAP kinase. It was then observed that WNV leads to neuron death causing fragmentation and shrinkage with loss of morphological structure and neuronal activity (Dhingra et al., 2005).

Analysis of protein expression during WNV infection in Vero cells showed that 93 proteins involved in transcription, translation, cytoskeletal and cell mobility, chaperones, energy pathways, apoptosis, lipid and nucleotide metabolism and regulation, binding and transport, and ubiquitin-proteasome pathway were present, with several of them suggesting different isoforms and possible post-translational modifications. Tubulin α and vimentin were up and down-regulated, respectively, and this is probably because flaviviruses and other viruses manipulate host cytoskeleton elements for viral infection and virion release. Also, infection requires organelle movement and cargo transportation which requires cytoskeleton rearrangement. Vimentin also has a cell-protective function for preventing viral entry in the cytoplasm and regulation of cell death via caspase-mediated cleavage, which could be related to the induction of apoptosis that WNV promotes in host cells. The heat-shock protein Hsp70 plays a role as a protector from cell apoptosis, which explains its abundance during WNV infection, while Hsp90 was down-regulated, probably as a protective mechanism against WNV (Pastorino et al., 2009). Upregulation of heterogeneous nuclear ribonucleoproteins (hnRNP)-L and (hnRNP)-U was identified in WNV infection of Vero cells, and this shows that these proteins, which participate in mRNA biogenesis, RNA binding and protein interactions are essential for WNV replication process along with the nonribosomal regulating protein nucleolin (Piñol-Roma 1997; Noisakran et al., 2008). And finally, the apoptosis-inducing factor mitochondrion-associated 1 (PDCD8/AIF) was also upregulated during infection which reflects WNV induction of apoptosis networks along with stress by disturbance of the endoplasmic reticulum membranes for viral assembly (Pastorino et al., 2009; Fraiser et al., 2013).

Avian host zebra finch transcriptional response to WNV infection was analyzed and similarities to mammalian hosts response were observed, as both the innate and adaptive immune response pathways are activated by the infection. The retinoic acid-inducible gene 1 (Rig-I)-like receptor (RLR) pathway plays a key role in viral clearance by recognizing viral particles and initiating interferon expression (Errett et al., 2013), and this study found that 5 genes that are related to this pathway were significantly upregulated in zebra finch response 4 days post-infection (dpi). Similarly, T-cell immunoglobulin mucin receptor 1 (TIM1) was upregulated 2 dpi in zebra finches. In human cells, this receptor is known for viral entry (Jemielity et al., 2013), which shows that possibly the same process occurs in avian hosts, as well as chemokine C-C motif production that was also upregulated in zebra finches and the upregulation of the apolipoprotein D (APOD) gene, which is linked to the neurodegenerative damage caused by WNV similar to humans and

mice studies (Newhouse et al., 2017). Despite many similarities, some immune genes also showed different expression levels in avian hosts when compared to mammals. Some of the genes were the cytokine pro-inflammatory IL18 (Interleukin 18), which was found to be upregulated in contrast to studies with human cell lines. The interferon regulatory factor 6 (IRF6) showed downregulation in this study along with the ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) that is associated with suppression of the pattern recognition receptor pathway (PRR) which leads to viral evasion in human cell-lines (Karim et al., 2013). IFIT5 (Interferon induced protein with tetratricopeptide repeats 5) and IFITM10 (Interferon induced transmembrane protein 10) genes implicated in restricting WNV entry were upregulated in avian hosts (Newhouse et al., 2017).

Transcriptomic analysis of mouse brain infected by WNV to evaluate neuroinvasive disease showed upregulation of apoptosis and pyroptosis markers such as caspase -1, -4, -7 and -8, IL-18, NOD-like receptor proteins such as NLRP3 and NLRC5 (NLR family pyrin domain containing 3 and NLR Family CARD Domain Containing 5, respectively, both important molecules for inflammasome formation), along with necroptosis and autophagy pathways. Also, cytokines (such as IL-1 β and TNF- α), chemokines (CXCL10 - C-X-C motif chemokine ligand 10, which is linked to apoptosis), and immune pathways were found to be differentially expressed during infection, with upregulation of inflammatory molecules such as TNFRSF1A (TNF Receptor Superfamily Member 1A), CXCR4 (C-X-C chemokine receptor type 4), CD47 (Cluster of Differentiation 47, also known as integrin associated protein - IAP), MAPK12 (Mitogen-activated protein kinase 12), and transcription factors FOX (forkhead box proteins), USF1 (Upstream Transcription Factor 1) and NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B cells). Altogether, the results showed evidence of cell death pathways, strong inflammation, and neurodegeneration during the neuroinvasive disease caused by WNV, and such results were similar to CHIKV (Chikungunya virus) infection and neuroinvasion, as it was also analyzed in the same study (Lim et al., 2017). Analysis of transcriptomic RNA profile of extracellular vesicles (EVs), that are secreted by cells for molecule and signal transfer, performed on infected and uninfected cells by WNV demonstrated significant alterations including increase and decrease in host miRNAs (microRNAs), small non-coding RNAs (sncRNAs) and mRNAs that were incorporated into these EVs and secreted. The results obtained showed an increase of EVs containing miRNAs, sncRNAs, and even mRNAs that were capable of regulating genes involved in inflammation, innate immune response, and virus-host interaction in general. Some of the increased mRNAs in EVs during WNV infection were well-known antiviral response molecules, such as cytokines (IFN- β , IL28, pattern recognition receptors - MDA-5 -melanoma differentiation-associated protein 5; and RIG-I - retinoic acid inducible gene 1), transcription factors (AP-1 - Activator protein 1, IRF-9 - Interferon Regulatory Factor 9, IRF-2 - Interferon Regulatory Factor 2, I κ B - nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor), regulators of type I IFN synthesis (TRIM25 - Tripartite motif-containing protein 25, DDX1 - DEAD-Box Helicase 1), as well as proteins such as Hsp90 (heat shock protein 90), actin, and others all related to stress/defense response and involved in pathways such as type I IFN signaling, Jak/STAT cascade, T cell receptor signaling, Wnt signaling, etc., therefore showing an increase and overall change in the content of EVs, containing molecules important for an antiviral response during infection (Slonchak et al., 2019).

Mouse brain proteomic analysis at early and late time-points of the infection presented 148 altered proteins linked to the neurological symptoms caused by WNV. Through bioinformatics analysis, four main functions were found to be altered due to infection: i) modifications of cytoskeleton-associated with virus circulation; ii) deregulation of the protein ubiquitination pathway; iii) inflammatory response modulation, and iv) modification of neurological development and neuronal death. This shows that a variety of pathways are altered due to viral infection and that some responses are highlights for future investigation and

studies on host physiology alteration and antiviral therapies (Fraisier et al., 2013).

¹H NMR metabolomics applied to cerebrospinal fluid (CSF) to differentiate biochemical responses in patients with distinct causes of encephalomyelitis (Lyme disease, WNV infection, multiple sclerosis, rabies, and fungal infection by *Histoplasma*) showed different patterns of response to the various diseases analyzed. WNV infected samples presented low levels of amino acids, citrate, and lactate alteration along with high levels of formate, pyroglutamate, glutamate, pyruvate, 2-oxoglutarate and acetate. The patterns common to the diseases involved high levels of ketone bodies, pyruvate, glutamate, carnitine, glycine, and 2-oxoglutarate, which suggests common alterations in energy metabolism, excitotoxicity, and antioxidant response. The evident factor that promoted significant variation between patient samples was age, which was represented by the 5–20 years in the control group and the 4–83 years range in the disease groups. More studies into prominent and unidentified peaks may bring more results indicating important disease biomarkers and a better understanding of disease mechanisms in hosts (French et al., 2018).

In a lipidomic analysis of infected Vero cells with a Kunjin virus strain (a subtype of WNV), some important phospholipids were found to be up or downregulated, specially Lyso-PChol (lysophosphatidylcholine) produced by the Phospholipase A2 (PLA2) enzyme from phosphatidylcholine, which plays a critical role in the assembly of the viral replication complex. Other lipids, such as phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), were also upregulated, and such increase of those lipids induced by WNV infection is supposedly used to convert them into lyso-PChol by the PLA2 enzyme and to contribute to the virus replication cycle. This hypothesis is supported by the fact that when exogenous lyso-PChol488 was added into the media, it was recruited in viral-induced membrane structures, which proved its requirement for efficient and successful replication (Liebscher et al., 2018).

2.7. Rocio virus

December of 1975, Rocio Virus (ROCV) was first isolated as the SPH 34,675 strain from the cerebellum and spinal cord of a 39-year-old farmer who died 5 days after the encephalitis diagnosis in the south-eastern rural part of Iguape, Sao Paulo, Brazil. A series of cases of encephalitis were reported between April and June around the Santista Lowlands and the Ribeira Valley and from which, 465 cases were reported with 61 deaths included (Lopes et al., 1978; Iversson 1988; Setoh et al., 2018). Outbreaks were no longer reported since then, however, seroprevalence reports have demonstrated the presence of antibodies against ROCV in humans, horses (Silva et al., 2014), birds and water buffaloes, which represents a potential for this neuroinvasive virus to reappear and cause new outbreaks and epidemics. Symptoms caused by ROCV include fever, severe headache, neurologic disorders such as meningeal irritation, consciousness alteration, motor abnormalities, malaise, weakness in lower extremities, and sequelae post encephalitis was reported in 20% of surviving patients (Setoh et al., 2018).

ROCV is phylogenetically associated with the Japanese Encephalitis serogroup along with West Nile virus, St. Louis Encephalitis virus, and Ilheus virus (Medeiros et al., 2007). *Psorophora ferox*, a day-flying mosquito mostly found in rural areas and near swamps or forests, was discovered as the vector of ROCV during the 1975 - 1976 outbreak in Brazil along with other potential mosquito vectors such as *Aedes serratus*, *A. scapularis*, and *Culex (Melanoconion)*. Birds were suggested as possible hosts and reservoirs of ROCV, especially after the virus was isolated from a rufous-collared sparrow (*Zonotrichia capensis*) in Sete Barras, Sao Paulo, Brazil, and other data that suggested the circulation of ROCV through other Brazilian regions by birds (Ellwanger et al., 2017). The genome organization shows the same pattern of all flaviviruses, including its cysteine residue distribution in genes such as prM, E, and NS1 that promotes good conformation due to disulfide bridges, and

several conserved amino acids or peptides (Kuno et al., 1998). ROCV is also phylogenetically closer to JEV serogroup viruses although it has some distinction, for instance, the CS (conserved sequence) organization in the 3' non-coding region which revealed a CS3-CS2-RCS2-CS1 pattern that could serve as a supplementary taxonomical marker for the viral group (Medeiros et al., 2007). Phylogenetic studies suggest that ROCV and Ilheus virus (ILHV), both Brazilian flaviviruses, segregated early and independently and that they form a lineage different from the lineage that turns into the JEV and Ntaya groups (Mertens et al., 2004). Although ROCV is still considered an ILHV subgroup, it has been already distinguished from it serologically, and nucleotide sequence identity of 72.5% along with the length of NS5 gene sequence proves they can be recognized as two distinct species. Furthermore, ROCV is mostly related to encephalitis epidemics, whereas ILHV cases are usually characterized by febrile illness with minor neurological alterations, showing differences in neurovirulence and transmission as well (Medeiros et al., 2007).

Evaluation of the inflammatory process caused by ROCV in animal model revealed that pro-inflammatory cytokines such as interferon α (IFN- α), interleukine-1- β (IL1- β), and tumor necrosis factor α (TNF- α) are increased along with inflammatory cells, degeneration of neurological cells, and apoptosis (Amarilla et al., 2017). Interferons α/β (IFN - α/β) are the major cytokines produced in response to viral infection in order to fight it. The ability to block or escape IFN α/β by viruses is possible because of the viral proteins and it characterizes their pathogenicity and virulence, which has been demonstrated in JEV, DENV, WNV, in Tick-borne encephalitis virus (TBEV) (Muñoz-Jordán et al., 2003; Best et al., 2005; Liu et al., 2005; Lin et al., 2006), and also in ROCV, where the prM-E proteins are the major virulence factors that could promote IFN α/β signaling inhibition (Amarilla et al., 2017). Pathogenesis of ROCV also demonstrated a high level of inflammatory macrophages in the brain, along with other immune cells that are recruited during infection. Migration of monocytes to the brain depends on CCL2 cytokine (chemokine C—C motif ligand 2) and its receptor CCR2 (C—C chemokine receptor type 2) on monocytes surface, and ROCV induces the production of CCL2, increasing CD8+ T lymphocytes and macrophages in the brain, and making CCR2 mandatory for the infiltration of these cells to reduce disease severity, as it was demonstrated in mice. CCL7 (chemokine C—C motif ligand 7) was also reported to be involved in the recruitment of CD8+ cells into the central nervous system (Bardina et al., 2015), although more studies should be carried out to evaluate and understand these processes, it can be concluded that ROCV promotes an increase in monocytes and macrophages into the brain and that CCL2 and CCR2 promote infiltration of these cells for antiviral response reducing the severity of the disease (Amarilla et al., 2019).

2.8. Murray Valley Encephalitis virus

Murray Valley Encephalitis virus (MVEV) is another encephalitis virus that belongs to the JEV serogroup and is endemic to Australia, Papua New Guinea, and Irian Jaya. It was first isolated in Australia from the brain of fatal human cases during its first outbreak in 1951, and later outbreaks happened in 1956 and 1974. Although these years were documented, it is suggested that MVEV caused early outbreaks in Australia in 1917 - 1925, when there was a suspicious outbreak of a so-called Australian "X" disease (Mackenzie and Broom 1995). In 2015, the first MVEV isolation from cerebrospinal fluid (CSF) of an 8-year-old patient from the Royal Darwin Hospital in Australia was performed, which provided full genome sequencing and genotype identification for better comprehension of MVEV infection (Russell et al., 2018). It is believed that MVEV is sustained in an enzootic cycle that involves waterbirds and *Culex annulirostris* and *Aedes normanensis* mosquitoes in the north of Western Australia and part of the Northern territory where most human cases have been registered since 1974. Other native and nonnative animals are also thought to be involved, although there is no clear role of the species described yet. Despite the infrequency of outbreaks, patterns of rainfall and climatic conditions such as floods are

thought to be intimately related to the disease, as it is very favorable for mosquito proliferation (Nicholls 1986; Bennett 2008; Floridis et al., 2018). The main symptoms caused by MVEV infection are fever over 40 °C and headache as well as nausea, vomiting, diarrhea, coughing, macular rashes, and neurological manifestations such as lethargy, irritability, confusion, and sometimes seizures. As the disease progresses, other severe symptoms can be observed and may be fatal or involve sequelae, for instance, prominent spinal cord involvement that can cause flaccid paralysis, cranial nerve involvement, tremor and encephalitis (Anderson 1952; Robertson and McLorinan 1952; Burrow et al., 1998).

MVEV presents amino acid sequence homology to YFV and JEV and conservation of *Flavivirus* structures (Dalgarno et al., 1986; Lee et al., 1990). NS3 amino acids His51, Asp75, and Ser135 are in conserved regions in MVEV and its central amino acid sequence has conserved sequence motifs that are found in helicases and NTP (Nucleoside triphosphate) proteins that are involved in unreeing dsRNA during viral replication. Currently, four MVEV genotypes have been identified, G1 to G4. G1 is the prevalent genotype that circulates in the Australian continent and has also been isolated from Papua New Guinea. Genotype 2 was isolated from mosquitoes in the Kimberley region of Western Australia, while genotypes 3 and 4 have been identified in Papua New Guinea as single strains. The complete genome sequence from genotype 1 was reported from strain MVE-1-51, G2 was taken from strain OR156 isolated from *Culex annulirostris* in 1973, G3 from strain NG156, a human brain isolate, and G4 from strain MK6684 isolated from *Culicinea* mosquitoes in 1966. Genomes from G1 and G4 are similarly 11,014 nucleotides long, whereas the G3 strain has 11,012 nucleotides and G2 has 10,953 nucleotides. The 3' UTR of strains from G4 and G2 has deleted and inserted regions, and in the 5' UTR of G4 and G3, a single uracil insertion was found at position 53, while double uracil was found inserted in G2 at the same position. The highest levels of identity were found between G4 and G3 (94%) and G2 was the least similar to the others (Coelen and Mackenzie 1988; Williams et al., 2014). Recently, G1 was found to have new circulating sublineages: G1A and G1B. G1A has been exclusively isolated from the Kimberley region in Western Australia, while G1B is widely distributed and the only genotype found outside northwestern Australia. G2 was found to be the oldest lineage among the rest and G3 and G4 are apparently confined to the New Guinea Island or may even have become extinct, as no new members of these two genotypes have been found so far (Williams et al., 2015). Unique prM and E proteins amino acids and motifs are responsible for genotypes differentiation and even their geographic distribution. For instance, G1 strains uniquely have V165, A229, and V240 in their E protein, which may have been selected to escape of host neutralization setting replicative advantage to this genotype (Shiu et al., 1992; Williams et al., 2015). G2 has unique residues at positions 72, 126, 275, 276, 330, and 369, which are presumably exposed on the viral surface being associated with biological function and antigenicity (Luca et al., 2012). Residues 126, 275, 276 and 330 serve as neutralizing epitopes and are possibly the ones that distinguish genotype 2 from other genotypes. G3 has four unique amino acids that are encoded in the prM and E proteins: H77, A187, V270, and I442, respectively, while G4 encodes a unique I64 amino acid in the E protein and is closely related to G3 (Williams et al., 2015).

3. Conclusion

This review aimed to provide information on arboviral infection-related aspects seen from "omics" approaches including the most prevalent mosquito-transmitted arboviruses from the *Flaviviridae* family and the *Flavivirus* genus. The tools and technology sensitivity that these omic approaches provide enable better understanding of systems biology and validate the potential effects that these viruses have on experimental animal models, cell culture, and, more importantly, on humans. The study integration of genes, to transcripts, proteins, and metabolites can

provide a holistic and unique perspective on biomarkers and biochemical fingerprints that are present in a specific host due to different conditions set by pathogens or diseases. Improving knowledge on how these viruses affect organisms, especially humans, is key for the development of future therapies or medications that aim to prevent or treat such relevant public health issues.

Author contributions

All authors contributed to the article and approved the submitted version.

Funding

This work was financed by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Process number 2020/03639-6) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Special thanks to Dr. Mônica Coronado for constantly reviewing and ideas for enrichment of the manuscript, Dr. Carolina Pacca, Dr. Raphael Eberle and Prof. Dr. Raghuvir K. Arni for their useful commentaries, contributions and thorough review of the article and topics.

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Review

A Review of Omics Studies on Arboviruses: Alphavirus, Orthobunyavirus and Phlebovirus

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Abstract: Since the intricate and complex steps in pathogenesis and host-viral interactions of arthropod-borne viruses or arboviruses are not completely understood, the multi-omics approaches, which encompass proteomics, transcriptomics, genomics and metabolomics network analysis, are of great importance. We have reviewed the omics studies on mosquito-borne viruses of the *Togaviridae*, *Peribunyaviridae* and *Phenuiviridae* families, specifically for Chikungunya, Mayaro, Oropouche and Rift Valley Fever viruses. Omics studies can potentially provide a new perspective on the pathophysiology of arboviruses, contributing to a better comprehension of these diseases and their effects and, hence, provide novel insights for the development of new antiviral drugs or therapies.

Keywords: arboviruses; omics; genomics; transcriptomics; proteomics; metabolomics



Citation: Peinado, R.d.S.; Eberle, R.J.; Arni, R.K.; Coronado, M.A. A Review of Omics Studies on Arboviruses: Alphavirus, Orthobunyavirus and Phlebovirus. *Viruses* **2022**, *14*, 2194. <https://doi.org/10.3390/v14102194>

Academic Editor: A. Lorena Passarelli

Received: 29 June 2022

Accepted: 3 October 2022

Published: 5 October 2022

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1. Introduction

Arboviruses form a diverse group consisting of over 500 viruses from different families that are transmitted by a variety of arthropod vectors, including mosquitoes, ticks, flies and midges, where they multiply, resulting in a high-titred load, especially in salivary glands, and are subsequently transmitted to humans and other vertebrate hosts through bites. Their transmission can be maintained through a sylvatic cycle, where viruses are stably transmitted from vectors, and wild animals with sporadic spillover to humans or domestic animals, or a human or domestic animal host infected can act as an amplifier to other humans and animal hosts and trigger epidemics, which is the case of the urban cycle [1]. Their emergence and re-emergence are of great public health importance. The clinical effects are associated with neurological, viscerotropic and hemorrhagic diseases. Their ability to adapt to new environments, new arthropods, and human hosts causes major health and socioeconomic issues. The great majority are classified as neglected diseases due to the lack of antiviral treatment, with only symptomatic treatments and prophylactic measures to contain the available vectors [2,3].

Considering the impact that arboviruses have throughout the world, a multi-omics approach to comprehend viruses, virus-host interactions, and the disease process is of significant relevance. Genomics, transcriptomics, proteomics and metabolomics have been widely used as technological advances made high-throughput and cost-efficient analysis available. From the identification of genetic variants of the diseases, characterization, RNA (transcript) levels, protein abundance, structure and interactions to the identification of small molecules derived from cellular metabolism, the disease process can be deciphered as well as disease biomarkers, which are of utmost importance for the development of future antiviral therapies and drugs [4]. Technological advancements in techniques such as DNA sequencing [5], transcriptomics via RNA-seq [6], SWATH (Sequential Windowed Acquisition of all Theoretical fragments) and Mass Spectrometry (MS)-based proteomics [7,8], Nuclear

Magnetic Resonance (NMR), and MS-based metabolomics [9,10] permit us to integrate data from these different areas, although still challenging, as the specific analytical tools for each omics must be well-suited for logical comparisons of the obtained results [11].

Here, we present a review of general viral characteristics along several omics studies applied to well-known mosquito-borne viruses within the *Togaviridae*, *Peribunyaviridae* and *Phenuiviridae* families, more specifically, the following arboviruses: Rift Valley Fever, Oropouche, Chikungunya and Mayaro virus [12,13].

2. An Overview of the Genera

2.1. *Togaviridae*—*Alphavirus*

Arboviruses from the *Alphavirus* genus are classified into three major clades, including several complexes. The Venezuelan equine encephalitis complex comprises the Venezuelan Equine Encephalitis virus (VEEV), and the Western Equine Encephalitis complex comprises the Western Equine Encephalitis virus (WEEV) and the Sindbis virus (SINV). Another complex is the Semliki Forest complex, in which we find the Semliki forest virus (SFV), Chikungunya virus (CHIKV), Mayaro virus (MAYV) and Ross River virus (RRV). Other known complexes are the Barmah Forest, the Eastern equine encephalitis complex, and the Middelburg and Nmdumu complexes [14]. *Alphaviruses* belong to the *Togaviridae* family, characterized as enveloped, spherical viruses with a single positive RNA strand. The *Alphavirus* viral particle is around 70 nm in diameter T = 4 icosahedral symmetry capsid, which presents 240 copies of the capsid protein in the nucleocapsid core (NC), coated by a host-derived lipid membrane with glycoprotein spike-like projections that consist of envelope E1 and E2 heterodimers associated as trimers [15]. The ~11 kb single-stranded RNA codes for structural and nonstructural proteins, as illustrated in Figure 1 [16], and contains a 5' 7-methyl-GpppA cap and a 3' poly(A) tail [17]. Genome organization can be summarized as 5' m⁷G-nsP1-nsP2-nsP3-nsP4-(junction)-C-E3-E2-6K/TF-E1-An3' [18]. Some conserved and repeated sequences are present in the 5' and 3' ends, which are important for RNA synthesis and replication regulation. Two open reading frames (ORFs) are present in the genome. The first ORF is responsible for translating directly from genomic RNA and encodes the four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) required for RNA synthesis [19]. The second ORF is expressed by producing a subgenomic mRNA generated by an internal promoter in the minus-strand RNA replication intermediate. It encodes the structural proteins C—capsid, E—envelope E3, E2, E1 and 6K/TF (transframe is a C-terminal extension of the 6K protein after a frameshift event), which are responsible for the assembly of new virion particles and their entry into the cells. The short 5' and 3' untranslated regions form stem-loop structures with repeats are presumed to be responsible for viral adaptation to a range of vectors and hosts. They are presumably responsible for viral gene expression, replication, and virus-host interactions [20]. The capsid protein (C) is the icosahedral nucleocapsid (NC) component and is present with 240 copies that compose the important structure for viral RNA packaging. The NC is formed by a host-derived lipid-bilayer where E1 and E2 glycoproteins are embedded along with smaller amounts of other membrane-associated proteins (6K/TF) that also form part of the viral particle [21]. As E1 and E2 interact, a rigid structure is formed across the viral membrane, creating the icosahedral format that surrounds the virus. E3 glycoprotein works as a signal sequence for insertion of the remaining polyprotein into the endoplasmic reticulum for later processing, and 6K seems to act as a signal sequence for processing the E1 protein [22,23].

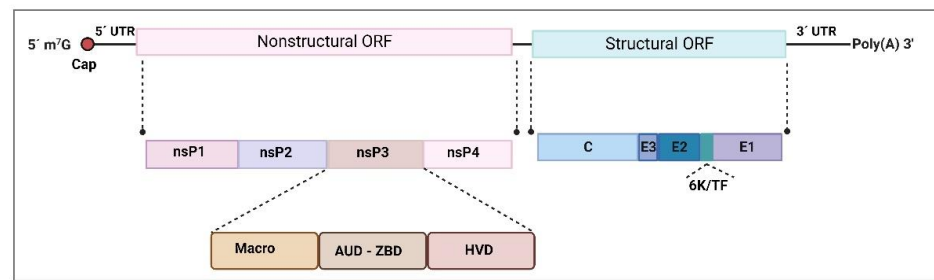


Figure 1. *Togaviridae* family. Genome structure of *Alphavirus* and nsP3 non-structural protein domains (the macro domain, the alphavirus unique domain (AUD) and the hypervariable region (HVD)). The genome organization can be summarized as 5'm⁷G Nonstructural ORF-(junction)-Structural ORF-An3', in which two open reading frames (ORFs) are present. The non-structural ORF (NS-ORF) encodes the four non-structural proteins (nsP1–nsP4) which are required for replication. The structural ORF, produced by a subgenomic mRNA, encodes the structural proteins in the viral particle (C, E3, E2, 6K/TF, E1). The genomic RNA with noncoding regions is represented as solid black lines and ORFs as open boxes. Based on Götte et al. (2018) [24] and created with BioRender.com (Accessed on 14 September 2022).

Viral entry in cells is mediated mainly by clathrin proteins through membrane invagination and scission to form a clathrin-coated vesicle that contains the viral particle, which is delivered into the cell and taken by endosomes with a low pH environment that facilitates E1/E2 glycoproteins fusion to the endosomal membrane due to putative binding sites (Figure 2). Multiple entry receptor pathways have been suggested, including membrane proteins prohibitins (PHB) [25], phosphatidylserine-mediated virus entry-enhancing receptors (PVEERs) [26], glycosaminoglycans (GAGs) [27], and others [28,29]. After fusion, the nucleocapsid is released and dismantled in the cytoplasm, and the viral genome is liberated (Figure 2). The translation of the RNA genome is a membrane-associated process that, in most *Alphavirus* and RNA viruses, induces host membranes into forming cytoplasmic structures known as type-1 cytopathic vacuoles (CPVs). The genomic RNA is translated into the P1234 polyprotein, which is then cleaved by nsP2 protease function and forms the minus strand replicase complex P123 and nsP4 [30]. This complex initiates the minus strand RNA synthesis that will be the template for the production of genomic and subgenomic RNAs. The P123 polyprotein is also cleaved into nsP1 and P23, forming the nsP1/P23/nsP4 replicase complex that synthesizes positive-strand RNAs from the minus strand RNAs previously produced for genomic and subgenomic production [31].

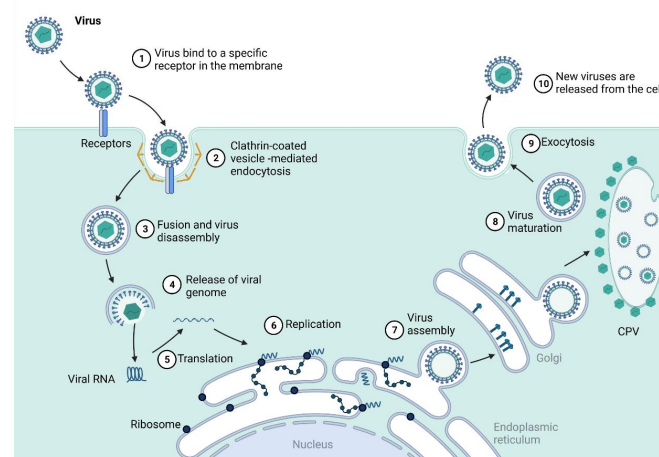


Figure 2. *Alphavirus* replication. After binding to specific receptors in cells (1), membrane invagination occurs, forming clathrin-coated vesicles with the viral particle inside (2) that are later taken by

endosomes, whose low pH environment promotes envelope proteins E1/E2 fusion to the endosomal membrane, and the nucleocapsid is released into the cytoplasm with further viral RNA liberation (3, 4). Translation of the non-structural proteins is initiated, and replication occurs associated with ER membranes forming the cytopathic vacuoles (CPVs) (5, 6). After the replication process and synthesis of structural proteins, viral assembly and maturation (7, 8) take place, and budding from the cell membrane occurs through interactions of the nucleocapsid proteins with membrane glycoproteins, and virion are released to infect new cells (9, 10). Based on: Garoff, Sjöberg and Cheng (2004) [32] and created with [BioRender.com](#) (Accessed on 14 September 2022).

After this process, the P23 portion, which is present only for a few seconds, is processed, yielding the fully cleaved replicase complex that promotes RNA synthesis with the individual nonstructural proteins nsP1, nsP2, nsP3 and nsP4 [19,33]. After forming the RNA replicase complex by the nonstructural proteins, the positive-stranded RNA is translated into a polyprotein that is cleaved into three structural proteins: C—the capsid protein, PE2, and E1. PE2 is then processed into E2 and E3, responsible for envelope assembly together with the E1 glycoprotein. The viral envelope comprises a lipid bilayer derived from the host cell membrane and the main E1 and E2 glycoproteins anchored as spikes in an orderly arrangement [34,35]. The structural proteins, essential for virion assembly, are translated from subgenomic RNA, as CHIKV shuts down cell mRNA translation to produce its mRNAs. Viral particle assemblage occurs on the membrane of infected cells, and nsP1 works as a defence mechanism against host antiviral effects at this stage [29]. As alphaviruses disseminate mostly through the lymphatics and microvasculature, the blood is loaded with virion particles and infected monocytes that target other organs. The liver and spleen are sites for further viral replication and dissemination (Figure 3). Subsequently, the viral particles can reach bones, muscles, and articular tissues, which characterizes the acute phase of the disease, where inflammatory infiltrate rich in monocytes, macrophages, natural killer cells, CD4+ and CD8+ lymphocytes are prevalent and affect joints and muscles, inducing the characteristic arthralgia and arthritis symptoms [36–38].

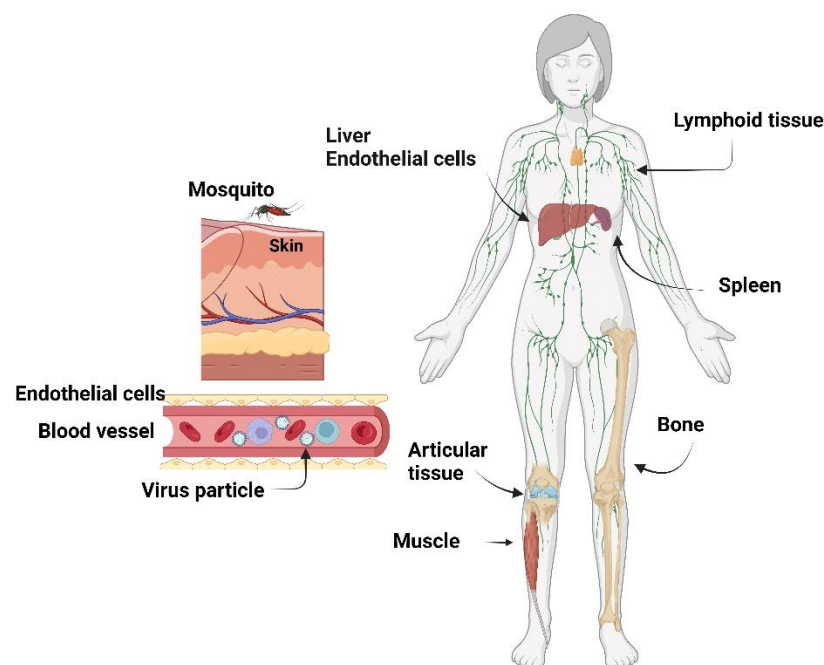


Figure 3. *Alphavirus* dissemination. Probable alphavirus dissemination in humans after inoculation of the virus through the mosquito vector bite. It propagates into the target tissues (muscle, articular tissue, liver, spleen, etc.) via the blood vessels. Created with [BioRender.com](#) (Accessed on 10 September 2022).

Regarding the nonstructural proteins, nsP1 presents both guanine-7-methyltransferase and guanylyl transferase activities required for the capping and methylation of new viral genomic and subgenomic RNA. It is also believed to anchor replication complexes to the cytoplasmic membrane, which is required to initiate and maintain the minus-strand replicative intermediates [22], along with membrane and cytoskeletal rearrangements [39,40]. The nsP2 protein possesses several enzymatic functions in its N-terminal regions, such as helicase, nucleoside triphosphatase (NTPase), and RNA-dependent 5'-triphosphatase activity. In contrast, the C-terminal region yields a viral cysteine protease required for nonstructural polyprotein processing [41]. Also, during infection, a portion of nsP2 remains in the nucleus. It is responsible for host-cell transcription shut-off and cytopathogenicity [42,43]. Along with E1 and E2, it has been shown to strongly antagonise IFN- β and other signalling molecules involved in the MDA5/RIG-I receptor signalling pathway [44].

Concerning nsP3, it is known to present three domains: the macro domain, the alphavirus unique domain (AUD), and the hypervariable region (Figure 1) [19]. The N-terminal region of nsP3 is highly conserved among alphaviruses and has a macrodomain that presents homologues across other domains of life [45]. The macrodomain has nucleic acid binding and phosphatase activities and has been found to bind to DNA, RNA and poly ADP-ribose [46,47]. The AUD comprises the central portion of nsP3 and has strong sequence homology among alphaviruses, and the C-terminal domain, which is the hypervariable region, presents poor conservation among the genus [48]. nsP3 associates with other nsPs as part of the replication complex and is required for replication due to its N-terminal region, which also presents an ADP-ribose 1-phosphate phosphatase activity region [24,49]. It is known that some part is also liberated and forms cytoplasmic aggregates that can interact with host factors such as G3BP (Ras-GTPase-activating protein (SH3 domain)-binding proteins) proteins and others, independent of but possibly enhancing replication [24,50]. In some alphaviruses, nsP3 can also act as an important neurovirulence factor [51], although E2 and other structural proteins are usually virulence determinants [24,52].

Finally, nsP4, the highly conserved polymerase among alphaviruses [53], is suggested to act as a scaffold for interaction with other host proteins or nsPs. When not integrated into the replication complex, it is targeted for degradation by a ubiquitin-dependent N-end rule pathway, depending on the N-terminal residue [54]. Its N-terminal domain presents adenylyl transferase activity (ATase), possibly indicating a polyadenylation function [55]. In contrast, the C-terminal region has an RNA-dependent RNA-polymerase (RdRp) function which is solely responsible for the RNA synthetic function of the replication complex associated with the P123 replication complex after the P1234 polyprotein cleavage [56,57].

2.2. Bunyavirales—Orthobunyavirus and Phlebovirus

The *Orthobunyavirus* (Oropouche Virus) and *Phlebovirus* (Rift Valley Fever Virus) genera belonged to the *Bunyaviridae* family until 2016, when the International Committee on Taxonomy of Viruses (ICTV) created the order *Bunyavirales* that replaced *Bunyaviridae* and placed the *Orthobunyavirus* and *Phlebovirus* into the *Peribunyaviridae* *Phenuiviridae* families, respectively [58]. These viruses are negative or ambisense RNA viruses that can infect a variety of animals, plants, and humans with the capacity to cause severe disease in hosts, including haemorrhagic fever, with over 350 viruses mainly transmitted by arthropod vectors [59,60]. They are lipid-enveloped viruses of 80 to 120 nm in diameter that include three ssRNA segments: large (L), medium (M) and small (S). These segments are surrounded by a helicoidal nucleocapsid and encode the RNA-dependent RNA polymerase (RdRp), the surface glycoproteins Gn and Gc arranged in spikes, and the nucleocapsid protein N, along with two other nonstructural proteins NSm and NSs that are encoded by the M and S segment, respectively, as shown in Figure 4 [60–62], which are mainly associated with virulence. The coding sequences of the three segments are flanked by two terminal non-translated regions (NTRs) 5' and 3' that present 11 nucleotides, highly conserved among the segments but with a variable number of nucleotides in length. Such regions

are complementary, allowing genomic RNA circularization to work as replication and transcription promoters [63,64].

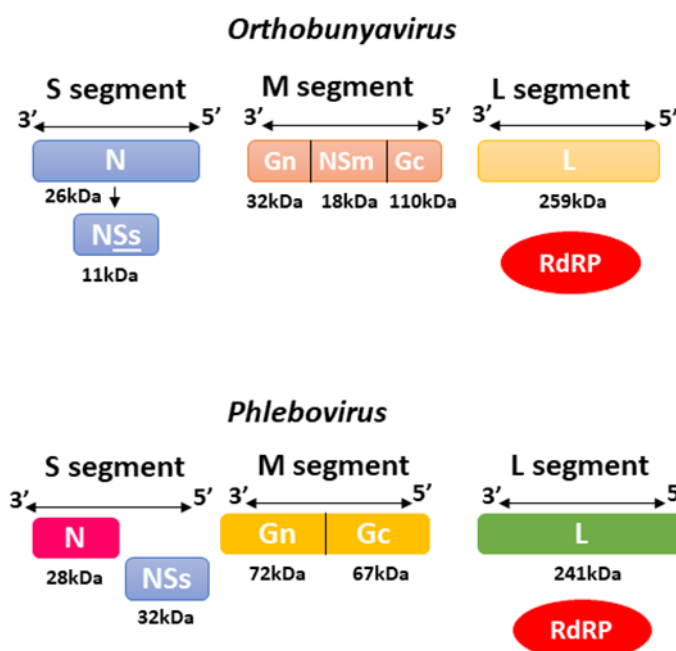


Figure 4. Genome segments of *Orthobunyavirus* and *Phlebovirus*. The three-segmented negative-sense single-strand RNA are nominated according to their size as L (large), M (medium) and S (small). The L segment encodes the multifunctional RNA-dependent RNA polymerase (RdRP) named L protein. The medium segment encodes the Gn and Gc glycoproteins, and the small segment encodes the nucleocapsid protein (N). Additional non-structural proteins such as NSm and NSs can also be encoded by segments M and S, respectively, which can be cleaved by cellular proteases during polyprotein maturation. Based on: Ferron et al. (2017) [65].

The small (S) RNA segment encodes the N protein, which presents regions involved in oligomerization, polymerase binding and RNP (ribonucleoprotein complex) assembly into virus particles and is also thought to promote virus template recognition by the RNA-dependent RNA polymerase [66]. The S segment also encodes the nonstructural protein NSs responsible for regulating host antiviral response by inhibiting host transcription and terminating interferon production, therefore working as a major virulence factor. Both proteins are translated from the same mRNA produced by the S segment [67]. The medium (M) segment yields a polyprotein precursor inserted into the endoplasmic reticulum membrane during translation. Later, it is cleaved by host proteases into two polypeptides called Gn and Gc [66,67]. Together they form the Gn-Gc heterodimer, which is retained in the Golgi apparatus due to glycosylation and promotes virus assembly associated with the RNP complex, viral particle formation, budding, attachment and entry into new host cells. Most orthobunyaviruses also produce the NSm protein from the same polyprotein that serves as a complex for translation initiation and polyprotein processing [68]; it is thought to participate in viral assembly as well as inter-cellular transmission. At the same time, for *Phlebovirus*, NSm is non-essential for replication, but there is speculation that it may work on apoptosis regulation mechanisms [69].

The viral RNA-dependent RNA polymerase (RdRP) is encoded by the large segment (L), the largest segment with ~250 kDa, for RNA replication and mRNA transcription. A comparison of several RdRPs from negative-strand RNA viruses shows a well-known polymerase module, including its motifs pre-A, A, B, C, D and E [68,70]. The RNA segments are templates for RNA replication and mRNA transcription. They are always associated with viral NPs (nucleoprotein complex) with at least one copy of the L protein assembled into the complex due to the negative polarity of the viral RNA (vRNA), and the one needed

first to transcribe their mRNAs and produce the viral proteins essential for replication and formation of new virion particles [65]. The synthesis of a complementary RNA (cRNA) with positive polarity starts with 5' nucleoside triphosphates for genomic replication, and it is the viral RNA segment's full-length complementary (antigenomic) copy. At the same time, transcription requires a host-cell derived cap primer to signalize initiation and ends at specific termination signals before the 3' end region without adding a poly-(A) tail [66,71,72].

Viral entry in the cell generally occurs through a clathrin-mediated endocytic pathway, with different pathways and interactions that have been proposed for different bunyaviruses, including members of the integrin family, and binding to filopodia or dendrites with the formation of non-coated vesicles [60] and glycosaminoglycans. After membrane fusion and endocytosis, the viral ribonucleoprotein complex (RNP) is released into the cytoplasm. It accumulates in the Golgi stacks, where so-called viral tubes are formed, composed of the host cell and viral components (NSm) [73], for the viral polymerase packed with the RNP to initiate primary transcription for the synthesis of mRNA. During the primary transcription, N mRNA and NSs mRNA are produced (~40 min after infection) from the S segment. Within two hours post-infection, replication of the viral RNA commences, increasing mRNAs and proteins. The RNP is probably packed into the virions by interaction with the cytoplasmic domains of Gn and Gc at the Golgi apparatus. The three RNA segments are co-packed in a manner where M and S segments may assist L segment packaging [74]. Finally, the new virions bud from the Golgi complex is released from the cells undergoing further morphological changes for maturation and total infectivity capacity (Figure 5) [60].

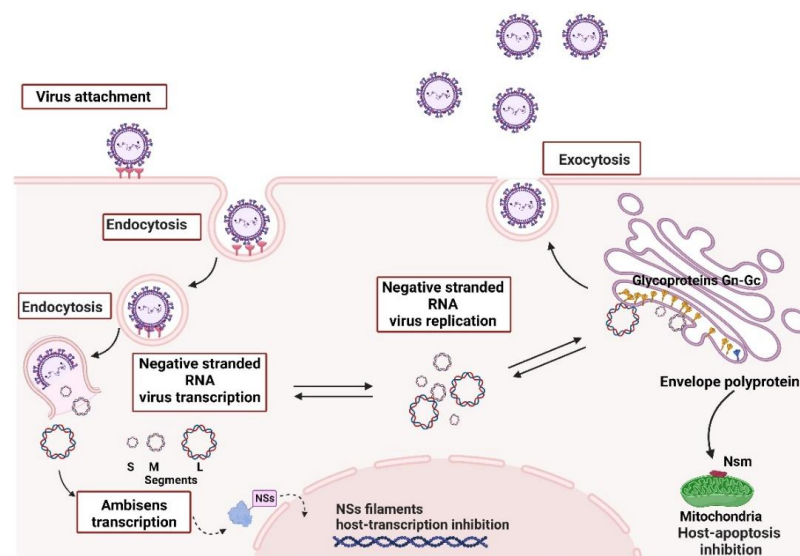


Figure 5. Replication process of *Orthobunyavirus* and *Phlebovirus*. The virus enters the cells through clathrin-mediated endocytosis, and endosome membrane fusion occurs at acidic pH facilitated by viral glycoproteins, uncoating and liberating the viral RNA into the cytoplasm. The viral polymerase L and the N protein catalyze transcription and replication of the viral genome in the cytoplasm. Translation and transcription are coupled processes due to the action of translocating ribosomes, which are able to prevent nascent mRNA interactions and premature termination, allowing transcription to occur up to the termination signals at the 5' end. In replication, antigenomic RNAs (cRNAs) are synthesised and used as templates for progeny genomic RNAs (vRNAs). The non-structural NSs and NSm proteins have been proposed to shut down host transcription and apoptosis for efficient viral replication. Interaction of the viral glycoproteins Gn and Gc in the Golgi apparatus allows packaging of the RNP complex into the viral particles. Further modifications and maturation steps occur until the new virions are released from the cells. Based on: Guu, Zheng and Tao (2012) [72]. Created with BioRender.com (Accessed on 11 September 2022).

3. Omic's Study across Members of the Togaviridae Family

3.1. Chikungunya Virus

Chikungunya virus (CHIKV) is the pathogen responsible for Chikungunya fever, mainly transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes. It was first recognised in an epidemic on the Makonde Plateau in the Southern Province of Tanganyika (Tanzania) from 1952 to 1953 [75], and since then has triggered several outbreaks and epidemics around the world and has been identified in over 40 countries [76,77]. The word “Chikungunya” belongs to the Makonde dialect and means “that which bends up”, which refers to the contorted posture of infected patients due to severe joint pain. The clinical manifestations of CHIKV may vary but are usually very debilitating and are characterized by sudden onset fever that can appear two days post-infection, skin rash, arthralgia and arthritis, joint and muscle pain, joint swelling, fatigue, nausea, vomiting, headaches and even conjunctivitis. Infected individuals may present protracted arthralgia even weeks, months, or years post-infection. Some cases observed from the 2006 outbreak on La Réunion Island included encephalitis, Guillain-Barré syndrome, myocarditis, and hepatitis [78].

It is known that the CHIKV lineages spread across the world derive both from African and Asian lineages (the Asian Urban lineage—AUL; the Indian Ocean lineage—IOL; the East, Central and South African lineage—ECSA; and the West African lineage—WA) in a series of genetic and evolutionary events that enabled adaptation and transmission in new environments and vector species. A recent investigation of CHIKV phylogeny with analysis of the RNA architecture of the UTRs (untranslated regions) from all CHIKV lineages, which are well-conserved evolutionarily with known elements related to viral pathogenesis, revealed some lineage-specific structured and unstructured repeat elements that are possibly involved in pathogenicity, vector adaptation and specificity, viral replication and host factor binding [79,80]. Based on the results and geographic distribution of CHIKV lineages, the authors suggested three major epidemic clades, the ECSA-MASA (ECSA + Middle Africa/South American lineages), the AUL-Am (Asian Urban + American lineages), and the ECSA-IOL (Eastern Africa + Indian Ocean Lineage). The main observation was that the ECSA lineage is no longer a single lineage due to the co-circulation of multiple lineages revealing the presence of ECSA subgroups. Composition and length of the 3'UTRs were also compared, revealing substantial differences among lineages and the characterization of five highly conserved regions into four structured and one non-structured element, including stem-loop elements. However, their functional roles remain to be determined [80].

CHIKV virus infection generates temporary dsRNA intermediates during the replication process that can engage with pathogen receptors in cells such as Toll-like receptor 3 (TLR3), TLR7, TLR8 and the retinoic-acid-inducible gene I (RIG-I) [81,82], which induces a cascade leading to the activation of type I interferons (IFNs), cytokines and chemokines production. IFNs activation is regulated by MAVS (mitochondrial antiviral-signalling protein), and IL-1 β production may be induced. In mouse models, the defence mechanism was also dependent on the TLR adaptor myeloid differentiation primary response protein 88 (MYD88), which can also act as an adaptor for the interleukin-1 β receptor (IL-1R) that may be activated by the secretion of IL-1 β from infected cells and activate type I IFN in cells that are not infected [77,83]. Moreover, it has been reported that CHIKV nsP2, E2 and E1 proteins are major suppressors of IFN- β activation through the MDA5-RIG-I pathway, and nsP2 has been specifically shown to induce host cell shut-off by inhibiting cellular transcription and therefore promoting a decrease in signalling molecules involved in the IFN- β pathway to effectively evade host immunity (Figure 6) [43,84].

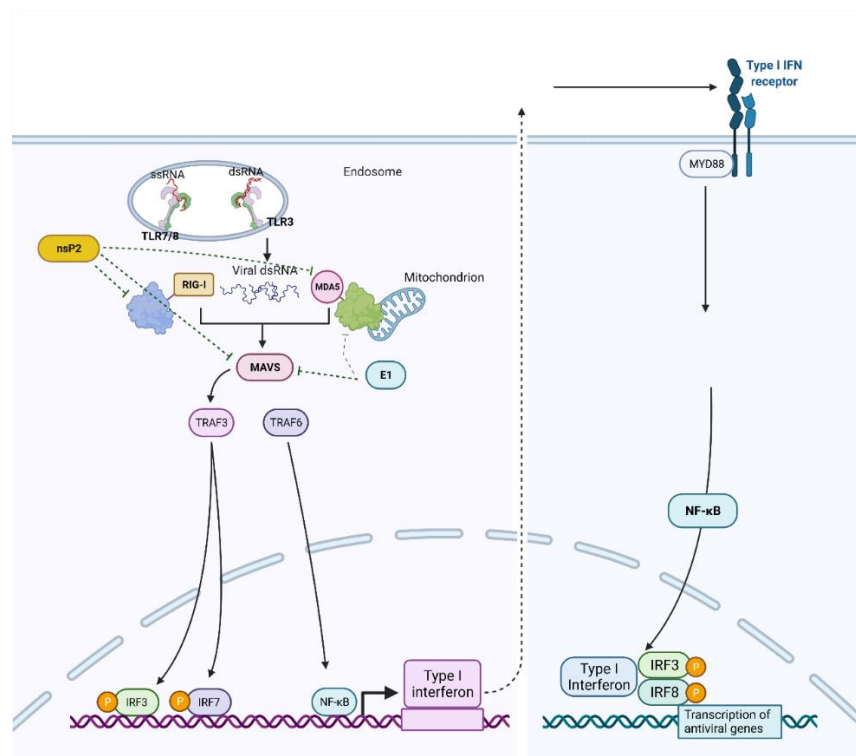


Figure 6. Proposed CHIKV signalling cascades. Engagement of the pathogen receptors in cells (TLR-X) and (RIG-I) induces a cascade that activates the type I interferons. Recognition of viral RNA in the endosomes of infected cells leads to the recruitment of adaptor proteins such as MyD88. This results in activating several signalling cascades and the phosphorylation of transcription factors: IRF3, IRF7 and NF-κB (Nuclear factor kappa B). When phosphorylated, these transcription factors can translocate from the cytosol into the nucleus and induce the transcription of type I interferons (IFN- α and IFN- β) and proinflammatory cytokines (IL-1b, IL-6, TNF- α). CHIK nsP2, E2 and E1 proteins suppress IFN- β through the MDA5-RIG-I pathway. Created with [BioRender.com](https://www.biorender.com) (Accessed on 11 September 2022).

Mammalian and human cells infected with CHIKV rapidly undergo apoptosis. It presents highly cytopathic effects due to the disturbance of cell physiological processes, inhibiting cell transcription and translation and redirecting cellular mechanisms and resources towards viral replication and virion production. However, innate immune response with type I and II IFNs can usually reduce viral content within a few days post-infection, reducing symptoms and viremia [85]. Numerous proinflammatory mediators are produced during infection, including IFN- α , IL-4, IL-10 and IFN- γ , with a high circulation of CD8+ and CD4+ lymphocytes, monocytes and leukocytes. During the early phases of the infection, infected monocyte/macrophages migrate to the synovial tissues of infected patients, contributing to the inflammation process and joint pain symptoms, which may persist for a long time after infection because these areas may become viral reservoirs, generating chronic arthralgia. In addition, these infected cells may also be responsible for viral dissemination in other tissues, such as the nervous system, and contribute to the development of other clinical features [86].

Using RNA-seq and Ribo-seq, Jungfleisch and colleagues showed that CHIKV infection induces codon-specific reprogramming of the host translation machinery in order to favour the translation of viral RNA genomes over host mRNAs. This reprogramming was mostly apparent at the endoplasmic reticulum, where CHIKV RNAs showed high ribosome occupancy. Mechanistically, it involves the CHIKV-induced overexpression of KIAA1456, an enzyme that modifies the wobble U34 position in the anticodon of tRNAs, which is required for the proper decoding of codons that are highly enriched in CHIKV RNAs [87].

Recently, defective viral genomes (DVGs) generated during infection in mammalian and mosquito cells revealed interesting antiviral effects that interfere with viral dissemination, especially in *Ae. aegypti* cells. It was shown that, although DVGs vary in type and abundance depending on the viral strain and host environments, most DVGs possessed promising inhibiting abilities resulting from the hijacking of the replication machinery and host resources in a competitive manner against non-defective genomes. Some DVGs may even function against other CHIKV strains as well as other alphaviruses. Such cross-reactivity may be used in favour of antiviral research for the development of future antiviral studies and therapies against arthritogenic alphaviruses. However, much research is still needed on identifying these DVGs and testing their antiviral functions, as well as developing efficient delivery systems [88]. Another inducer of antiviral response discovered in a recent transcriptomic RNA-seq analysis was IL27 (interleukin 27). The results of this study demonstrated that CHIKV-infected human monocytes-derived macrophages (MDMs) with recombinant expressed IL27 lead to the activation of JAK-STAT signalling, inducing a pro-inflammatory and antiviral response in order to control viral replication. Furthermore, treatment of cells with IL27 inhibits CHIKV in a dose-dependent manner [89].

Proteomic analyses of liver and brain tissues from mock and CHIKV infected newborn mice by two-dimensional electrophoresis (2-DGE) revealed differentially expressed proteins involved in iron metabolism, with upregulated transferrin levels (mostly related to defence against infection), dysregulation of the urea cycle and fatty acid oxidation. Furthermore, many proteins identified were related to cellular stress, such as catalase, peroxiredoxin-6, molecular chaperone proteins (Hspd1 and DNA K chaperonin), and heat shock proteins (HSPs), all related to stress response, including oxidative stress, inflammation and apoptosis. Other related pathways affected by CHIKV were energy metabolism (decrease in glycolytic enzymes), urea cycle enzymes (carbamoyl phosphate synthase, arginase, ornithine carbamoyltransferase, glutamate dehydrogenase—leading to accumulation of ammonia). CHIKV infection results in the rise of nitrogenous metabolites, cytoskeletal proteins (coronin 1A), fatty acid metabolism (increased levels of apolipoprotein A-IV and decreased Acox1), apoptosis (with upregulated levels of prohibitin) and overall inflammation in both tissues, with a clear demonstration of CHIKV neurotropism and neuroinvasiveness potential [90]. Comparatively, proteomic profiles of WRL-68 cells (a human hepatic cell line) by 2-DGE and MALDI-TOF/TOF (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) during early infection by CHIKV revealed alteration in the expression of proteins related to mRNA processing and translation, energy and cellular metabolism, ubiquitin-proteasome pathway (UPP), and cell cycle regulation, with cyclin-dependent kinase 1 (CDK1) regulation for efficient viral replication, survival and transmission [91].

Treffers and collaborators, using SILAC LC/MS/MS (Liquid Chromatography Tandem Mass Spectrometry) in a temporal approach—8, 10 and 12 h post-infection, identified over 4700 proteins, and the majority showed decreased abundance over time [92]. In agreement to host shut-off caused by the virus, RNA polymerase II complex subunits presented progressive degradation. Four other proteins, namely Rho family GTPase 3 (Rnd3), DEAD box helicase 56 (DDX56), polo-like kinase 1 (Plk1) and ubiquitin-conjugating enzyme E2C (UbcH10), which are likely overexpressed in order to reduce cell susceptibility to CHIKV infection, also presented downregulation during infection as a mechanism for efficient viral replication [92]. Moreover, in a 2-DGE proteomic analysis combined with MALDI-TOF MS of human muscle cells, the expression of proteins involved in the synthesis and metabolism of biomolecules, cell signalling, reorganization, cellular stress, and gene silencing via siRNA was observed. Interestingly, the authors also observed an interaction of CHIKV nsP3 with vimentin filaments, suggesting that this association plays an essential role in the anchorage and trafficking of replication complexes (RCs) to ensure efficient viral replication [93].

A recent TimsTOF Pro Based LC/MS/MS proteomic study of *Ae. aegypti* mosquitoes with the oral acquisition of CHIKV demonstrated exquisite but minor changes in the mosquito proteome, possibly due to the adaptation of the vector to the infection. Overall, enrichment of pathways such as oxidative phosphorylation, endocytosis and ribosome

biogenesis was observed. At the same time, the attenuation of cellular RNA machinery related to RNA transport, RNA polymerase and Aminoacyl-tRNA biosynthesis was reported, possibly for viral RNA protection from degradation in the mosquito and for maintaining infection, or even for host defence as a means to decrease viral replication and consequent titer yields in cells. Likewise, cytoskeleton proteins such as syntenin, actin, twinfilin and the vesicle trafficking MON1-CCZ1 complex-like protein were modulated by infection, with decreased levels as infection progressed (from 24 h post-infection onward), possibly as a host response to avoid de novo cell and tissue infection. Finally, immune-related proteins (RNA interference—RNAi; immune deficiency factor—IMD; Janus kinase-signal transducer and activator of transcription—JAK-STAT; and defensin C) and serine-type endopeptidases and metalloproteinases related to cell entry, signalling, viral synthesis, maturation and release were also affected during infection, although further studies are essential to properly assess their functions related to the infection itself [94].

An isobaric tag-based high-resolution MS (mass spectrometry) proteomic study of infected *Ae. aegypti* salivary glands by CHIKV, comparing the effects of other two arboviruses (Dengue and Zika virus), showed virus-specific regulation patterns related to immunity, blood-feeding and cellular machinery processes, with a total of 58, 27 and 29 regulated proteins (by Dengue, Zika and Chikungunya viruses, respectively) and four upregulated proteins common to the three infections all related to antiviral functions (ADA—adenosine deaminase, SGS1—salivary gland surface protein 1, GILT-like protein—gamma-interferon responsive lysosomal thiol-like protein, and SGBAP—salivary-gland broad-spectrum antiviral protein). Results showed that the ADA, a saliva protein that was upregulated in all three infections, exerted an anti-CHIKV effect, possibly through regulating immune activation and repression. The SGBAP had an antiviral potential against all three viruses by inhibiting viral propagation. However, its mechanisms remain to be discovered through homily investigation [95].

Moreover, a very recent label-free quantitative proteomic analysis of *Ae. aegypti* Aag-2 cells revealed a total of 196 regulated proteins related to energy metabolism, protein synthesis, signalling pathways and apoptosis, with antiviral and proviral effects. A known mechanism to control viral propagation was observed with the shutoff of general protein synthesis in a 48 h post-infection period and the induction of autophagy by ROS (Reactive oxygen species) production in mitochondrial nets to delay apoptosis and prolong survival of the infected cells before apoptosis [96].

In order to comprehend the mechanisms involved in CHIKV-induced arthralgia, a proteomic analysis of primary human fibroblast-like synoviocytes (HFLS), which play a pivotal role in joint damage in arthritic disorders, was performed through gel-enhanced chromatography-mass spectrometry (GeLC-MS/MS). The study was conducted 12 and 24 h post-infection, and 259 and 241 proteins were identified, respectively. Investigation of protein functions revealed mechanisms such as cytoskeletal organization (MAEA—macrophage erythroblast attacher; NEFL—neurofilament light polypeptide and FLG—filaggrin) and cellular trafficking (Rab-8B—Ras-related protein; DNAH5 and DNAH12—dynein heavy chain 5 and 12; VPS13D—vacuolar protein sorting-associated protein 13D and NSFL1C—NSFL1 cofactor 47), cellular homeostasis, immune response (HLA-A, HLA-B and HLA-G—MHC class I antigen proteins; MAPKAPK2—MAP kinase-activated protein kinase-2 and TRAF3IPI—TRAF3-interacting protein 1), protein modifications and metabolic processes (SLC27A3—long-chain fatty acid transport protein-3 and APOL1—apolipoprotein L1, both involved in lipid metabolism). The findings included some commonly known proteins involved in arthralgia and arthritis (i.e., MAEA; NEFL; FLG as well as COL23A1—collagen alpha 1 XXIII chain; DMGDH—dimethylglycine dehydrogenase and PDE3A—cGMP-inhibited 3',5'-cyclic phosphodiesterase A) and also in cell death (such as USP30—ubiquitin carboxy-terminal hydrolase 30) [97].

Transcriptomics analysis of infected mice showed, as predicted, many pathways associated with viral infection with an upregulation of transcription factors involved in inflammatory response such as NFκB, USF1 (Upstream Transcription Factor 1), FOX (Forkhead box

transcription factors), class I antigen presentation and complement pathways, cytokines and chemokines in general. Similarly, cell-death pathways were upregulated with strong apoptosis evidence along with necroptosis-related genes (such as MLKL—Pseudokinase mixed lineage kinase domain-like protein, RIPK—Receptor-interacting serine/threonine-protein kinase 1 and 3) in early and late stages of infection and BIRC2 and BIRC3 (Baculoviral IAP repeat-containing proteins) necroptosis inhibitors upregulation as well. Apoptosis genes such as caspase-3 and 9, TNF- α and pyroptosis-related genes caspase-1, IL-18 and IL-1 β mRNAs showed increased expression during infection [98].

AN analysis of the *Ae. aegypti* transcriptome response to CHIKV infection showed a variety of differentially expressed transcripts from different functional categories such as binding, catalytic activity, cellular process, immune response, metabolic alterations, stimulus-response, biological process regulation, structural molecular activity, transporter activity, signal transducer activity and development in a total of 1299 upregulated and 1217 downregulated genes 3 h post-infection [99]. Orally acquired CHIKV by *Ae. aegypti* mosquitoes through a mixture of infected cells in solution revealed gene expression patterns in the midgut of the fed mosquitoes. Genes related to immunity were upregulated along with other genes not related to immune response, such as genes that encoded three synaptic vesicle protein genes, which seem to be important in viral assembly and/or budding [100], the cysteine-rich venom protein gene that contains a trypsin inhibitor-like domain (TIL) and may function as serine protease inhibitors, and two C-type lectins (CTL), which have been supposed to function as ligands, recognizing specific extracellular glycans that facilitate mosquito infection and promote gut microbiome homeostasis [101]. Other genes that were also significantly upregulated were the cytochrome p450 gene, the juvenile hormone inducible protein gene, the heat shock protein gene and a microtubule protein gene. Besides, genes that could be related to midgut escape of CHIKV when it crosses the midgut basal lamina (BL) were identified and included serine collagenases, glutamate carboxypeptidase, M1 zinc metalloproteases, serine-type endopeptidases and ten trypsins, all related to BL degradation/remodeling due to infection [102–104]. Comparatively, RNA-seq analysis of *Ae. albopictus* viral disseminating body parts (heads and thorax, 8 days post-infection) showed the differential regulation of various biological processes, including RNA and mRNA binding, lysosomal pathways and also the down-regulation of defensin genes, possibly as a result of either mosquito immune response or the viral modulation of immunity, which had also been observed in a previous transcriptomic analysis of *Ae. albopictus* midguts by the same authors [105]. In addition, the differential expression of two odorant binding proteins (OBPs) was observed, which could be related to reducing chemosensory and transmission mosquito abilities. Interestingly, upregulation of the BTKi, an inhibitor of Bruton's tyrosine kinase (Btk), which is involved in several innate immune response mechanisms, was identified, and these results correlate with the effects of Btk inhibition in higher-level organisms in a protective manner, ameliorating the excessive inflammatory response [106].

Finally, NMR metabolomics of the patient's serum analysis showed alteration in glycine, serine, threonine, galactose metabolism and TCA (*tricarboxylic acid cycle*), similar to DENV infection [107,108]. Alteration in the TCA cycle is possible because CHIKV requires high energy for the rapid multiplication and generation of lipids, proteins, and RNA components. In addition, joint damage and arthralgia were correlated with high hypoxanthine and 4-hydroxyphenylpyruvic acid levels and carbon metabolism alterations. Sorbitol, 2-ketobutyric acid and sarcosine are biomarkers that differentiate CHIKV and DENV co-infections [107,108].

3.2. Mayaro Virus

The Mayaro virus (MAYV) is a neglected tropical arbovirus responsible for causing a mild febrile illness that may be accompanied by severe incapacitating arthralgia. It was first isolated from the serum of a forest worker in 1954 in Mayaro County on Trinidad Island. Since then, its presence has been reported in several countries within the tropical

regions of South and Central America. It is highly underestimated as a neglected arbovirus that affects mostly poor regions in tropical and subtropical areas. Little investment has been made for epidemiological studies of it, which is considered dangerous, as it could become a public health issue due to its high urbanization potential and remarkable host plasticity [22].

MAYV comprises an enzootic cycle involving *Haemagogus* mosquitoes and monkeys as reservoirs. Secondary vectors such as *Ae. aegypti*, *Ae. albopictus* and *Ae. scapularis* have shown potential for viral infection and spread and secondary hosts such as humans, which poses a significant risk for urban outbreaks and epidemics [109]. The virus is, by phylogenetic analysis, a member of the Semliki complex that consists of seven other viruses: CHIKV, Bebaru virus (BEBV), Getah virus (GETV), Semliki Forest virus (SFV), Ross River virus (RRV), O'nyong-nyong virus (ONNV) and the Una virus (UNAV). The group is characterized by some common antigenic sites that generate polyclonal immune sera cross-reactivity among species and common disease manifestations such as fever, arthritis and skin rash [110]. Phylogenetic studies also classify MAYV into three genotypes: D, widely spread in South America; L, limited to Brazil; and N, recently discovered in Peru [111,112].

As a response to MAYV infection in the human hepatocyte cell line (hepG2), reactive oxygen species (ROS) were produced, causing relevant oxidative stress with increased protein carbonyl and malondialdehyde (MDA) levels, namely biomarkers of lipid peroxidation and protein oxidative modification [113]. There was also a decrease in reduced versus oxidized glutathione (GSH/GSSG) ratio and higher levels of antioxidant activity by CAT (Catalase) and SOD (Superoxide dismutase) enzymatic systems, which was not efficient in restoring the normal redox status due to high oxidative stress levels. As the infection progressed, decreased antioxidant activity and glutathione contents proved that ROS accumulation, such as H_2O_2 , was enhanced [114]. Similarly, MAYV infection was linked to oxidative stress biomarkers in mouse models, such as malondialdehyde, carbonyl protein, myeloperoxidase (MPO) and GSH/GSSG ratio, as well as SOD and CAT activity in the liver. Liver damage was also observed by increased aspartate and alanine aminotransferases (AST/ALT) and high levels of inflammatory cells [115].

Research searching for prominent epitopes for the development of antiviral vaccines is essential, as most of these arboviruses still have no vaccine available. In a recent study for vaccine candidate epitopes conserved and homologous among CHIKV and MAYV, where identification of CD8+ T cell epitopes (CTL) was accomplished from the antigenic structural polyprotein (KPGDSGRPI, TCTMGHFIL, ALSVVTWNK, KPGRRERMC and GRRERM-CMK), two other epitopes were identified for CD4+ T cells (HTL) (MCMKIENDCIFEVKH and DRTLLSQSGNVKIT) and one for B cell (BTL) (GGRFTIPTGAGKPGDSGRPI). All epitopes demonstrated high population coverage, and screening for toxicity, allergenicity, and antigenicity yielded high safety and efficacy results. Using docking analyses, the binding affinity of HTL and CTL epitopes to HLA alleles (MHC class I and II alleles) resulted in high affinity and binding potential. However, further studies are still necessary for structural modification and stability prior to developing vaccine candidates, as these epitopes are short and could easily be degraded by host enzymes [116].

Proteomic analysis of *Ae. aegypti* Aag-2 cells infected with MAYV by label-free mass spectrometry resulted in identifying 5330 peptides and mapping several protein groups within the periods prior to and post-infection (0, 12 and 48 hpi). A total of 161 proteins were identified as differentially expressed. Proteins such as ATP synthase, heat shock proteins (HSP-20 and HSP-60), enolase phosphatase E1 (ENOPH1), membrane-related proteins (such as prohibitin, an important viral entry receptor), vesicle-associated membrane proteins (VAMPs), and overall proteins related to redox, energy metabolism, oxidative stress and viral particle maturation were identified, mostly with increased abundance over time of infection. Glycolytic enzymes related to the energy demand and manipulation of the host metabolism were identified as upregulated. An increased abundance of intermediate metabolites such as glyceraldehyde-3-phosphate was observed, demonstrating high modulation of the glycolysis pathway during infection [117].

An analysis of transcriptomic and small RNA responses to MAYV infection at 2, 7 and 14 days post-infection in *Anopheles stephensi* mosquitoes infected by blood meal resulted in 487 regulated transcripts, with 78 identified miRNAs and a siRNA response towards the MAYV genome. Enrichment of serine proteases related to the activation of the Toll pathway for an innate humoral response during infection was observed from 2 to 7 days post-infection. Moreover, later during infection, results showed the depletion of autophagic and apoptotic pathways, with induction of JNK and MPK cascades as well as repression of JAK/STAT signalling pathways due to repression of MAPK signalling, indicating that such mechanisms are part of mosquito defences against viral replication and the production of new viral particles at late stages of infection. Several differentially expressed miRNAs were identified, such as miR-286b, miR-2944a, miR-2944b, miR-307, miR-309, and novel miRNAs such as mirNOV10 and mirNOV17, and pathway analyses revealed effects in reduced oocyte viability, protein binding and signalling functions, ion channels and transport, actin filament binding and gene transcription. In addition, the identification of piRNA-like small RNAs revealed an antiviral function against replication in the mosquito, along with an increase of siRNAs during infection time related to an increased innate immune response from early to later stages of infection [118].

Bengue and collaborators' studies on the human brain cells revealed the modulation of the immune gene expression profile in MAYV-infected astrocytes, analyzed at 48 hpi (hours post-infection) that PRRs (pattern recognition receptors) such as TLRs are present in brain cells, and the expression of TLR3, and not TLR7 (as for CHIKV infection), was found to be upregulated by MAYV, they also observed a strong induction of the PRRs IFIH1 and DDX58 transcripts by MAYV infection. The chemokines CXCL10, CXCL11 and CCL5, known to be expressed in the CNS during various viral infections, including those by encephalitic arboviruses, were upregulated by the MAYV virus, pointing to the induction of a strong inflammatory response [119].

Metabolic alterations in MAYV infected Vero cells' exometabolome showed a variety of altered components, such as amino acids, organic acids (gamma-keto acid, beta hydroxy acid, carboxylic acid, dicarboxylic acid and phenylpropanoic acid), guanidine compounds (polyamine), monoamine (phenol), carbohydrates (monosaccharides) and lipids, all involved in glycolysis, TCA cycle, phosphate-pentose pathway or lipid β -oxidation. Therefore, alterations were observed in different periods of infection; for instance, in a 2 hpi period, metabolites such as tryptophan, 3-phenylpropionate, valine, carnitine, 3-hydroxyisobutyrate, 2-oxoglutarate and pyruvate presented high levels, whereas, in a 6 hpi period, tyramine, galactose, glucose, creatine, phosphate creatine, galactarate, serine and methyl guanidine were elevated. These results demonstrate that the alteration of metabolites directly influences biochemical reactions in response to viral infection and are generally related to amino acid metabolism, energetic metabolism and lipid metabolism that can be the focus of the future investigation of specific pathways and how viruses affect their hosts metabolically [120].

4. Omic's Study across Members of Bunyavirales Order—*Orthobunyavirus* and *Phlebovirus*

4.1. Rift Valley Fever Virus

Rift Valley virus (RVFV) is a mosquito-borne virus known for causing Rift Valley Fever in humans and livestock throughout Africa and the Arabian Peninsula. It was first described in 1931 and isolated from inoculated lambs with infected sheep serum near Lake Naivasha in Kenya's Rift Valley. Since then, it has caused several economically devastating epizootics with high death ratios among sheep and cattle. Outbreaks were reported in Kenya in 1968, 1978–1979 and 1997–1998, and in Southern Africa, Zambia, South Africa and Zimbabwe. In West and Central Africa, it was only isolated in 1974 and caused severe outbreaks in Mauritania in 1987 and 1998. Other countries such as Senegal, Mali, Guinea, Egypt, Tanzania, Somalia and Madagascar have also reported RVFV outbreaks throughout the years [121,122]. Transmission mainly occurs via infected mosquitoes of the genus *Aedes*,

Culex, *Anopheles*, *Eretmapodites* and *Mansonia*, as well as *Culicoides* (biting midges), *Simuli* (black flies) and even ticks (*Rhipicephalus*). Direct contact with an infected animal's body fluids, tissues or aerosols can also be a risk factor for the infection. Usually, veterinarians and workers who deal with livestock are at significant risk [122].

Based on molecular analysis of the Gn glycoprotein gene sequence, the classified virus isolates from 16 countries into 15 lineages (from A to O) [123]. The RVFV genome is highly conserved, with no defined serotypes and genetic differences that are mainly random single-site mutations with variable regions that are not well defined. Although differences in virulence of strains exist, genetic diversity is considerably low, and multiple strains can co-circulate during endemic periods; co-circulation, as well as co-infection with more than one viral strain, can lead to viral reassortment, a common mechanism in segmented RNA viruses, where exchange of genomic segments occurs in the host cell, resulting in either viable or non-viable reassortants [58]. Technologies for genetic surveillance are essential to tracing outbreaks' origins and their effects. As of very recently, Juma and associates developed a web-based computational tool in order to improve RVFV genomic surveillance. The efficiency was validated by large datasets containing whole genome sequences of the L, M and S segments and the Gn glycoprotein gene sequence with optimal classification results of all 15 lineages at a phylogenetic level, proving to be an effective tool to aid in RVFV surveillance [124].

RVFV outbreaks outside the endemic regions can cause severe public health and agro-economic issues. In humans, the disease is mainly characterized by fever. However, it can progress to a more severe state, including encephalitis, blindness, fulminant hepatitis, retinitis, thrombosis or hemorrhagic syndrome, and the mortality rates are near 20% [121]. RVFV also has a high probability of vertical transmission, as observed in Saudi Arabia in 2000, where pregnant women experienced a series of symptoms such as fever, headaches, dizziness, and myalgia and presented IgG specific to RVFV [125]. In animals, symptoms can also vary from fever, viremia, diarrhea, hemorrhage, lethargy, and death. However, there is a high chance of post-infection sequelae such as limb paralysis and severe hepatitis. In newborn lambs, the virus causes fatal disease and causes symptoms such as high fever (40–41 °C), loss of appetite, lethargy and prostration just a few hours before they die [74].

In the 1960s, a formalin-inactivated vaccine was elaborated to be tested in humans generating neutralizing antibody response [126], and ever since, several formalin-inactivated vaccines have been developed, but meagre quantities still exist and are administered and studied. In a study with seven individuals (37 to 61 years old, four males and three females) that received one to six doses of the RVFV vaccine (ranging from 7–24 years ago), T-cell responses, which have been shown to modulate and eliminate the RVFV disease, could still be detected through the ELISA and neutralization assays. This is due to the many doses of the vaccine received, although with varying responses, probably due to the many years of past vaccination. The study also presented a panel of peptides from N, Gn and Gc antigens that could be applied to further vaccination studies to investigate vaccine effects, T-cell responses and patient long-lived immune protection [127]. Other types of vaccines, such as live-attenuated and new generation vaccines, are still being investigated and developed as human and veterinary vaccines [128].

During infection, RVFV inhibits host cellular RNA synthesis through NSs protein targeting of the transcription factor TFIIF, decreasing its cellular concentration by interacting with its p44 and XPB subunits, inhibiting TFIIF subunits assembly and formation as a means of evading host response, as TFIIF is crucial for host transcription (Figure 7) [129]. Some aspects of innate and adaptive immune response to RVFV were analysed in goats. They developed viremia one-day post-infection (dpi), which persisted for at least four days. The detection of IL-6, IL-12, IL-1 β , IFN- γ , and TNF- α was observed, which resulted in neutralising antibodies post viremia. The absence of IFN- α , which is one crucial antiviral cytokine, was detected [130] and, in agreement with other experiments that established that the NSs protein might also have an essential role in blocking immune response by inhibiting IFN (interferon) α/β production and action, therefore promoting evasion from host de-

fences and presenting more virulent and pathogenic characteristics [131]. Furthermore, NSs seem to promote the downregulation of the double-stranded RNA (dsRNA)-dependent protein kinase (PKR)-mediated eukaryotic initiation factor (eIF)2a phosphorylation in order to secure efficient viral translation, as this mechanism suppresses RVFV translation when NSs is not present [132]. Another virulence factor, the RVFV NSm protein, was identified as an apoptosis inhibitor by suppressing caspase-8, a death-receptor-mediated apoptotic pathway, along with caspase-9 and caspase-3, which are downstream caspases involved in apoptotic pathways, and this makes NSm the first *Phlebovirus* protein identified with antiapoptotic function [133].

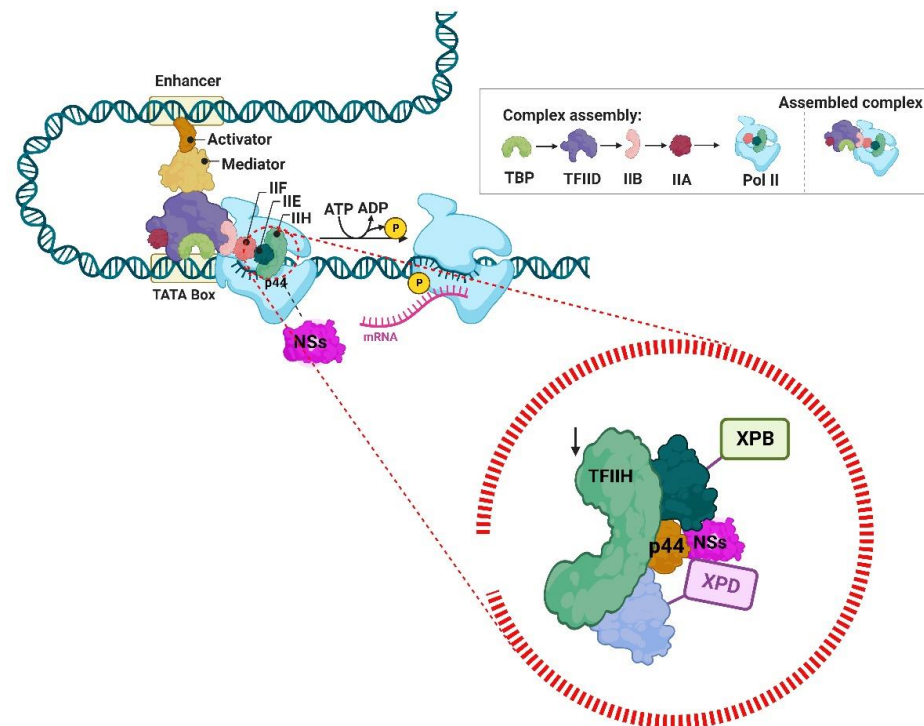


Figure 7. Schematics of RVFV NSs-mediated TFIID suppression. The top portion illustrates the transcription factor of the RNA. At the same time, NSs protein (pink) binds to p44 and XPB, inhibiting TFIID subunits assembly and the formation of the complex to evade host response and shut off host transcription. Created with [BioRender.com](https://www.biorender.com/) (Accessed on 14 September 2022).

LC/MS/MS proteomic analysis of RVFV (MP-12 strain) detected various virion and host-derived protein interactions during infection. Host cytoskeleton protein association to RVFV, such as actin filaments and integrins, was identified, probably for the entry and exit of infected cells and the Golgi viral tubes formed during viral production. The Ras protein superfamily members were also involved, suggesting that they may participate in viral entry. Chaperones have also been observed from heat shock HSP70 and HSP90 protein families, such as HSPA5 (BiP) and HSPA8, subunits of the T-complex protein chaperone (TCP)—CCT2 and CCT6A, and others, working either as host factors used to promote viral replication (BiP) or with antiviral roles to prevent it (HSPA8), which makes these proteins potential targets against RVFV infection [134].

In a reverse phase protein array (RPPA) of attenuated and virulent RVFV strains infecting human cells, changes in phospho-signalling cascades were identified, especially in Smad transcription factors, which are primarily phosphorylated by and transducers of the transforming growth factor-beta (TGF- β) superfamily receptors. Increased phosphorylation of the Smad proteins was related to RVFV replication, and further analysis of transcripts altered by RVFV infection identified 913 genes that contained a Smad-response element. It was related to axonal guidance cell-signalling pathways for cellular adhesion/migration, hepatic fibrosis, cytoskeletal reorganization and calcium influx. A Smad complex on the

interleukin-1 receptor type 2 (ILIR2) promoter was identified, which may be responsible for the disruption of IL-1 receptor activation and the generation of an inflammatory response during infection [135]. In human small airway epithelial cells, RVFV infection increased phosphorylation and activation of MAPK—Mitogen-activated protein kinases (with activation of p38 as a protective cellular response and control of viral replication by ERK—extracellular signal-regulated kinase) and transcription factors such as STAT1 (Signal transducer and activator of transcription 1), AFT2 (Activating transcription factor 2), MSK1 (Mitogen- and stress-activated protein kinase 1), CREB (cAMP response element binding protein) and NF- κ B (Figure 8). The activation of p53 was correlated to increased levels of apoptotic pathways (caspases -3, -6 and -9) and the downregulation of antiapoptotic pathway regulator AKT (also known as PKB—protein kinase B) and apoptosis-inhibitor XIAP (X-linked inhibitor of apoptosis protein) [136].

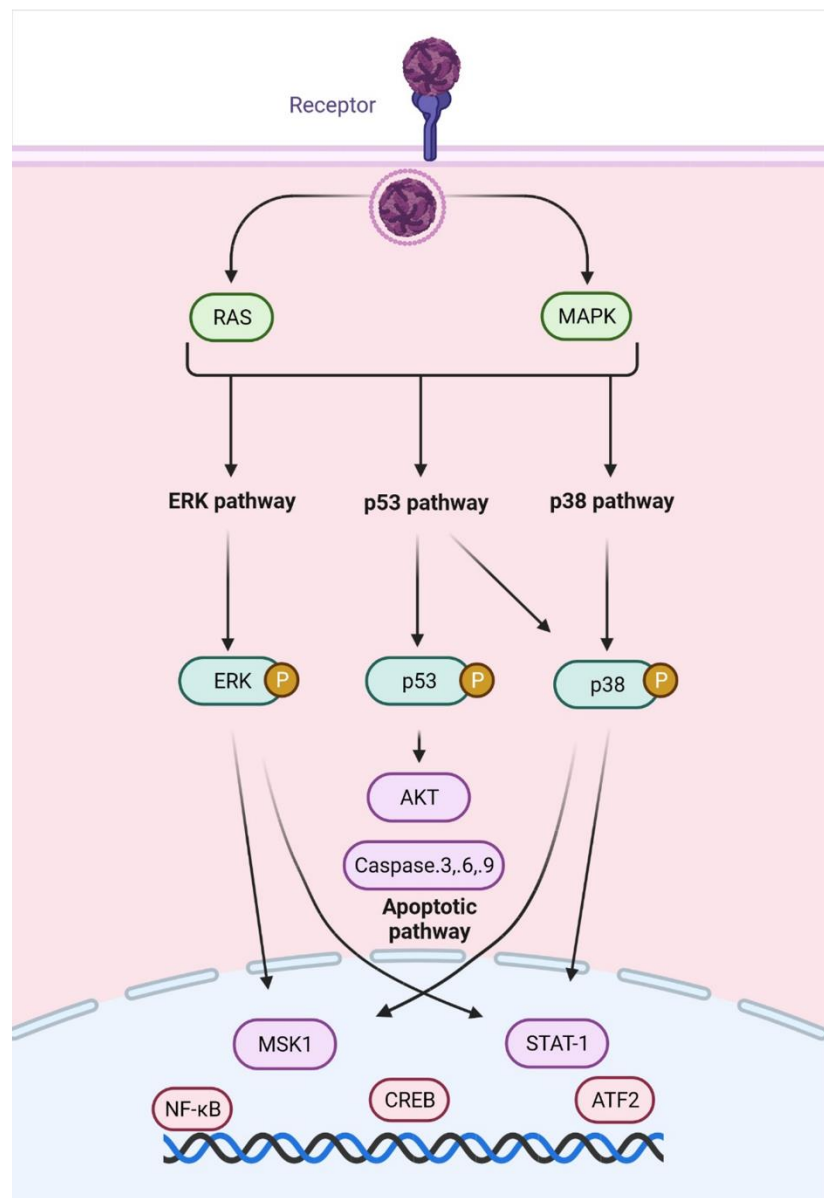


Figure 8. RRVV infection leads to the induction of ERK, p38, MAPK, and p53 phosphorylation. The activation of p53 escorts transcriptional upregulation of the apoptotic genes correlated with the increased levels of cleaved effector caspase -3, -6 and -9 and apoptotic pathways. Created with [BioRender.com](https://www.biorender.com) (Accessed on 13 September 2022).

Transcriptome analysis of human HEK293 cells infected with a vaccine attenuated strain of RVFV resulted in 2090 upregulated genes and 216 downregulated genes. The most upregulated genes were ISGs (interferon-stimulated genes), particularly IFIT2 (Interferon-induced protein with tetratricopeptide repeats 2) and OASL (2'-5'-Oligoadenylate Synthetase Like), both at 18 and 48 hpi, along with other 2'-5' oligoadenylate synthase (OAS) family members and inflammatory cytokines and chemokines such as CXCL10 (C-X-C motif chemokine ligand 10), CCL5 (C-C motif chemokine 5), IL16 (Interleukin 16), ANPT2 (Angiopoietin 2) and CXCL11 (C-X-C motif chemokine ligand 11). Other upregulated genes were related to cell adhesion (ITGAM—Integrin alpha M), microtubule activity (DNAH12—Dynein axonemal heavy chain 12), Wnt signalling (FAP), membrane transport (NPC1L1—Niemann-Pick C1-Like 1), metabolism (CPA2—Cation-proton antiporter 2) and apoptosis (XAF—X-linked inhibitor of *apoptosis* (XIAP)-associated factor 1). Among the downregulated genes, MIR210HG, a microRNA involved in oxidative stress, was very relevant, possibly due to oxidative stress caused by RVFV infection, as NSs are also known to affect mitochondria function and cause redox imbalance generating reactive oxygen species, besides transcription inhibition and other virulent functions [137]. Other significantly downregulated genes were ionotropic glutamate receptors (GRIA3, GRID2), kinesin family member 12 (KIF12) and several noncoding RNAs [138].

In general, pathway analysis showed many genes involved in type I IFN response (NF- κ B—nuclear factor kappa-light-chain-enhancer of activated B cells signalling, TNF—tumour necrosis factor signalling, toll-like-receptor signalling, RIG-I—retinoic acid-inducible gene-I-like receptor signalling and cytosolic DNA sensing) post RVFV infection. The key features include activating cytokine-mediated inflammatory response, cytokine-cytokine receptor interaction, chemokine signalling, and NOD-like receptor signalling. In addition, pathways involved in linoleic acid and arachidonic acid metabolism were altered, indicating their influence on immune and inflammatory responses during infection. Other pathways altered post RVFV infections were PI3K/AKT/mTOR signalling and extracellular matrix (ECM) receptor interaction, probably involved in viral entry and cell-to-cell spread of the virus [138], G2/M DNA damage checkpoint, ATM signalling, mitochondrial dysfunction and ILK signalling (also involved in cell adhesion, cytoskeletal reorganisation and cell mobility) [139–141].

The role of exosomes in modulating the immune response to bacterial and viral infections remains to be fully elucidated, although its importance has been recognized. How these exosomes affect viral replication in infected cells and regulate antiviral responses is still not fully clear. Alem and collaborators demonstrated that exosomes released from RVFV infected cells (Exi-RVFV) containing sequences of the viral genome but lacking the L polymerase (RdRp) or the NSs protein play a protective role against infection by inducing the RIG-I dependent activation of IFN expression leading to autophagy of infected cells in order to resist subsequent viral dissemination [142].

RNA-seq transcriptomic analysis of *Ae. aegypti* cell lines infected by RVFV and DENV (Dengue virus) revealed the upregulation of 39 genes in early (27 genes), late (22) or both early/late (10) responses. Upregulated genes from both infections and early and late responses included proteins such as PGRP (Peptidoglycan recognition protein), GNBP (Gram-negative binding protein), CTLMA 13 and 14 (C-type lectins-mannose binding) and transferrin that are involved in antiviral immune response, and family B and D clip-domain serine proteases (CLIP-B15, CLIP-B34, CLIPB-35, CLIP-B46 and CLIP-D1), which might have roles in defence responses such as hemolymph coagulation, antimicrobial peptide synthesis as well as the melanization of pathogen surfaces [143]. Comparatively, exploring *Culex pipiens* mosquitoes-RVFV molecular interactions through RNA-seq de novo transcriptomic analysis, Nuñez and collaborators identified a total of 451 differentially expressed genes (DEGs), mainly at an early stage of infection and corresponding to metabolic and cellular processes. Forty-eight were characterized by focusing on the immune response-related genes, mostly related to Toll, IMD (Immune deficiency) and RNAi

(RNA interference) pathways, and a great majority were involved in apoptosis or the UPP pathway (Ubiquitin-proteasome pathway), even at an early stage of RVFV infection [144].

Metabolomic studies to evaluate the effects of RVFV infection are still not available in the literature. However, a metabolic profile of anti-RVFV medicinal plants was characterized using ^1H NMR and chemometric analytical techniques along with UHPLC-qTOF-MS (Ultra-High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry) in order to confirm the compounds that are common in plants with antiviral activity. Compounds with significant discriminating results and potential antiviral activity were selected and included chlorogenic acid, vanillic acid, ferulic acid and trigonelline, all of which have already been reported as potent antivirals. Other compounds such as flavonoids (rutin, kaempferol, flavonol glucoside and kaempferol 3-O-rutinoside) were also identified, along with two hydroxylated fatty acids (13S-Hydroxy-9Z,11E,15Z-octadecatrienoic acid and 13-Hydroxy-9Z,11E-octadecadienoic acid), which were identified for the first time in the antiviral plants' samples [145].

4.2. Oropouche Virus

Oropouche virus (OROV) is the agent of oropouche fever transmitted mainly by *Culicoides paraensis* midges that circulate in South and Central America and has caused over 30 epidemics with more than half a million cases over the past 60 years in Brazil—being one of the most critical arboviruses in the Amazon region—Peru, Tobago, Trinidad and Panama. It is characterized by acute febrile illness similar to dengue fever with symptoms such as fever, headaches, skin rash, and muscle and joint pain. In severe cases, it may develop into meningitis or encephalitis [146]. In 1955, OROV was first isolated from a forest worker in Vega de Oropouche in Trinidad [147]. In Brazil, its first isolation occurred in 1960 from a blood sample of a sloth (*Bradypus trydactylus*) seized during the Belem-Brasilia Highway construction. Since then, it has circulated in the Amazon region (South and Central America) and Latin America. More recently, due to climate change, the expansion of the arthropod vectors, globalization and its potential public health significance, it has attracted research attention along with other emerging viruses [61].

OROV is a member of the *Bunyaviridae* family belonging to the *Orthobunyavirus* genus and is a member of the Simbu serogroup that comprises 22 other virus species according to serological evaluation [63]. According to genome sequence analysis, its diversity comprises four genotypes (I–IV) that are spread throughout the regions where OROV is present [148]. Genotype I was reported only in Trinidad and Tobago, while genotype II was restricted to Peru and genotype III was found only in Panama. In Brazil, both genotypes I and II were found, the former restricted to the eastern Amazon and the latest to the western Amazon region. Genotype III was found in Acre and the Minas Gerais States, and genotype IV was exclusively isolated from an outbreak in Manaus in the 1980s and is restricted to Brazil [63,149]. Genetic reassortment is also common among bunyaviruses (as in segmented RNA viruses), making genetic analyses of all three segments crucial for identifying such reassortants, which has yielded a variety of results classifying OROV reassortants based on high similarity of L, S and M segments and its proteins with at least four different viruses (Jatobal virus—JATV, Iqtos virus—IQTV, Madre de Dios virus—MDDV and Perdoes virus—PERDV) [150].

OROV infection in HeLa cells is mediated via clathrin-coated pits, and in an acidification-dependent endocytic pathway, it reaches late endosomes in about an hour. It also causes cell apoptosis via an intracellular pathway that involves mitochondria and is dependent on viral replication and protein expression [61,151]. In mice, OROV infection triggers the activation of mitochondrial antiviral-signalling protein MAVS, interferon regulatory factors such as IRF-3, IRF-7, IRF-1 and IRF-5, which regulate expression and production of type I IFN and ISGs (Interferon-stimulated genes) for host defence and viral control in non-myeloid cells [152]. High levels of liver transaminases and low glucose levels were found, which have also been reported in human serum analysis [153]. Severe liver damage was observed after OROV infection in immunocompromised mice, mainly due to high

levels of cytokines and chemokines such as IL-6, IL-12, p40, G-CSF, KC and others, and immune targeting of infected cells by natural killer and/or cytotoxic T cells, as observed in Flavivirus infection [154]. In addition, IRF-5 has been suggested to play a significant role in modulating host response in peripheral organs controlling OROV dissemination in the central nervous system (CNS), inhibiting the neuroinvasive disease manifestation and also viral replication in the liver, blood, and spleen in mice in early stages of the disease [155].

During OROV infection, IFN-induced responses and TNF signalling components such as TNF-Receptor Associated Factor 3 (TRAF3) and Stimulator of Interferon Genes (STING) are essential for innate immune control. As the infection advances, the virus down-modulates these mediators, trying to escape antiviral pathways, increasing short noncoding miRNAs and type-I IFN [156]. High levels of IFN- α were present in early and late seroconverter patients, as it was considered a universal biomarker of human OROV infection. Early seroconverters presented high levels of CXCL8 (C-X-C Motif Chemokine Ligand 8) and IL-5 through the disease, consistent with the role of IL-5 in B cell activation for antibody production of plasma cells. Late seroconverters produced high levels of CXCL10 and IL-17 along with CCL2 (C-C motif ligand 2), which could be potential complementary biomarkers of OROV fever that precede seroconversion in patients [157,158]. Human peripheral blood mononuclear cells (PBMCs) were susceptible to OROV, where expression of IFNs I, II and III was increased at 24 and 48 hpi, associated with an increase in Mx1 and IFIT1 and decreased supernatant viral titers. Similarly, an increase in RIG-I and MDA5 expression was observed, which are two essential proteins for viral recognition in the cytoplasm (Figure 6), all corroborating for suppression of viral replication in these cells along with an overall increase of IFNs and ISGs, as immunodeficient environments created by blocking IFN response resulted in efficient viral replication [159].

Using sliced cultures from an adult human brain, Almeida and colleagues investigated OROV infection of mature human neural cells, which resulted in the release of TNF- α (pro-inflammatory cytokine tumour necrosis factor- α) and the reduction of cell viability and tissue damage in a 48-h period post-infection, which could generate further neuronal dysfunctions and neurological symptoms in human infections. Although OROV infects both neurons and microglia, the latter were the preferred central nervous system (CNS) cells infected by the virus, again demonstrating that OROV infection may lead to neuronal complications, as microglia cells are the primary defence cells in the CNS that might even serve as viral reservoirs in the brain [160]. OROV infection has also been studied in human blood mononuclear cells, and results demonstrated that T CD4⁺ lymphocytes (Jurkat cells) were more susceptible to infection than other lymphoid lineages. Moreover, CD3⁺, CD14⁺ and other cell populations also showed productive infection, and the presence of a viral genome throughout a few subculturing events is indicative of persistent infection [159].

Hepatocarcinoma cell line HuH-7 infected with OROV submitted to RT-qPCR-based screening detected 13 differentially expressed miRNAs, known for regulating gene expression post-transcriptionally and playing important roles in viral infections [161]. These results demonstrated that miR-217 and miR-576-3p were upregulated during infection, and gene targets involved in apoptosis, type I interferon-mediated response, and antiviral restriction factors were associated with those miRNAs. Also, miR-217 and miR-576-3p are described as inhibitors of IFN- β antiviral response, leading to a decrease in antiviral response as the infection continues, and the NSs protein regulates innate host immunity response, modulating the IFN pathway [156,162].

An investigation of genome-wide transcriptomic alterations of human primary astrocytes infected by the Oropouche, Zika, Chikungunya and Mayaro viruses presented different pathways affected by each virus, but with common signatures related to the down-regulation of innate immune pathways, antiviral response and interferon-associated inflammatory cytokines. Examples of common down-regulated genes/protein expression were DDHX58 (RIG-I), IFN- β and transcription factors related to interferon pathways such as IRF-7. OROV infection alone revealed the up-regulation of pathways related to cell division and cell cycle, DNA maintenance and replication, along with extracellular

matrix remodeling (SPARC/Osteonectin/SPOCK3; ITGAM—Integrin Subunit Alpha M; and COL17A1—Collagen Type XX Alpha 1 Chain; COL20A1—Collagen Type XX Alpha 1 Chain). Interference with cell cycle, DNA condensation and cellular transcription has been observed with orthobunyaviruses. Cell cycle arrest might account for the increased availability of capped mRNAs to act as donors for translation, the cap-snatching mechanism [163,164]. The down-regulation of interferon response and genes with antiviral activities was also observed (RSAD2—Viperin; OASL and OAS1—2′5′-Oligoadenylate Synthetase, MX1/2—MX Dynamin-like GTPase 1; IFIT2—Interferon-induced Protein with Tetratricopeptide Repeats 2), and evidence of the down-modulation of ion channels, transmembrane solute carriers and synapse regulation pathways was also identified, revealing manifestations of OROV neuropathology [165].

As no vaccination is available, studies on immunogenic and antigenic epitopes are fundamental for developing antiviral therapies. Through immunoinformatic techniques, analyses of OROV proteome for the search of T-cell and B-cell epitopes were performed along with finding inhibitor-binding sites by docking simulation of the putative OROV polyprotein, which was identified as the most antigenic protein with a total of 128 epitopes with the highest antigenic scores. By narrowing criteria (antigenicity, immunogenicity and conservancy), a total of 18 highly conserved T-cell epitopes were identified as potential vaccine candidates, with significant population coverage and with one particular epitope (LAIDTGCLY) defined as a putative vaccine candidate due to its binding affinity to both MHC-I and MHC-II alleles identified through docking experiments. Moreover, five highly conserved B-cell epitopes were also identified, and one in particular (HHYKP-TKNLPHVVPRYH) presented promising results. The 3D OROV polyprotein structure presented ten possible ligand-binding pockets, and its amino acid residues were identified, supporting its potential as an antiviral target for future research against OROV infection [166].

5. Remarks and Conclusions

As arboviruses are the causative agents of recurrent epidemics worldwide, and the infection of humans may result in long-term complications and chronic conditions, understanding the viral structure, mechanisms and interactions with hosts is a key step for the development of antiviral approaches. Among the nearly 540 arboviral species comprised within several families and genera are the mosquito-borne Chikungunya, Mayaro, Oropouche and Rift Valley viruses which belong to the *Alphavirus*, *Orthobunyavirus* and *Phlebovirus* genera, respectively, and which were the main focus of this review, which aims to provide information on arboviral mosquito infection-related aspects obtained from "omics" studies and approaches. Herein, we aimed to display how some of the tools and technology sensitivity of omic approaches provide a better understanding of biological systems and validate the potential effects these viruses have on experimental animal models, cell culture, and, more importantly, humans. The study integration of genes to transcripts, proteins and metabolites can provide a holistic and unique perspective on biomarkers and biochemical fingerprints present in a specific host due to different conditions set by pathogens or diseases. Improving knowledge of how these viruses affect organisms, especially humans, is critical for developing future therapies or medications to prevent or treat such relevant public health issues. Although the combination of several omics approaches and the integration of these results for biological interpretation is still a challenge, thanks to technology advances and improvement of online databases, future studies regarding these viruses or other pathogens under the multi-omics approach might be able to offer new perspectives on systems biology to elucidate several aspects of such recurrent human pathogeneses.

Author Contributions: Conceptualization: M.A.C., writing-original draft preparation: R.d.S.P., figures: M.A.C., writing-review and revision: R.d.S.P., R.J.E., R.K.A. and M.A.C., supervision: M.A.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Process numbers 2020/03639-6 and 2020/08615-8), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Process number 309940/2019-2) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: M.A.C. and R.J.E. like to thank the support of the Institute of Biological Information Processing, Structural Biochemistry (IBI-7) Forschungszentrum Jülich, Germany.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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Article

HR-MAS NMR Metabolomics Profile of Vero Cells under the Influence of Virus Infection and nsP2 Inhibitor: A Chikungunya Case Study

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Citation: Peinado, R.d.S.; Martins, L.G.; Pacca, C.C.; Saivish, M.V.; Borsatto, K.C.; Nogueira, M.L.; Tasic, L.; Arni, R.K.; Eberle, R.J.; Coronado, M.A. HR-MAS NMR Metabolomics Profile of Vero Cells under the Influence of Virus Infection and nsP2 Inhibitor: A Chikungunya Case Study. *Int. J. Mol. Sci.* **2024**, *25*, 1414. <https://doi.org/10.3390/ijms25031414>

Academic Editor: Luigi Russo

Received: 16 December 2023

Revised: 11 January 2024

Accepted: 16 January 2024

Published: 24 January 2024



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Abstract: The arbovirus Chikungunya (CHIKV) is transmitted by *Aedes* mosquitoes in urban environments, and in humans, it triggers debilitating symptoms involving long-term complications, including arthritis and Guillain-Barré syndrome. The development of antiviral therapies is relevant, as no efficacious vaccine or drug has yet been approved for clinical application. As a detailed map of molecules underlying the viral infection can be obtained from the metabolome, we validated the metabolic signatures of Vero E6 cells prior to infection (CC), following CHIKV infection (CV) and also upon the inclusion of the nsP2 protease inhibitor wedelolactone (CWV), a coumestan which inhibits viral replication processes. The metabolome groups evidenced significant changes in the levels of lactate, myo-inositol, phosphocholine, glucose, betaine and a few specific amino acids. This study forms a preliminary basis for identifying metabolites through HR-MAS NMR (High Resolution Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy) and proposing the affected metabolic pathways of cells following viral infection and upon incorporation of putative antiviral molecules.

Keywords: arbovirus; Chikungunya; metabolomics; ¹H-NMR; Vero cells; wedelolactone

1. Introduction

Chikungunya virus (CHIKV) is a reemerging arbovirus (arthropod-borne virus) from the *Togaviridae* family and *Alphavirus* genus mainly transmitted by mosquitoes of the *Aedes* genus (*Ae. aegypti* and *Ae. albopictus*), which along with other arboviruses such as Dengue, Zika and yellow fever virus are a public global health concern, as they are spread worldwide [1] and are responsible for significant global human morbidity, mortality and recurrent epidemics [2]. The aforementioned viruses belong to neglected tropical diseases (NTD), which, although manageable, affect more and more of the world's population [3]. Low investment in the already fragile healthcare systems and a lack of potent vaccines or antiviral drugs make it difficult to control the spread of these infectious diseases [4,5].

Acute symptoms of CHIKV infection include high fever, myalgia, arthralgia, skin rashes, nausea, headaches, intense asthenia and conjunctivitis. Eventually, CHIKV may

also cause long-term debilitating conditions and complications, such as arthritis, meningoencephalitis, encephalopathy, hepatitis, myocarditis and Guillain-Barré syndrome [6,7].

CHIKV is a positive-sense RNA virus (12 kb) with two open reading frames that encode four nonstructural proteins (nsP1–nsP4), which are primarily responsible for viral replication, transcription and host evasion, and five structural proteins (C—capsid, E3, E2, 6K/TF and E1—envelope), which are encoded by subgenomic RNA in infected cells [8]. Among the nonstructural proteins, nsP2 plays diverse roles in replication, propagation and host gene expression shutdown, displaying proteolytic, NTPase and RNA helicase activities within its C- and N-terminal domains, making nsP2 a potential target for antiviral research [9,10].

Clinically approved vaccines or antiviral drugs are not yet available against CHIKV. Thus, disease prevention still relies solely on vector control and the treatment of symptoms with anti-inflammatory, analgesic and antipyretic drugs. Globally, scientific efforts have focused on the discovery of novel antiviral drugs and the repurposing of existing drugs such as chloroquine [11], ribavirin [12], favipiravir [13], immunotherapy [14] and other inhibitor molecules [15], but further research is vitally important [16]. In this context, wedelolactone (WDL), a coumestan derived from *Eclipta prostrata* (*Asteraceae*), has demonstrated potential as an antioxidant [17], anti-inflammatory [18–21], hepatoprotective [22–24] and anticancer [25–30] molecule and also as an inhibitor of HIV-I (human immunodeficiency virus type I) [31], hepatitis C virus RNA polymerase [32] and replication of HCMV (human cytomegalovirus) [33]. However, no studies have been reported on its activity toward CHIKV nsP2 nor CHIKV infection in cellular models.

Understanding how viruses hijack the host cellular machinery and how potential antiviral drugs affect cell metabolism when placed in the cellular environment is imperative. HR-MAS ^1H -NMR metabolomics permits the identification of metabolites with experimental reproducibility and relatively rapid sample preparation, which makes it well-suited for diverse solid/semi-solid sample types [34]. The spectral data obtained represent unique chemical fingerprints that, combined with statistical methods, provide valuable information on metabolic alterations caused by different conditions, such as diseases, pathogens and inhibitor molecules [35–37].

In the present study, we explored the inhibitory potential of wedelolactone against the nonstructural protein 2 of CHIKV as well as the metabolic disturbances in model mammalian cultured cells (Vero E6—African green monkey kidney cells). Vero E6 cells are highly permissive and commonly used in viral propagation and viral assays, and while they lack interferon response genes, our primary objective was to explore the impact of the virus's presence and potential inhibitors on their metabolism. We explored the changes caused by both CHIKV and WDL using HR-MAS ^1H -NMR spectroscopy methods. We suggest that metabolomics studies of viruses and inhibitors may represent a rapid and efficient approach for unveiling viral and inhibitor mechanisms that serve as the basis for further investigation of metabolic pathways and drug development through omics integration.

2. Results

2.1. In Vitro Inhibition Assay of the CHIKV ns2 Protease (nsP2) by Wedelolactone

Experiments employing a wide range of WDL concentrations (ranging from 0 to 50 μM) were performed, and the titration of the molecule demonstrated 100% protease inhibition at 25 μM WDL. Using the titration data, the half-maximal inhibitory concentration (IC_{50}) of WDL toward nsP2 was determined to be $2.3 \pm 0.4 \mu\text{M}$ (Figure 1). This value is in the same range as previously reported flavonoids and small molecules targeting CHIKV nsP2: hesperetin (2.5 μM) and hesperidin (7.1 μM) [38] and 1,3-thiazolbenzamide derivatives (between 8.3 and 13.1 μM) [39].

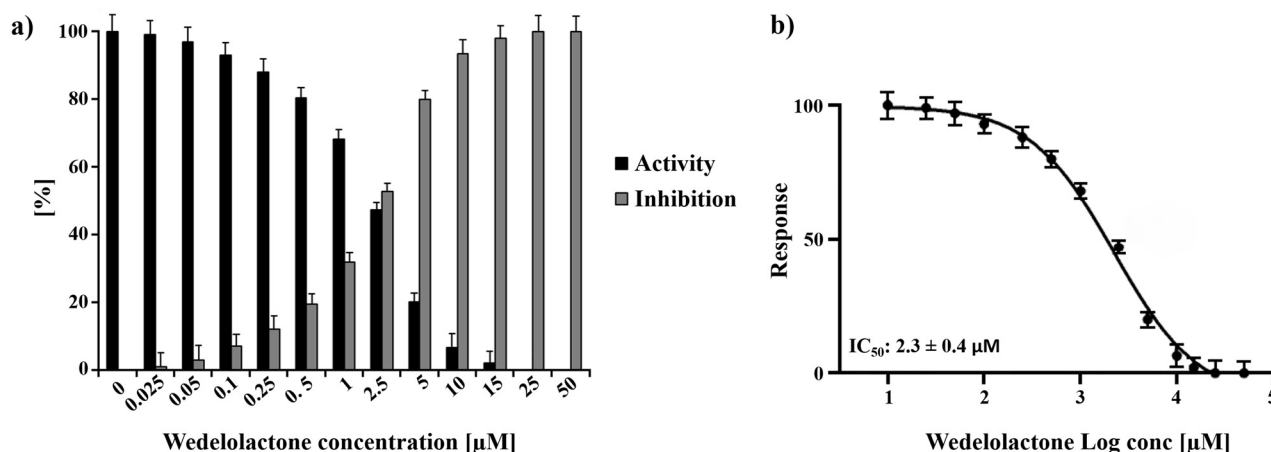


Figure 1. Normalised activity and inhibitory effects of WDL on nsP2 protease. (a) Inhibition of nsP2 by WDL with the concentrations ranging from 0 to 50 μM. (b) Dose response curve of WDL and nsP2 depicting the the half-maximal inhibitory concentration (IC₅₀) of 2.3 ± 0.4 μM. The experiments were performed as triplicates and the results are shown as mean ± standard deviation (SD).

2.2. Assessment of Wedelolactone Cytotoxicity in Vero E6 Cells

A cytotoxicity assay of WDL was performed on Vero E6 cells to obtain the CC₅₀ value. Cells were treated with different concentrations of WDL and DMSO (negative control) for an incubation period of 48 h. Living cells were quantified through the MTT assay by assessing the production and solubilisation of formazan crystals [40]. The results yielded a CC₅₀ value of 373.5 ± 154.2 μM, calculated using GraphPad Prism (8.0) from a normalised dose–response curve fitting (Figure 2). Cell viability was also calculated for each concentration used and is available at Table S1.

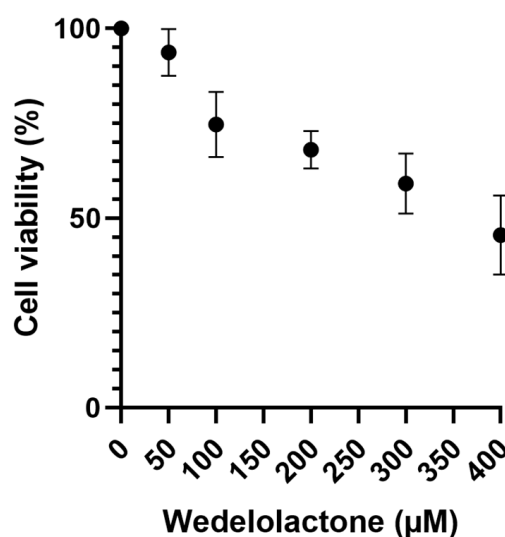


Figure 2. Vero E6 cell viability MTT assay using a range of WDL concentrations from 50 to 400 μM where live cells were quantified. Cell viability percentages are shown on the y axis, while WDL concentrations (μM) are on the x axis. Error bars represent standard deviations. Values are the mean ± standard error obtained from three independent experiments.

2.3. Effects of Wedelolactone on CHIKV-Infected Cells

To evaluate the in vitro inhibitory potential of WDL on CHIKV infection, we treated infected cells with different concentrations of WDL (5, 10, 15, 20 and 30 μM) based on our nsP2 inhibition assay results. This was performed to evaluate the antiviral effects of the molecule on postentry steps, including replication, virion assembly and release, as these

were the focus of our metabolomics analyses conducted during the postinfection period. After a 24 h period postinfection and treatment, supernatants were collected, and viral titration was performed. We observed a slight decrease (ANOVA, Dunnett's test, $p < 0.0001$) in viral titres with concentrations of 20 μM and 30 μM , with 0.44 and 0.45 Log_{10} units decreases in viral titres, respectively, compared to the viral progeny titres in controls (6.92 Log_{10} units, corresponding to approximately 10^7 PFU/mL); see Figure 3. The IC_{50} for WDL in this assay was calculated and determined as $22.9 \pm 6.7 \mu\text{M}$.

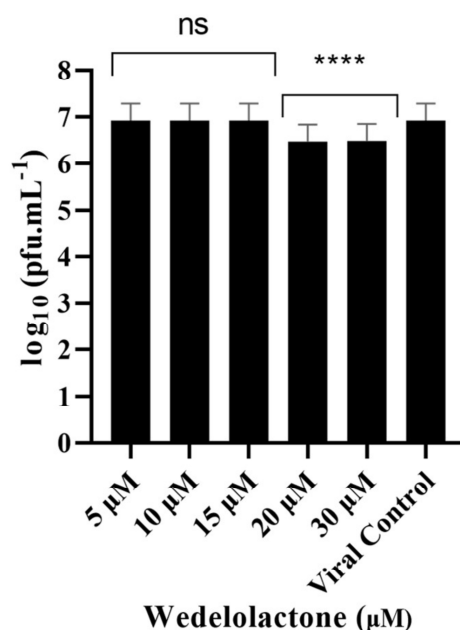


Figure 3. Dose-dependent inhibitory effects of WDL on CHIKV titre yields. Cells were infected with CHIKV at MOI of 0.1 and treated with different concentrations of WDL ranging from 5 μM to 30 μM . The reduction in the viral titre was determined by plaque-forming assay after collection and titration of the supernatants 24 h postinfection and treatment. PFU infectivity titration of CHIKV is shown on the left vertical axis. Error bars represent standard deviations. Values are the mean $\pm$ standard error obtained from three independent experiments. Asterisks indicate statistical significance between the control and each group as determined by two-way ANOVA and subsequent Dunnett's test (ns, not significant; ****, $p < 0.0001$).

2.4. HR-MAS NMR Spectroscopy Applied to the Analysis of Vero E6 Cells

Twenty-nine metabolites were assigned in the ^1H -NMR spectra of the Vero E6 cell control group (Figure 4, Table S2). The 2D-HSQC (Figure S2) and 2D-TOCSY experiments were used to identify and assign the metabolites accurately.

Representative NMR spectra of each group with identified metabolites are shown in Figure S1. The T_2 -edited (CPMG) ^1H NMR spectra highlighted the most relevant differences in the aliphatic region (0.5–4.55 ppm).

2.5. Chemometric Analysis

We first used an unsupervised discriminant model (PCA) to explore intrinsic groupings and reduce dimensionality of datasets from the different conditions. The clustering tendency between the control samples and the other groups reflects the overall changes affected by infection or the inhibitor (Figure S3). The postscattering between the first two components of NMR data of CC $\times$ CW, PC1 (27.2%) and PC2 (18.7%), presented a net variance of 45.9% (Figure S3a). The other groups presented a net variance of 56.1% (CC $\times$ CV), 60.9% (CC $\times$ CWV) and 60.3% (CV $\times$ CWV) (Figure S3b–d), which indicates significant variability before and after the infection and following the addition of the WDL. PCA analysis was performed on the data matrices of all groups, indicating that the control cells and cells treated with the inhibitor (CC and CW) were similar among

themselves, and as expected, similarities among the infected cells (CV and CWV) were also observed (Figure S4a).

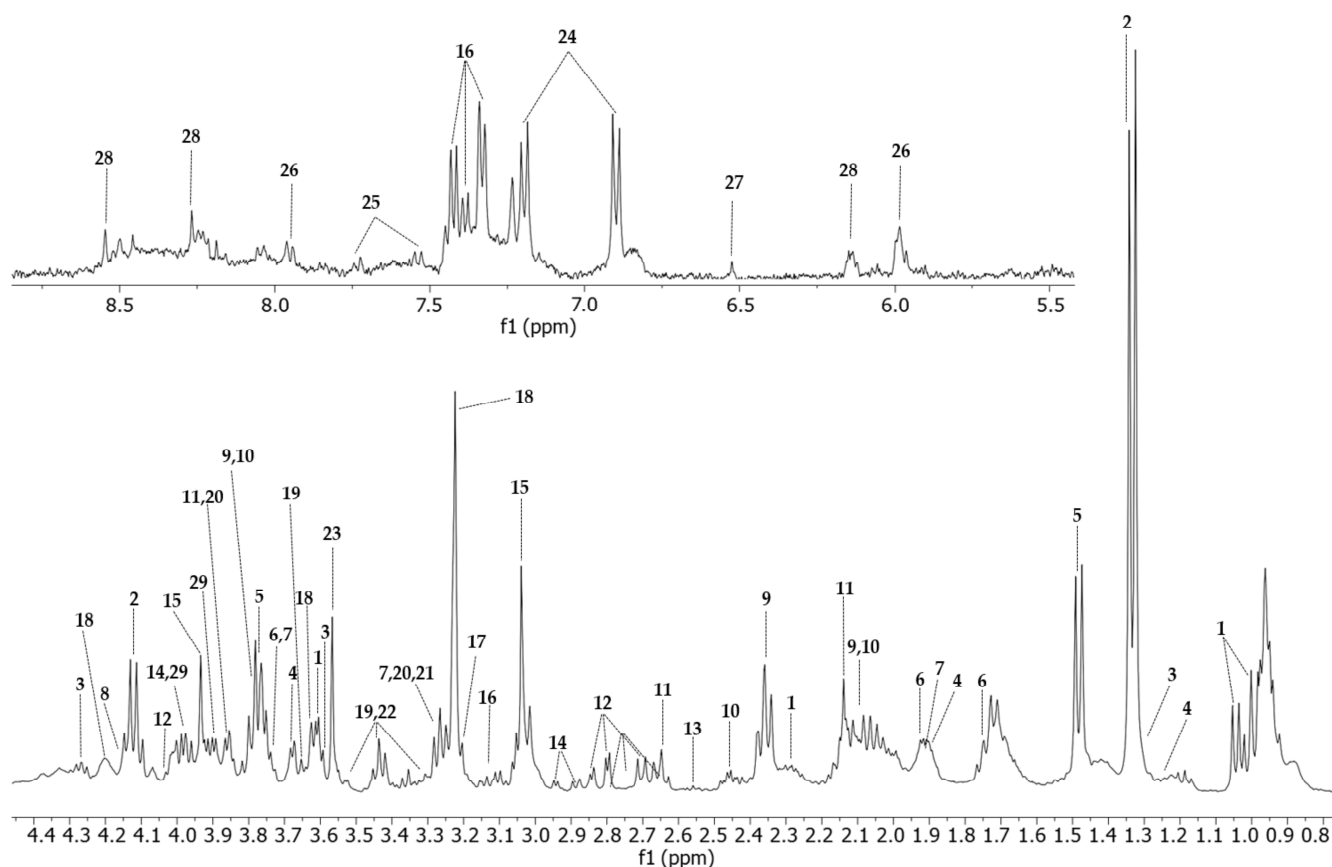


Figure 4. HR-MAS ^1H -NMR T_2 -edited (CPMG) spectra (400 MHz) of all four conditions. Vero E6 control sample is represented in green (CC), cells treated with WDL (CW) are in blue, cells infected and treated with the molecule (CWV) are represented in purple and CHIKV-infected cells are represented in red (CV). Identified metabolites are Val (valine); Lac (lactate); Iso (isoleucine); Pro (proline); Met (methionine); Asp (aspartate); Cre (creatine); Phe (phenylalanine); Pho (phosphocholine); Glu (glucose); Bet (betaine); Myo (myo-inositol). HR-MAS ^1H -NMR with T_2 filter spectrum of a Vero E6 cell sample acquired with a Bruker Avance III 400 MHz using a CPMG (*cpmgpr1d*) pulse sequence. The upper image of the spectrum comprises the region between 5.0 and 9.00 ppm, amplified 20 times, and the lower image shows the 0.80 to 4.55 ppm region. All peaks assigned to a particular metabolite are numbered. 1: valine; 2: lactate; 3: threonine; 4: isoleucine; 5: alanine; 6: lysine; 7: arginine; 8: proline; 9: glutamate; 10: glutamine; 11: methionine; 12: aspartate; 13: hypotaurine; 14: asparagine; 15: creatine; 16: phenylalanine; 17: choline; 18: phosphocholine; 19: glucose; 20: betaine; 21: trimethylamine-N-oxide; 22: myo-inositol; 23: glycine; 24: tyrosine; 25: tryptophan; 26: UDP-N-acetylglucosamine, UDP-glucose, UDP-glucuronate; 27: fumarate; 28: ATP; 29: serine.

To focus our attention on the influence of the virus and the inhibitor on the metabolomic profiles of the Vero E6 cells, we assessed the significance of the results using a supervised PLS-DA model. As presented in Figure 5a–d, the PLS-DA class discrimination model maximised the difference between the groups and obtained better separation effects than PCA, demonstrating substantial differences in cellular metabolism among the analysed conditions and presenting adequate cross-validation results of the model through values of accuracy, R^2 and Q^2 (Table 1).

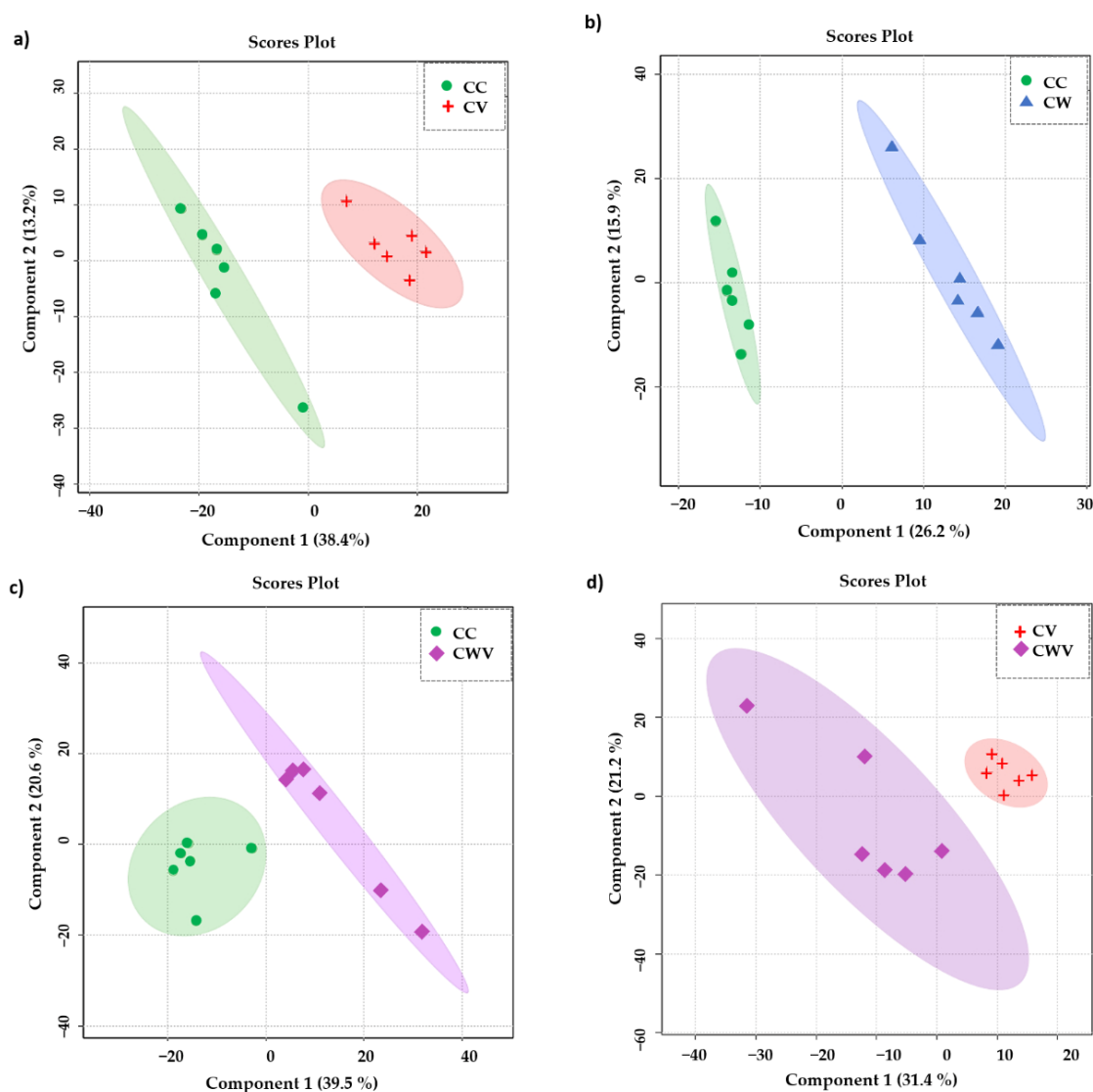


Figure 5. PLS-DA scores plot in 2D of the HR-MAS ^1H -NMR data showing the discrimination between the healthy cell control (CC, green circles) and the other conditions: (a) CC $\times$ CV (red crosses); (b) CC $\times$ CW (blue triangles); (c) CC $\times$ CWV (purple diamonds); (d) CV $\times$ CWV. The explained variance of the models with the two first latent variables is represented on the x and y axes.

Table 1. Accuracy, R^2 and Q^2 values of the best performance PLS-DA models calculated by LOOCV.

Classes	Accuracy	R^2	Q^2
CC $\times$ CV	0.91	0.99	0.78
CC $\times$ CW	1.0	0.99	0.90
CC $\times$ CWV	1.0	0.99	0.85
CV $\times$ CWV	0.91	0.99	0.72
CC $\times$ CV $\times$ CW $\times$ CWV	0.87	0.98	0.85

2.6. Metabolite Discrimination

The most significant variables contributing to class discrimination in the PLS-DA models were identified through VIP graphs. Metabolites with VIP scores > 1.5 in the PLS-DA model (Figure S5) and $p < 0.05$ in the t -test were selected as differential metabolites. Box and whisker plots show relative concentrations for the most significantly altered metabolites ($p < 0.05$) in cell control (CC, green) and CV (red), CW (blue) and CWV (purple)

(Figure 6). False discovery rate (FDR) estimates and the p -value threshold are presented in Tables S3–S6.

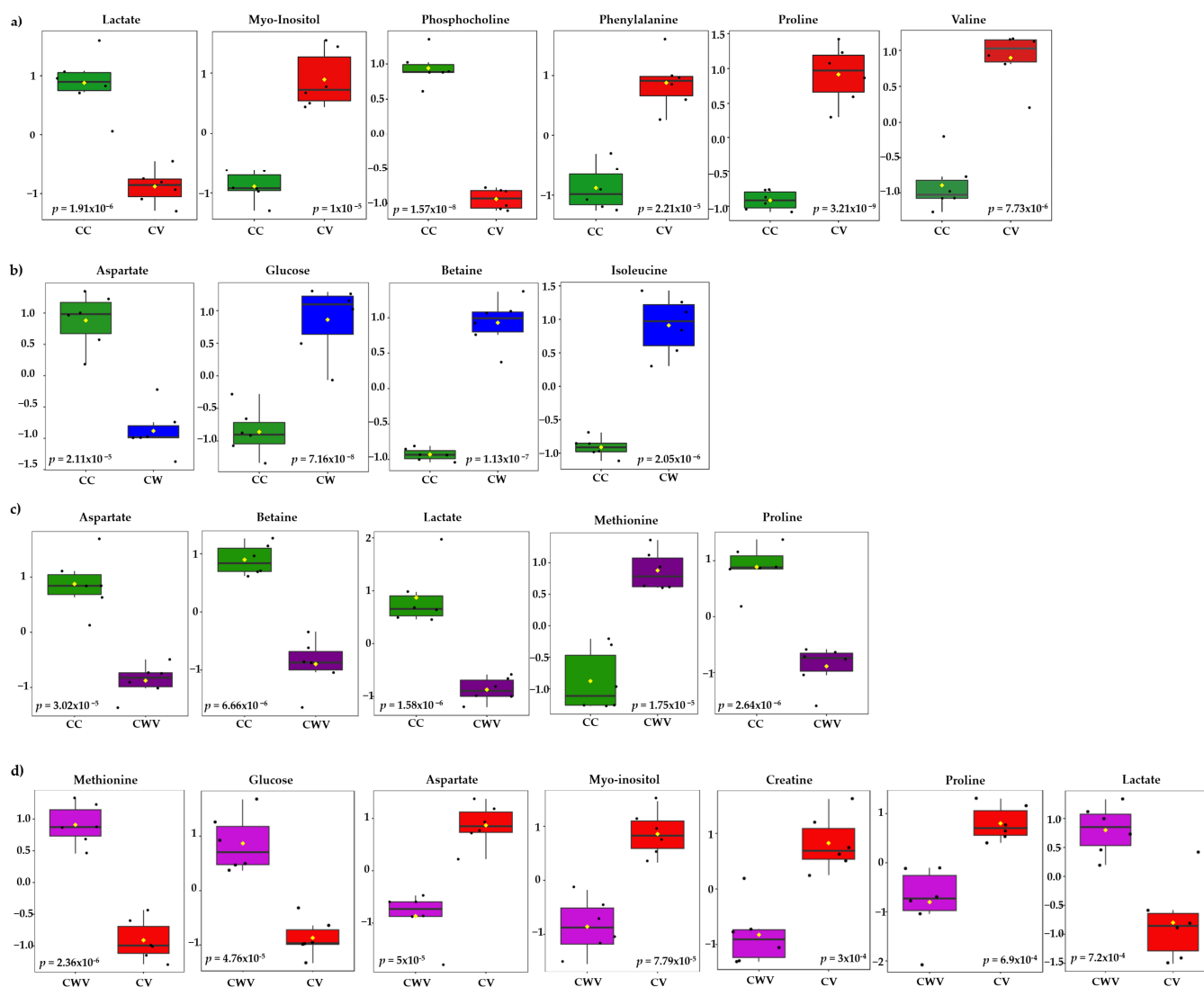


Figure 6. Box plots of discriminant metabolites identified in the univariate analysis (t -test, $p < 0.05$) of the (a) CC × CV, (b) CC × CW, (c) CC × CWV, (d) CV × CWV groups. Relative metabolite levels between the groups are shown (measured as peak intensities). The black dots represent metabolite levels in the samples, and the yellow diamond represents the average value for the group, as indicated on the y axis. Groups are CC, green; CV, red; CW, blue; CWV, purple.

Six of the twenty-nine identified metabolites showed significant differences between CC × CV; they are proline (δ 4.14), phosphocholine (δ 4.22), myo-inositol (δ 3.52), lactate (δ 1.33), valine (δ 2.28) and phenylalanine (δ 3.11) (Figure 6a). The cells treated with WDL (Figure 6b) presented differences in four metabolite levels: glucose (δ 3.54), betaine (δ 3.89), isoleucine (δ 3.67) and aspartate (δ 2.68). The corresponding CWV samples showed similar tendencies with five significant metabolite differences between the compared groups (CC × CWV): methionine (δ 3.85), lactate (δ 1.33), proline (δ 4.14), betaine (δ 3.89) and aspartate (δ 2.68) (Figure 6c). Lastly, we compared the infected cells (CV) with the infected WDL-treated cells (CWV), and the most significant metabolites were methionine (δ 3.85), glucose (δ 3.54), aspartate (δ 2.68), myo-inositol (δ 3.52), creatine (δ 3.93), proline (δ 2.36) and lactate (δ 1.33) (Figure 6d).

2.7. Heat Map Analysis of Metabolites

Hierarchical clustering analyses (HCA) of the PLS-DA data were performed to explore the substantial changes in each metabolite among the groups. The heat maps visually present the relative increase or decrease in differential metabolite levels in Vero E6 cells in each condition described in the previous analyses (Figure 7).

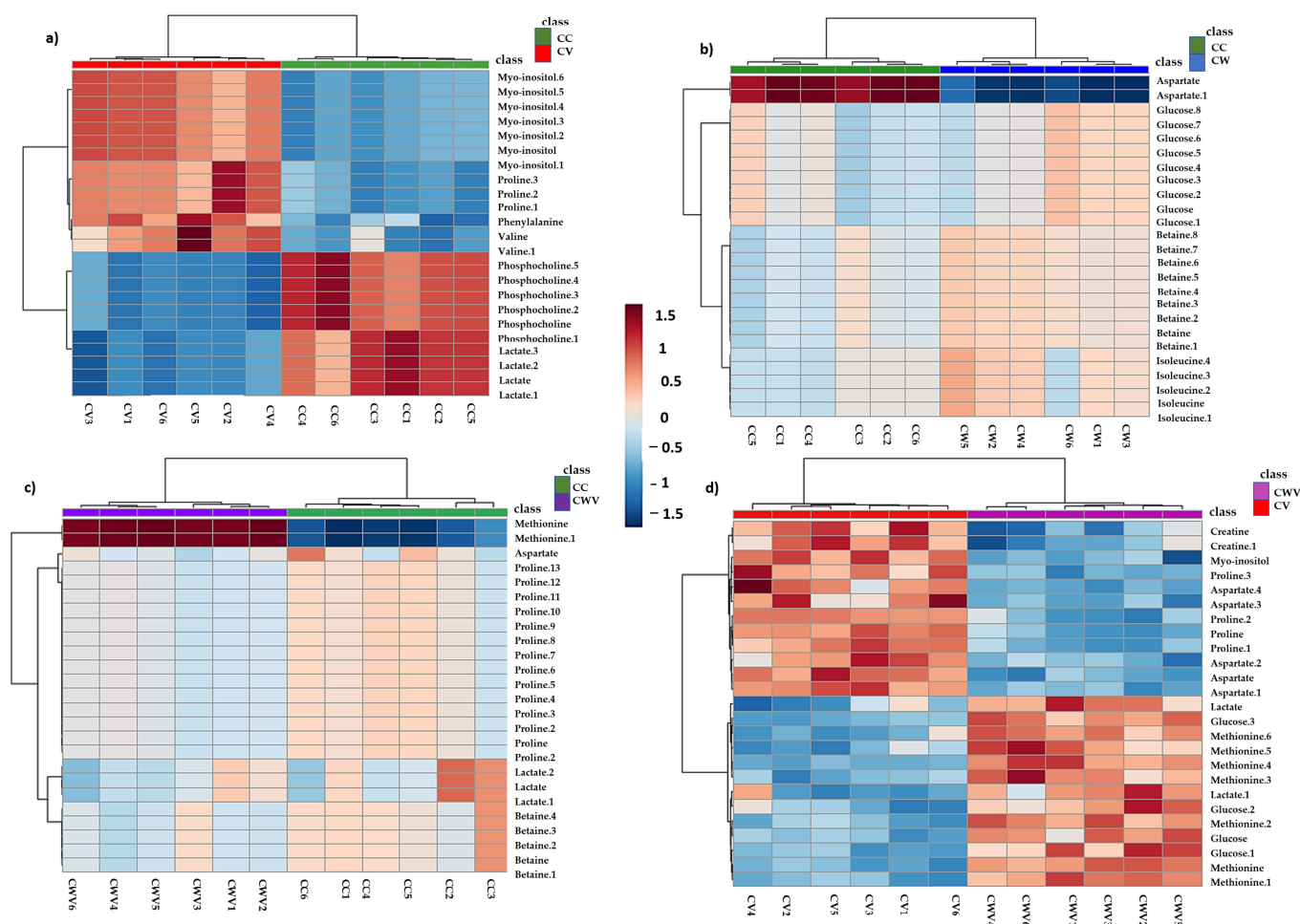


Figure 7. Hierarchical clustering through heat maps analysis using Euclidean distance. The lines represent the discriminant variables from the PLS-DA analysis for (a) CC × CV, (b) CC × CW, (c) CC × CWV and (d) CV × CWV. The columns represent the samples, and the colour bars on the right represent the relative concentrations of the discriminant metabolites (red: higher relative concentration; blue: lower relative concentration).

The heat map displayed significant differences and clustering of groups along with differences in metabolites' relative concentrations, demonstrating the overall metabolic variation in the different conditions. In CHIKV-infected cells, levels of myo-inositol and amino acids (proline, phenylalanine and valine) increased, while decreased levels of phosphocholine and lactate were observed (Figure 7a). Cell treatment with WDL decreased relative levels of aspartate, whereas levels of glucose, betaine and isoleucine increased (Figure 7b). Furthermore, the CHIKV-infected cells treated with WDL showed increased methionine levels, while aspartate, betaine, lactate and proline showed lower intensities (Figure 7c). When comparing the CV × CWV results, we observed a decrease in metabolites that were increased during viral infection (CV); they were myo-inositol and proline. Moreover, similar to the CW samples, an increase in glucose, methionine and lactate and decreased aspartate levels were observed. Interestingly, here we also identified an alteration in creatine, which presented higher levels in CHIKV-infected cells and lower levels when

the infected cells were treated with WDL (Figure 7d). Figure S4b shows the heat map with all conditions, and the metabolic alterations will be discussed further.

3. Discussion

Our results showed that WDL possesses an inhibitory potential towards CHIKV nsP2 protease in vitro in the low μM concentration range (IC_{50} value of $2.3 \pm 0.4 \mu\text{M}$). Our value is similar to IC_{50} values reported previously for flavonoids targeting CHIKV nsP2 (HST: $2.5 \pm 0.4 \mu\text{M}$, HSD: $7.1 \pm 1.1 \mu\text{M}$) [38]. In contrast, we observed a slight antiviral effect in CHIKV-infected Vero cell cultures. A major problem of the development and discovery of potent antiviral molecules is an efficient cellular delivery; molecules with a poor permeability possess a reduced bioavailability [41]. Additionally, the molecules can be stored in cellular compartments where they are not available for the virus target proteins. However, the treatment of CHIKV-infected Vero cells with WDL demonstrated metabolic alterations that we could observe through our metabolomic experiments.

As virus formation depends on the host cell's metabolic capacity to provide the required low-molecular-weight metabolites (nucleotides, amino acids and fatty acids (FAs)/lipids) and energy in favour of viral infection, the current study speculates about the metabolic changes of Vero E6 cells induced by CHIKV infection and the inclusion of an nsP2 inhibitor (WDL) exclusively through the HR-MAS ^1H NMR method.

In our findings, we observed a significant reduction in lactate production in infected cells (CV) when compared to control cells (Figure 6a). This stands in contrast to the findings in MAYV-infected cells, where lactate production exhibited an increase 8–10 h postinfection [42], suggesting a dynamic shift in glycolytic activity throughout the infection process. The sustained decrease in lactate production over a prolonged period (24 h postinfection) leads us to hypothesise that glucose may be metabolised into pyruvate, subsequently entering the TCA cycle. Alternatively, it is plausible that lactate is repurposed as a gluconeogenic agent, serving as a vital energy source [43]. It is important to emphasise the role of the TCA cycle in synthesising amino acids, lipids, nucleotides (purine and pyrimidines) and nucleic acids [44–48] and its significant contribution to ATP synthesis, which is crucial to meet the high-energy requirements during infection. Additionally, overall evidence of glycolysis requirement for efficient replication of alphaviruses has been reported for Semliki, Sindbis and Mayaro viruses, where glycolysis inhibitors substantially diminish viral yields, indicating that glycolysis plays a pivotal role during infection [42,49].

In cultured cells, amino acids from culture media are assimilated and synthesised to form the necessary pools crucial for sustaining normal metabolism, biosynthetic activities and overall cellular homeostasis, and they also play a pivotal role during viral infection [50–52]. Accordingly, our results showed that valine, phenylalanine and proline were increased during CHIKV infection. These findings demonstrate broad alterations in glycolysis and TCA cycle metabolism to actively support and facilitate the infection process [53] (Figures 6a and 8).

We also observed elevated levels of myo-inositol in response to CHIKV infection (Figures 6a and 8). Myo-inositol serves as a major component of phospholipids and inositol phosphate derivatives, such as phosphatidylinositol [54]. Beyond its primary role in sensing metabolic shifts and energy demands through inositol phosphate signalling pathways [55], there is a presumed involvement in the assembly and maintenance of the integrity of replication complexes along with recruitment of host factors or viral components, as described by Zhang and collaborators [56].

Viral infection also regulates cellular lipid metabolism [57]. Accordingly, our results demonstrated perturbation of phospholipid metabolism with decreased phosphocholine levels during infection (Figures 6a and 8). Choline and phosphocholine are substrates for the synthesis of phosphatidylcholine (PC), which constitutes approximately half of the phospholipid species in most mammalian cells [58] and is a biosynthetic precursor for lipid-signalling molecules and lipoproteins [59]. PC synthesis is enhanced during the infection of numerous viruses, including Dengue virus [60], poliovirus [61] and Brome

mosaic virus (BMV), with a high accumulation of PC and choline metabolism enzymes for PC synthesis within the viral replication sites to sustain viral replication complexes (VRCs) and stimulate viral replication [62].

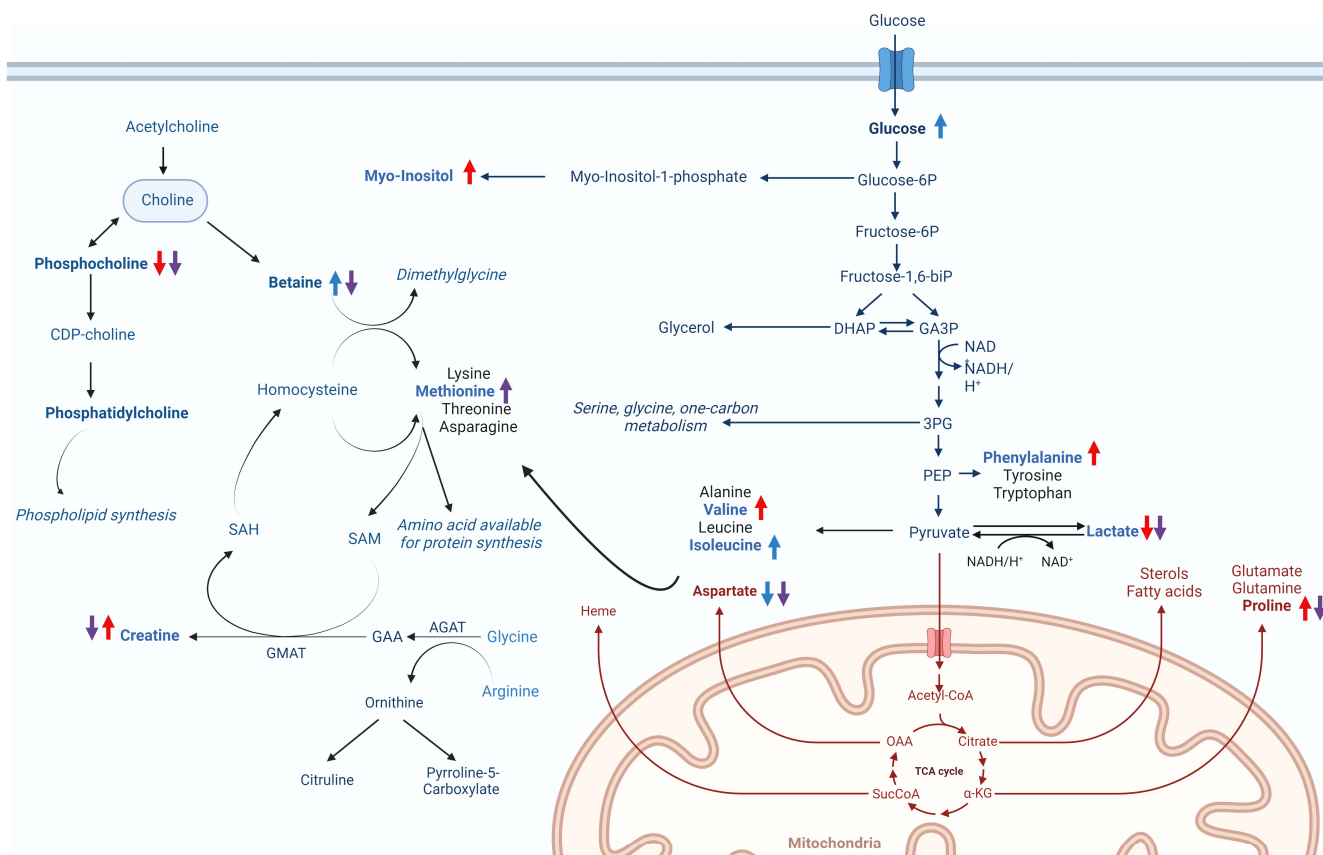


Figure 8. Hypothesised changes to metabolic pathways in Vero E6 cells caused by CHIKV infection and/or WDL treatment. The coloured arrows indicate increased (upwards) or decreased (downwards) metabolite levels for the different conditions analysed: CV (red), CW (blue) and CWV (purple).

The relevance of understanding the metabolic changes induced by a CHIKV nsP2 protease inhibitor may favour future studies on drug development. The inhibitor is introduced into an intricate environment and undergoes metabolism by cells. In this condition (CW), alterations in central energy metabolism were observed upon WDL addition. The reduction in aspartate, a critical building block for several biomolecules and a glucogenic amino acid within the TCA cycle [63], may reflect the promotion of gluconeogenesis upon the addition of WDL, a phenomenon further supported by the elevated glucose levels (Figures 6 and 8).

Metabolic changes upon adding WDL (CW) also indicates higher levels of betaine (Figures 6 and 8). Betaine, derived from choline oxidation, is an essential osmoregulatory metabolite and a cofactor of methylation during the methionine–homo-cysteine cycle [64,65]. Interestingly, betaine acts as an antiviral and anti-inflammatory compound that may exert functions through epigenetic regulation and mitigation of oxidative and ER (endoplasmic reticulum) stress and cell apoptosis [66]. A notable decrease in betaine levels followed by elevated levels of the sulphur-containing amino acid methionine were observed in our CC × CWV analysis (Figure 6c). As previously mentioned, betaine serves as a methyl donor for methionine synthesis through the activity of the betaine homocysteine S-methyltransferase enzyme [67]. The subsequent steps in the methionine cycle generate a pivotal methylating agent, SAM (S-adenosylmethionine), a cofactor essential for various cellular processes [68] (Figure 8). Both methionine and SAM can be helpful in oxidative stress conditions, as induced by CHIKV infection [69], and the betaine–methionine interaction

exerts an antioxidant function by maintaining cellular SAM:SAH ratios through increased methionine and SAM synthesis [66,70]. Furthermore, methionine and SAM synthesis are inducers of anabolic pathways such as amino acid and nucleotide biosynthesis to support cells under nutritional stress conditions (i.e., viral infection), which might also be correlated to the presence of WDL in the cellular environment during infection [71].

WDL consistently appears in the literature as possessing antioxidant effects, demonstrated by its ability to diminish levels of reactive oxygen species (ROS) and mitigate oxidative stress damage [21], promoting free radical scavenging [17] and alleviating oxidative stress and mitochondrial dysfunction [72]. Due to the limitations of our study, further research is necessary to elucidate these effects and mechanisms and investigate whether WDL might play a role against oxidative stress and cell damage caused by CHIKV infection.

Lastly, overall metabolic alterations in the CV × CWV analysis represented similar changes induced by the molecule in the CC × CW conditions, possibly enhancing energy production even amidst viral infection. Nevertheless, we noted decreased levels of myo-inositol and proline, aligning with diminished viral production. This observation contrasts with the observed increase in these metabolites to support viral infection, as previously discussed in the CC × CV results. Interestingly, exclusively in this analysis, creatine was found to be significantly decreased in CWV samples (Figure 6d). Creatine synthesis requires glycine, arginine and methionine [73], and creatine and phosphocreatine are known to regulate mitochondrial ATP synthesis during high-energy-demanding periods to help maintain ATP efficiency in cells [74–76]. Furthermore, it was elucidated that creatine kinase B (CKB), a pivotal ATP-generating enzyme responsible for modulating ATP levels within subcellular compartments, plays a crucial role in facilitating the efficient replication of the HCV genome and the subsequent propagation of the infectious virus [75]. Specifically, the recruitment of CKB to the HCV replication complex (RC) compartment, characterised by high and fluctuating energy demands, is paramount for the optimised replication of the viral genome. This recruitment is facilitated through its interaction with NS4A, underscoring the significance of this molecular interplay in the intricate process of viral replication [75]. Based on these findings, Parte superior do formulário we speculate that the decreased levels observed in CWV when compared to the CV samples may be linked to diminished energetic demands, a consequence of the lower yields in viral progeny.

4. Materials and Methods

4.1. Cloning, Expression and Purification of CHIKV nsP2^{pro}

The codon-optimised cDNA encoding CHIKV nsP2^{pro} (GenBank Protein Accession number AAN05101.1, strain S27-African prototype) was cloned, expressed and purified as described previously [38,77].

4.2. Viral Protease Inhibition Assay

The CHIKV nsP2 protease activity assay was performed as described previously [38,77], using DABCYL-Arg-Ala-Gly-Gly-Tyr-Ile-Phe-Ser-EDANS (BACHEM, Bubendorf, Switzerland) as substrate. The inhibition assay was performed in Corning 96-well plates (Merck, Darmstadt, Germany). An amount of 10 µM CHIKV nsP2^{pro} was incubated with 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25 and 50 µM WDL for 10 min at RT—the measurement started by adding the substrate with a final concentration of 5 µM. The fluorescence intensities were measured at 60 s intervals over 20 min at 37 °C using an Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). The excitation and emission wavelengths were 340 nm and 490 nm, respectively.

Activity and inhibition of the investigated protease was calculated using Equation (1):

$$\% \text{ protease activity: } (\text{Intensity of enzyme activity} - \text{intensity left after inhibition}) / \text{Intensity of enzyme activity} \quad (1)$$

The experiments were performed in triplicate and the results are shown as mean ± standard deviation (SD). Each experiment was performed with freshly purified

protein. The half-maximal inhibitor concentration (IC_{50}) value of WDL was calculated using GraphPad Prism 5 software (San Diego, CA, USA).

4.3. Cell, Virus and Reagents

Vero E6 cells (African green monkey kidney-derived cells—*Cercopithecus aethiops*, clone E6, ATCC C1008) were grown and maintained at 37 °C and 5% CO₂ in a humidified incubator in Eagle's Minimum Essential Medium (MEM—Gibco, Waltham, MA, USA) supplemented with 10% (*v/v*) foetal bovine serum (FBS—Gibco, Waltham, MA, USA) and the addition of 100 U/mL of penicillin (Hyclone Laboratories, Logan, UT, USA), 0.1 mg/mL of streptomycin and 0.5 µg/mL of amphotericin B (Gibco, Waltham, MA, USA). CHIKV (BHI3762/H804917 access number [78]) stocks were propagated and titrated in Vero cells using plaque-forming units assay (PFU). Wedelolactone ("WDL", 7-methoxy 5,11,12-trihydroxy-coumestan—Sigma Aldrich, MA, USA, purity ≥ 98%) was dissolved in DMSO (dimethyl sulfoxide, Merck, NJ, USA, 10 mg/mL) and used for the cell viability and antiviral assays as well as in HR-MAS ¹H NMR sample preparation.

4.4. Cytotoxicity Assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Aldrich, MA, USA) assay according to Mosmann [79] with slight alterations. Vero E6 cells at a 10⁴ density were grown in 96-well plates and treated with different concentrations of WDL ranging from 50 to 400 µM for 48 h at 37 °C with 5% CO₂, and DMSO was used as the vehicle control. After this period, molecule-containing media were removed, and MTT solution at 1 mg/mL was added to each well, incubated for 1 h and later replaced with 100 µL of DMSO for formazan crystals solubilisation. Absorbance was measured at 550 nm by a Spectramax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The CC₅₀ (cytotoxic effects on 50% of cultured cells) was defined as the concentration that reduced the viability of cells to 50% when compared to controls. The MTT assay was performed in triplicate, and the results were calculated in GraphPad Prism 8 software (San Diego, CA, USA) using a normalised dose–response curve fitting.

4.5. Virus Infection and Titration

A CHIKV stock was used to infect Vero E6 cells in a T-75 culture flask (ThermoFisher, Waltham, MA, USA) at 80% confluency. To determine the viral titre, we seeded 10⁵ Vero E6 cells in each well of a 24-well plate 24 h prior to infection. Cells were then infected with 10-fold serial dilutions of CHIKV and incubated at 37 °C for 1 h with gentle agitation every 15 min. The virus inoculum was removed, and overlay media containing 4% *v/v* carboxymethylcellulose (CMC—Synth, Sao Paulo, Brazil) and 1% FBS were added to the wells, followed by incubation for two days at 37 °C and 5% CO₂ in a humidified incubator. Finally, cells were fixated with 10% formaldehyde and stained with 4% violet crystal, and viral plaques were counted to determine the virus titre, expressed as plaque-forming units, PFU/mL.

4.6. Antiviral In Vitro Activity Assay

A postinfection treatment assay was performed to evaluate the in vitro antiviral activity of WDL. A 48-well tissue culture plate with 6 × 10⁴ Vero E6 cells/well was prepared and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. After that, media were removed, and cells were infected with CHIKV at a MOI (multiplicity of infection) of 0.1 and incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO₂. The inoculum was removed, five different diluted concentrations of WDL in MEM 1% FBS were added (30, 20, 15, 10 and 5 µM) to the wells and cells were incubated for a 24 h period at 37 °C in a humidified atmosphere with 5% CO₂. Supernatants were collected, and viral titration was performed and revealed through the plaque formation assay to evaluate plaque reduction and antiviral activity. The assay was performed in triplicate, and data were analysed by a

four-parameter dose–response curve fitting using GraphPad Prism 8 software (San Diego, CA, USA).

4.7. HR-MAS ^1H -NMR Sample Preparation

An amount of 6×10^6 Vero E6 cells were seeded in 100 mm culture dishes 24 h prior to infection and treatment. Six independent samples of each of the following treatment conditions were produced: healthy cell control (CC), cell-WDL (CW), cell-CHIKV (CV) and cell-WDL-CHIKV (CWV).

First, cells were infected with CHIKV at 0.1 MOI (as we identified high cell death rates with higher MOIs) and incubated for 1 h at 37 °C and 5% CO_2 with gentle agitation every 15 min. The virus inoculum was removed, and MEM 1% FBS was added to the CC and CV conditions. On the other hand, for the CW and CWV conditions, MEM supplemented with 1% FBS and the established diluted concentration of WDL (30 μM) was added. Cells were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 . Culture media were removed, and cells were washed twice with cold phosphate-buffered saline (PBS) and harvested by trypsinisation, and a subsequent addition was made of cold MEM supplemented with 10% FBS for trypsin neutralisation and collection of cells. Centrifugation was performed, and the supernatant was removed and discarded. The cell pellets were then washed with cold PBS, followed by another centrifugation step and complete removal of the supernatant for subsequent HR-MAS ^1H -NMR analysis.

4.8. HR-MAS ^1H -NMR and 2D Analysis

All HR-MAS ^1H NMR spectra were acquired on a Bruker Avance spectrometer (Bruker Biospin, Germany) operating at 400 MHz for ^1H , equipped with a triple nuclei 4 mm g-HR-MAS 400 SB BL4 probe and performing at a magic-angle spinning frequency of 3.5 kHz and 298 K. Cell pellets were resuspended in 12 μL D_2O (deuterated water) for the experiments. One-dimensional water-suppressed ^1H -NMR spectra were acquired with the noesygppr1d (NOESY 1D) pulse sequence. T_2 -edited spectra were obtained using the cpmgpr1d (Carr–Purcell–Melboom–Gill, CPMG) pulse sequence, both with 256 transients and a recycling delay of 4 s. Two-dimensional ^1H - ^1H total correlation spectroscopy (TOCSY) experiments were recorded using the mlevgpphw5 pulse sequence, $n_s = 110$, mixing time = 60 ms, and the ^1H - ^{13}C heteronuclear single quantum coherence (HSQC, hsqcetgpprsisp2.2 pulse sequence, $n_s = 384$) experiments were also performed on randomly selected samples.

4.9. Data Preprocessing, Statistical Analysis and Metabolite Identification

The HR-MAS ^1H -NMR cell spectra obtained were manually processed for phase and baseline corrections using MestReNova 12.0 software. Lactate (3H, δ 1.33, $J = 7.0$ Hz, doublet) was used as a chemical shift reference. The spectra were binned (0.005 ppm) and exported as csv matrices. Spectra regions below δ 0.5 and water region (δ 4.55–5.10) and above δ 8.7 were excluded from the analysis. Normalisation to a constant sum (100) of the spectra's intensities was performed to reduce concentration differences. Assignment of the metabolites was based on chemical shifts, correlation assignments (TOCSY and HSQC) and databases, such as the Human Metabolome Database [80] and Biological Magnetic Resonance Data Bank [81].

All statistical analyses were performed using the MetaboAnalyst online platform [82]. Multivariate analyses were performed using the matrices with a total of 810 variables for the parts of the aliphatic regions of water suppression (noesy1d) and T_2 -edited (CPMG) spectra and constructed for all conditions ($\text{CC} \times \text{CW} \times \text{CWV} \times \text{CV}$, with 24 spectra in total), as well as matrices containing each condition compared to the healthy control cells (CC), namely, $\text{CC} \times \text{CW}$, $\text{CC} \times \text{CWV}$, $\text{CC} \times \text{CV}$, and also compared to the viral control ($\text{CV} \times \text{CWV}$). Data were normalised by median and autoscaling.

Principal component analyses (PCA) were performed for all spectra (all conditions, separately for data obtained by the noesygppr1d and cpmgpr1d pulse sequences) to explore intrinsic groupings within samples, reduce data dimensionality and identify outliers [83].

Partial least squares discriminant analysis (PLS-DA) models were generated to classify samples from the four conditions evaluated together and compared to the healthy control (CC) and identify the essential variables [83,84]. Model validation was assessed by leave-one-out cross validation (LOOCV) to evaluate its accuracy and performance, and variable of importance in projection (VIP) scores were analysed to identify the most different chemical shifts among samples. The validated models' accuracy, specificity and sensitivity values were computed [85]. Univariate analysis was performed to confirm the discriminant metabolites identified by multivariate analysis (PLS-DA) between two conditions (CC $\times$ CV; CC $\times$ CW; CC $\times$ CWV; CV $\times$ CWV) and for visualisation of the relative metabolite levels between the groups, measured as peak intensities, using a *t*-test with $p < 0.05$ as the threshold for statistically significant difference.

5. Conclusions

With recurrent epidemics worldwide and the risk of developing long-term complications in human hosts, the effects of arboviral infections by Chikungunya and other viruses are concerning. To date, no licensed antiviral drugs or vaccines are available. Although studies have provided numerous promising results in drug and vaccine development, research contributing to understanding viral mechanisms is crucial.

In this study, we aimed to explore the potential of HR-MAS ^1H NMR to understand the metabolic effects of viral infection and viral inhibition by WDL with in vitro and metabolomic assays. Our results demonstrate that WDL inhibits viral nsP2 protease with considerably low IC_{50} values. At the same time, in cell culture, a much more complex environment, WDL slightly impairs the post-viral-entry steps in Vero E6 cells in a dose-dependent manner, where, perhaps, higher dosages of the molecule might have been more efficient in inhibiting viral replication in vitro, which were limitations in our study.

To date and to our knowledge, there are no HR-MAS NMR metabolomic studies regarding arboviruses of the *Togaviridae* family. HR-MAS NMR and chemometric analysis tools allowed us to trace a metabolic profile of Vero cells, along with metabolite disturbances triggered by Chikungunya virus infection, which mainly affected central energy, amino acid and phospholipid metabolism. We could also observe that WDL contributes to glucose synthesis in Vero cells. During infection, it might support anabolic pathways, lowering oxidative stress and cell damage, although it is speculative and provides further insights into future studies regarding these allegations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25031414/s1>.

Author Contributions: M.A.C., R.J.E., R.K.A. and R.d.S.P. conceived the concepts and designed experiments; R.d.S.P. and L.G.M. performed the experiments; R.d.S.P. and K.C.B. performed the NMR-based metabolomics analysis with aid from L.G.M. and L.T.; R.d.S.P. and M.V.S. performed the in-cell experiments with aid from C.C.P. and M.L.N.; R.J.E. and M.A.C. performed the enzymatic activity test; M.A.C., R.d.S.P. and L.G.M. wrote the original draft; and R.d.S.P., L.G.M., C.C.P., M.V.S., K.C.B., M.L.N., R.J.E., L.T., R.K.A. and M.A.C. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: R.d.S.P., M.V.S., C.C.P., M.L.N. and L.T. acknowledge the financial support from FAPESP (Project Nr. 2020/03639-6; 2020/12875-5; 2023/09590-7; 2017/13341-1; 2018/15083-2; 2013/21719-3; 2018/24069-3; 2018/06510-4). R.K.A. is grateful to FAPESP (Project Nr. 2020/08615-8) and CNPq (Project Nr. 309940/2019-2). M.L.N. thanks the partial support from the Centers for Research in Emerging Infectious Disease "The Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics (CREATE-NEO)" grant U01AI151807 awarded by the National Institutes of Health (NIH/USA) and INCT Viral Genomic Surveillance and One Health (grant 405786/2022-0).

Data Availability Statement: The data presented in this study are available in the article and its supplementary material.

Acknowledgments: M.A.C. and R.J.E. would like to thank Dieter Willbold and the Institute of Biological Information Processing (IBI-7) Forschungszentrum Jülich, Germany, for their support and also Andrew Dingley for the revisions and suggestions.

Conflicts of Interest: The authors declare no conflicts of interest.

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