



**HAL**  
open science

# Infection of the human testis by emergent viruses: the case of Zika virus and SARS-CoV-2

Salomé Bourgeau

► **To cite this version:**

Salomé Bourgeau. Infection of the human testis by emergent viruses: the case of Zika virus and SARS-CoV-2. Human health and pathology. Université de Rennes; Académie chinoise des sciences (Pékin, Chine), 2022. English. NNT : 2022REN1B030 . tel-04132042

**HAL Id: tel-04132042**

**<https://theses.hal.science/tel-04132042v1>**

Submitted on 18 Jun 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES 1

ECOLE DOCTORALE N° 605

*Biologie Santé*

Spécialité : « *Microbiologie, Virologie, Parasitologie* »

en cotutelle avec la CHINESE ACADEMY OF SCIENCES

Par

**Salomé BOURGEOU**

« **Infection of the human testis by emerging viruses:  
the case of Zika virus and SARS-CoV-2** »

Thèse présentée et soutenue à Rennes, le 17 Juin 2022

Unités de recherche :

IRSET - Inserm U1085 & Institut Pasteur de Shanghai

Equipes de recherche :

« Physiologie et physiopathologie du tractus urogénital »

& « Arbovirus interspecies transmission and therapeutics »

## Rapporteurs avant soutenance :

Béatrice GRASLAND  
Pierre ROQUES

Directrice de recherches, ANSES Ploufragan  
Directeur de recherches, Institut Pasteur de Guinée

## Composition du Jury :

Président du jury  
Vincent THIBAUT

Professeur des Universités - Praticien Hospitalier, CHU Rennes

Rapporteurs  
Béatrice GRASLAND  
Pierre ROQUES

Directrice de recherche, ANSES Ploufragan  
Directeur de recherche, Institut Pasteur de Guinée

Examineurs  
Xiaozhen LIANG  
Jin ZHONG

Professeur d'Université, Institut Pasteur de Shanghai  
Professeur d'Université, Institut Pasteur de Shanghai

Directrice de thèse  
Nathalie DEJUCQ-RAINSFORD

Directrice de recherche, Université de Rennes 1

Co-directeur de thèse  
Dimitri LAVILLETTE

Directeur de recherche, Institut Pasteur de Shanghai

« Il y aura donc des maladies nouvelles. C'est un fait fatal.

Un autre fait, aussi fatal, est que nous ne saurons jamais les dépister dès leur origine. Lorsque nous aurons notion de ces maladies, elles seront déjà toutes formées, adultes pourrait-on dire. Elles apparaîtront comme Athéna parut, sortant toute armée du cerveau de Zeus. Comment les reconnaitrons-nous, ces maladies nouvelles, comment soupçonnerions-nous leur existence avant qu'elles n'aient revêtu leurs costumes de symptômes ?

Il faut bien se résigner à l'ignorance des premiers cas évidents. Ils seront méconnus, confondus avec des maladies déjà existantes et ce n'est qu'après une longue période de tâtonnements que l'on dégagera le nouveau type pathologique du tableau des affections déjà classées. »

- *Charles Nicolle*, 1933

A Mamie,

Et à tous ceux à qui nous n'avons pu dire au revoir au cours de cette pandémie.

**« Start where you are. Use what you have. Do what you can. »**

- *Arthur Ashe*  
*via Tamara Milosevic*

# Table of contents

Table of contents.....	3
Abbreviations .....	7
Virus abbreviations.....	9
Résumé en Français.....	10
Introduction.....	25
I. Emerging Infections.....	25
A. Generalities .....	25
B. Emergence processes .....	26
C. Transmission routes .....	27
D. Factors of viral emergence.....	28
II. The male genital tract .....	31
A. The Testis.....	31
A.1. Morphology and function.....	31
A.2. The interstitial tissue .....	33
A.2.a. The Leydig cells .....	33
A.2.b. The Macrophages.....	34
A.2.c. Other interstitial cell populations .....	36
A.3. The seminiferous tubules .....	37
A.3.a. The Peritubular Myoid Cells.....	37
A.3.b. The Germ cells.....	37
A.3.c. The Sertoli cells.....	41
A.4. The blood-testis barrier .....	42
A.4.a. The physical barrier.....	42
A.4.b. The molecular barrier .....	44

B. The semen .....	44
B.1. Cell composition, function and origin.....	45
B.2. Seminal plasma origin, composition and function .....	47
C. Viral infections of the MGT and their consequences .....	48
C.1. Public Health Importance .....	48
C.2. Overview of the viruses detected in the MGT.....	49
C.3. Viral dissemination into the MGT .....	50
C.4. Consequences of MGT infection.....	50
C.4.a. Alteration of the hormonal and sperm parameters .....	51
C.4.b. Cancers.....	52
C.4.c. Vertical transmission and endogenization .....	52
C.4.d. Viral persistence in the MGT and sexual transmission .....	53
III. Zika Virus .....	57
A. Taxonomy and emergence .....	57
B. Molecular biology and evolution.....	59
B.1. Structural and genomic organization .....	59
B.2. Replication cycle .....	62
B.3. Cellular receptors involved in viral entry.....	64
C. Transmission modes .....	66
C.1. Vector-borne transmission .....	66
C.2. Vertical transmission .....	66
C.3. Sexual transmission .....	67
C.4. Auxiliary transmission .....	68
D. Physiopathology of the infection .....	69
D.1. Generalities.....	69
D.2. Complications in adults and newborns .....	69

D.3. Zika virus in the Male Genital Tract.....	70
IV. SARS-CoV-2 Virus .....	72
A. Taxonomy and emergence .....	72
B. Molecular biology and evolution.....	73
B.1. Structural and genomic organization .....	73
B.2. Replication cycle .....	79
B.3. Cellular receptors involved in viral entry.....	82
C. Transmission modes .....	84
D. Physiopathology of the infection .....	85
D.1. Generalities.....	85
D.2. Complications .....	86
D.3. The male predisposition and role of the renin-angiotensin system .....	88
D.4. SARS-CoV-2 in the Male Genital Tract.....	89
Objectives.....	92
Results .....	94
I. Viral persistence of ZIKV in the testis.....	94
II. Viral replication of SARS-CoV-2 in the testis .....	97
III. Viral entry factors of emerging RNA viruses in the testis .....	132
General Discussion and Perspectives.....	159
I. Experimental approaches.....	159
A. Cell lines and isolated primary cells .....	159
B. Organotypic cultures .....	162
C. Animal models .....	163
D. Clinical studies.....	165
E. Contribution of the conventional approaches .....	166
F. Limits of the conventional approaches .....	167

II. In Silico approaches.....	167
A. Unveiling viral entry factors expression in the testis through scRNA-seq analysis .....	167
B. Gaps and flaws of the scRNA-seq approach.....	168
C. Complementary experiments to infer tropism .....	170
C.1. Confirmation of the entry factor protein expression .....	170
C.2. Confirmation of the entry factor use.....	170
C.3. Investigation of the replication and restriction factors.....	172
Conclusion .....	174
Annexes .....	175
References.....	190
Acknowledgments.....	221



# Abbreviations

AA: Amino Acid  
Ab: Antibodies  
ACE2: Angiotensin-Converting Enzyme 2  
ADE: Antibody-Dependent Enhancement  
ALT: Alanine Transaminase  
AMO: Anucleate Mass with Organelles  
Ang: Angiotensin  
AR: Androgen Receptor  
ARDS: Acute Respiratory Distress Symptoms  
AST: Aspartate transaminase  
AT2R: Angiotensin II receptor type 2  
BHK-21: Baby Hamster Kidney fibroblasts  
BSG: Basigin  
BTB: Blood-Testis-Barrier  
C: Capsid protein  
cAMP: cyclic Adenosine Monophosphate  
CCL2/5: C-C Motif Chemokine Ligand 2/5  
CD: Cluster of Differentiation  
CDC: Centers for Disease Control and Prevention  
COVID-19: Coronavirus Induced Disease  
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats  
CS: Cytokine Storm  
CSF1: Colony Stimulating Factor 1  
CTD: C-terminal domain  
CTL: Cytotoxic T Lymphocyte  
CTSB/L: Cathepsin B/L  
CXCL10: C-X-C Motif Chemokine Ligand 10  
DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin  
DHT: Dihydrotestosterone  
DNA: Deoxyribonucleic Acid  
dpi: days post infection  
dps: days post symptom  
dsRNA: double-stranded RNA  
E: Envelop protein  
ECDC: European Centre for Disease Prevention and Control  
EGFR: Epidermal Growth Factor Receptor  
ELISA: Enzyme-Linked Immunosorbent Assay  
ER: endoplasmic reticulum  
ERGIC: ER-Golgi Intermediate Compartment

ERV: Endogenous Retroviral Elements  
ES: Ectoplasmic Specialization  
FasL: Fas Ligand  
FcγR: Fragment crystallizable region of Ab-receptor  
FCS: Furin Cleavage Site  
FSH: Follicle Stimulating Hormone  
Gas6: Growth arrest-specific Gene 6  
GBS: Guillain-Barré Syndrome  
GDNF: Glial cell line-Derived Neurotropic Factor  
GnRH: Gonadotropin-Releasing Hormone  
gRNA: genomic RNA  
HAVCR1: Hepatitis A Virus Cellular Receptor 1  
HEK293T: Human Embryonic Kidney 293 Cells  
HPG: Hypothalamic-Pituitary-Gonadal  
HSD-17β: 17 β-steroid dehydrogenases  
HSD-3β: 3β-steroid dehydrogenase  
HSP: Heat Shock Protein  
HSPG: Heparan Sulfate Proteoglycans  
ICTV: International Committee on Taxonomy of Viruses  
ICU: Intensive Care Unit  
IDO: indoleamine 2,3-dioxygenase  
IE: Immune Escaping  
IFIT: Interferon Induced Protein with Tetratricopeptide Repeats  
IFN: Interferon  
Ig: Immunoglobulin  
IL: Interleukin  
INSL3: insulin like factor 3  
iPS: Induced Pluripotent Stem Cells  
ISG: Interferon Stimulated Genes  
ITG: Integrin  
iTM: Interstitial Testicular Macrophages  
JAK: Janus Kinase  
JAM-A/B: Junctional adhesion molecule-A/B  
kb: kilobases  
KO: knockout  
LH: Luteinizing Hormone  
LIF: Leukemia Inhibitory Factor  
LINE-1: Long Interspersed Element-1  
L-SIGN: Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin  
M: Membrane protein  
MAGI: Male Accessory Gland Infections  
MasR: Mas receptor

MDA5: Melanoma Differentiation-Associated Protein 5

MGT: Male Genital Tract

MHC: Major Histocompatibility Complex

MRC1: Multidrug Resistance Protein 1

mRNA: messenger RNA

msgRNA: messaging sgRNA

MX1: MX Dynamin Like GTPase 1

NAb: Neutralizing antibody

NC: Nucleocapsid

NCAM1: Neural Cell Adhesion Molecule

NCR: Non-Coding Region

NHP: Non-Human Primates

NK: Natural Killer

NRP1: Neuropilin 1

NSC: Non-Sperm Cells

NSP: Non-Structural Proteins

NTR: Non-Translated Region

OAS1/2: 2'-5'-Oligoadenylate Synthetase 1/2

ORF: Open Reading Frame

PAHO: Pan American Health Organization

PCR: Polymerase Chain Reaction

PCSK1: Proteases including PC1

PEDF: Pigment Epithelium Derived Factor

PET/CT: Positron emission tomography / Computed Tomography

P-gp: P-glycoprotein,

PKA: Protein Kinase A

PMC: Peritubular Myoid Cells

prM: precursor of Membrane protein

PRSS1/2/3: Serine Protease 1/2/3

PS: Phosphatidyl Serines

pTM: Peritubular Testicular Macrophages

R&D: Research and Development

RA: Retinoic Acid

RAS: Renin-Angiotensin System

RBD: Receptor Binding Domain

RBM: Receptor Binding Motif

RdRP: RNA-dependent RNA polymerase

RLR: RIG-I Like Receptors

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rRNA: replicative RNA

RSAD2: Radical S-Adenosyl Methionine Domain Containing 2

RTC: Replication and Transcription Complex

RT-qPCR: Real-Time quantitative PCR

SARS: Severe Acute Respiratory Syndrome

S: Spike protein

SC: Sertoli Cells

SCB: Sertoli Cell Barrier

SCF: Stem Cell Factor

SE: Seminiferous Epithelium

SERPINF1: Serpin Family F Member 1

SFTS: Severe Fever with Thrombocytopenia Syndrome

SPC: Spermatocytes

SPG: Spermatogonia

SPT: Spermatids

SRC: SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase

SSC: Spermatogenic Stem Cells

ssRNA: single-strand RNA

ST: Seminiferous Tubules

ST14: ST14 Transmembrane Serine Protease Matriptase

STAR: Steroidogenic Acute Regulatory

STAT: Signal Transducer and Activator of Transcription

STI: Sexually Transmitted Infections

STRA8: Stimulated by Retinoic Acid 8

T: Testosterone

TGC: Testicular Germ Cells

TGF: Transforming Growth Factor

TGN: Trans Golgi Network

TAM: TYRO3, AXL, MERTK

TIM-1: T-cell Immunoglobulin Mucin Domain

TJ: Tight-junctions

TLR: Toll Like Receptor

TMD: Transmembrane Domain

TMPRSS: Transmembrane Serine Protease

TNF: Tumor Necrosis Factor

Treg: Regulatory T Cells

TSPO: Translocator Proteins

USA: United States of America

VLE: Vascular and Lymphatic Endothelium

VOC: Variants of Concern

vRNA: viral RNA

WHO: World Health Organization

ZO: Zonula occludens

# Virus abbreviations

AAV: Adeno-associated viruses  
ANDV: Andes orthohantavirus  
BKPyV: BK polyomavirus  
CCHFV: Crimean-Congo hemorrhagic fever virus  
CHIKV: Chikungunya virus  
CMV: Cytomegalovirus  
CV-A: Coxsackievirus A  
CV-B: Coxsackievirus B  
CV-C: Coxsackievirus C  
DENV: Dengue virus  
EBOV: Ebola virus  
EBV: Epstein-Barr virus  
EEEV: Eastern Equine Encephalitis Virus  
EV-A71: Enterovirus A71  
EV-D68: Enterovirus D68  
EV-D70: Enterovirus D70  
HBV: Hepatitis B virus  
HCoV-229E: Human coronavirus 229 E  
HCV: Hepatitis C virus  
HDV: Hepatitis D virus  
HeV: Hendra virus  
HGV: Hepatitis G virus  
HHV-6: Human herpesvirus 6  
HHV-7: Human herpesvirus 7  
HIV: Human Immunodeficiency Virus  
HKU1: Human coronavirus HKU1  
HPV: Human papillomavirus  
HRV-A: Human rhinovirus A  
HRV-B: Human rhinovirus B  
HRV-C: Human rhinovirus C  
HSV: Herpes simplex virus  
HTLV: Human T-lymphotropic virus  
HTNV: Hantaan virus  
JCPyV: JC polyomavirus  
JEV: Japanese encephalitis virus  
JUNV: Junín mammarenavirus  
KSHV: Kaposi's sarcoma-associated herpesvirus  
LASV: Lassa mammarenavirus  
LCMV: Lymphocytic choriomeningitis  
mammarenavirus  
LFV: Lassa fever virus  
LUJV: Lujo virus

MARV: Marburg viruses  
MAYV: Mayaro virus  
MCPyV: Merkel cell polyomavirus  
MCV: Molluscum Contagiosum Virus  
MERS-CoV: Middle East Respiratory Syndrome  
coronavirus  
MuV: Mumps virus  
NiV: Nipah virus  
NL63: Human coronavirus NL63  
OC43: Human coronavirus OC43  
ONNV: O'nyong nyong virus  
PoV: Poliovirus  
PTV: Punta Toro virus  
PUUV: Puumala orthohantavirus  
RRV: Ross River virus  
RUV: Rubella virus  
RVFV: Ross Valley Fever Virus  
SARS: Severe Acute Respiratory Syndrome  
SARS-CoV-1/2: Severe Acute Respiratory Syndrome-  
related Coronavirus 1/2  
SFV: Semliki Forest virus  
SINV: Sindbis virus  
SIV: Simian Immunodeficiency Virus  
SNV: Sin Nombre orthohantavirus  
SV40: Simian virus 40  
TOSV: Toscana virus  
VZV: Varicella-zoster virus  
VEEV: Venezuelan equine encephalitis virus  
WNV: West Nile virus  
YFV: Yellow Fever virus  
ZIKV: Zika virus

# Résumé en Français

## INTRODUCTION

### I- Infections émergentes

Les infections émergentes sont définies comme "des infections qui sont nouvellement apparues dans une population ou qui existaient déjà mais dont l'incidence ou la portée géographique augmente rapidement", et "qui posent des problèmes de santé publique, au niveau local ou international". Depuis les années 1970, plus de 1400 nouveaux agents pathogènes ont été identifiés, dont 13% sont considérés comme émergents ou réémergent. Parmi les agents pathogènes émergents depuis les années 1980, environ 75 % proviennent d'animaux, et la majorité d'entre eux sont des virus.

Le processus d'émergence se déroule en trois grandes étapes :

L'introduction d'un agent pathogène dans une nouvelle espèce hôte, qui dépend de la capacité de l'agent à se lier à différents récepteurs cellulaires, déterminant la gamme d'hôtes.

L'établissement de l'agent pathogène dans l'hôte, qui dépend de sa capacité à compléter son cycle de reproduction et à échapper aux réponses immunitaires pour une réplication efficace.

La dissémination de l'agent pathogène au sein de la population hôte. Elle dépend de sa capacité à se répliquer à une charge virale efficace dans les tissus clés (déterminant ainsi la voie de transmission) afin de fonder une population réservoir dans laquelle l'agent pathogène circule en permanence.

Les voies de transmission jouent un rôle clé dans l'efficacité de la dissémination et peuvent se produire par différents modes, classés selon la dichotomie verticale versus horizontale : transmission du parent à la progéniture versus transmission sexuelle ou non sexuelle.

Pour qu'un micro-organisme apparaisse dans la population humaine, plusieurs facteurs sont impliqués à chaque étape de l'émergence et impliquent une évolution rapide du pathogène pour s'adapter à la pression sélective d'un nouvel hôte ou environnement. À cet égard, les virus à ARN sont particulièrement préoccupants et représentent jusqu'à 44 % des maladies émergentes du XXI<sup>e</sup> siècle, en raison de leur taux d'évolution plus rapide que celui de tous les autres organismes vivants.

## II- Le tractus génital masculin

Le tractus génital masculin (TGM) est composé d'une série d'organes et de conduits qui assurent la production, le stockage et le transport des gamètes mâles. Les testicules endossent le double rôle de production de gamètes (la spermatogenèse) et d'hormones sexuelles, ces dernières étant responsables du maintien du phénotype corporel masculin et des fonctions de reproduction. Le testicule est morphologiquement divisé en deux compartiments :

Le tissu interstitiel contient des vaisseaux sanguins et lymphatiques ainsi que divers types de cellules. Parmi elles, les cellules de Leydig sont productrices de testostérone ; et les macrophages sont le type de cellules immunitaires le plus nombreux dans le testicule, ils jouent un rôle clé dans l'homéostasie testiculaire.

Les tubules séminifères sont entourés par les cellules myoïdes périvitubulaires. Elles englobent les cellules de Sertoli polarisées, qui soutiennent les cellules germinales, depuis les cellules souches spermatogoniales localisées à la membrane basale du tubule, jusqu'aux spermatozoïdes vers la lumière. Leur fonction sécrétoire assure un rôle nourricier pour les cellules germinales en développement, tandis que leurs jonctions serrées constituent la partie physique de la barrière hémato-testiculaire (BHT), assurant un rôle protecteur contre les agressions systémiques.

La production des gamètes mâles est contrôlée par un système hormonal endocrinien qui implique plusieurs organes composant l'axe hypothalamo-pituitaire-gonadique (HPG). La LH et la FSH sont les deux principales hormones sécrétées dans la circulation. La LH active les cellules de Leydig pour initier la synthèse de la testostérone tandis que la FSH potentialise la spermatogenèse en régulant l'expression génétique des cellules de Sertoli.

Le sperme est composé d'une fraction acellulaire, le plasma séminal, principalement produite par les glandes reproductrices accessoires (les vésicules séminales et la prostate principalement); et d'une fraction cellulaire, majoritairement (85%) composée de spermatozoïdes provenant du testicule. Parmi les 15 % restants, de cellules non spermatiques (CNS), la majorité sont des éléments germinaux immatures exfoliés du testicule. Des leucocytes (principalement des neutrophiles, monocytes et macrophages) constituent la deuxième population la plus représentée (13 %), suivie des cellules épithéliales (2,3 %) et des cellules de Sertoli exfoliées du testicule (0,7 %). À l'exception des cellules germinales et des cellules de Sertoli, l'origine des cellules non spermatiques, et en particulier des CNS infectées dans le sperme est largement inconnue et doit faire l'objet de recherches supplémentaires.

En 2021, plus d'un million d'infections sexuellement transmissibles (IST) ont été contractées chaque jour dans le monde, représentant 374 millions de nouveaux cas par an, la majorité étant asymptomatique. Notamment, plus de trente agents pathogènes différents peuvent être transmis sexuellement, parmi lesquels seize sont des virus, et quatre sont incurables (VIH, HSV, HBV, HPV). Les IST se transmettent par contacts sexuels (vaginaux, anaux et oraux) et ont un impact direct sur la santé sexuelle et reproductive (altération des paramètres hormonaux et spermatiques, inflammation) entraînant infertilités, cancers et complications de grossesse. Certaines sont également transmises verticalement et entraînent entre autres des fausses-couches et anomalies congénitales. Notamment, l'infertilité touche 8 à 12% des couples et l'infertilité masculine est responsable de 20 à 50% des cas. Les événements infectieux représentent 6 à 15% de toutes les causes et sont ainsi considérés contributeurs importants.

Les préoccupations concernant la transmission sexuelle des virus ont augmenté avec le début de la pandémie du VIH dans les années 1980. Les virus en question (HSV, CMV...) établissent une infection chronique chez leur hôte et leur persistance dans le TGM et le sperme n'était donc pas inattendue. Cependant, la transmission sexuelle des virus Zika et Ebola par des hommes guéris lors des récentes épidémies a été une surprise et a révélé que le TGM constitue également un réservoir de virus dits « aigus », en principe éliminés de la circulation périphérique. À l'heure actuelle, 32 virus ont été détectés dans le sperme humain, certains ayant été retrouvés plusieurs jours, mois voire années après la clairance systémique et/ou ont été impliqués dans des cas de transmission sexuelle. Notamment, la transmission sexuelle du virus Ebola par des patients jusqu'à 5 ans après guérison a été associée à la résurgence de foyers épidémiques, soulignant les conséquences dramatiques de la persistance virale dans le sperme, et le besoin crucial de l'anticiper. Les virus à ARN sont les plus répandus parmi les pathogènes émergents et il apparaît de plus en plus clairement, tant chez l'homme que chez l'animal, que plusieurs d'entre eux sont excrétés dans le sperme et peuvent infecter les cellules testiculaires. De plus, l'environnement immunoprivilégié du testicule suggère que cet organe pourrait être impliqué dans la persistance de plusieurs virus dans le TGM. Il est donc crucial de comprendre les mécanismes qui sous-tendent l'infection à long terme de cet organe et l'excrétion séminale prolongée qui pourrait en découler.

### III- Le virus Zika

Le virus Zika (ZIKV) appartient à la famille des Flaviviridae et au genre Flavivirus, comme le sont les virus YFV, DENV, WNV et JEV. ZIKV a été découvert en 1947 en Ouganda et fut responsable d'épidémies récurrentes à travers le Pacifique jusqu'à atteindre le Brésil en 2015, causant la dernière grande épidémie de ZIKV à travers le continent américain.

ZIKV présente un génome à ARN monocaténaire codant pour une poly-protéine qui sera divisée en protéines structurales (C, M, E), formant la particule virale, et en protéines non structurales responsables de la réplication, la maturation et la virulence du virus.

Bien que la plupart des infections soient asymptomatiques, des complications peuvent survenir chez les adultes, notamment sous la forme du syndrome de Guillain Barré ; et chez les nouveau-nés, notamment sous la forme de microcéphalies.

En tant qu'arbovirus, ZIKV est généralement transmis par les piqûres de moustiques ou par transfusion sanguine. Cependant, au cours des dernières épidémies, ZIKV fut également transmis pendant la grossesse (provoquant les microcéphalies) et lors de relations sexuelles, principalement des patients masculins vers leurs partenaires. Ceci fut notamment dû à la persistance du ZIKV dans le sperme des patients, à la fois dans le plasma séminal et dans la fraction cellulaire. La présence de ZIKV dans le sperme a été rapportée jusqu'à 69 jours après l'apparition des symptômes pour les particules infectieuses, et jusqu'à 414 jours pour l'ARN viral, dépassant ainsi la clairance dans le sang. La transmission sexuelle a notamment été rapportée jusqu'à 44 jours après l'apparition des symptômes, dans un total de 14 pays en dehors de la zone d'endémie du moustique vecteur.

Dans les testicules, notre équipe a montré que ZIKV se répliquait efficacement dans des explants humains en culture *ex vivo*, infectant un large éventail de cellules somatiques testiculaires tels que les macrophages résidents, les cellules péritubulaires et, dans une moindre mesure, les cellules de Leydig et les cellules de Sertoli. Notamment, ZIKV infecte de manière productive les cellules germinales immatures et matures, qui furent également retrouvées exfoliées *in vivo* dans le sperme d'hommes séropositifs au ZIKV jusqu'à 11 jours après apparitions des symptômes. Après infection *ex vivo*, la structure histologique, la viabilité cellulaire et la fonction hormonale du testicule sont restées inchangées, et seule une réponse pro-inflammatoire limitée fut induite, sans production d'interféron nécessaire à une défense antivirale efficace.

Ces observations suggèrent que le testicule pourrait être un « réservoir viral » de ZIKV et une source de persistance du virus dans le sperme. Cependant, des preuves suggèrent que ZIKV pourrait

persister dans plusieurs organes du tractus, qui pourraient tous être impliqués dans l'excrétion à long terme du virus. La caractérisation des cellules infectées dans le sperme permettrait donc de déterminer la ou les sources exactes de cette excrétion prolongée.

#### IV- Le SRAS-CoV-2

Le SRAS-CoV-2 est apparu pour la première fois en décembre 2019 à Wuhan, en Chine, et s'est rapidement propagé dans le monde entier, provoquant la pandémie de COVID-19. Il s'agit d'un bêta-coronavirus dont le génome à ARN simple brin code pour les protéines structurelles formant la particule virale, ainsi que 16 protéines non structurelles et 11 protéines accessoires impliquées dans la réplication virale et la virulence. La protéine S, pour Spike, est particulièrement importante pour l'entrée virale car c'est elle qui se lie au récepteur cellulaire (ACE2 notamment) et qui sera clivée par une protéase cellulaire pour révéler son peptide de fusion permettant la jonction des membranes cellulaire et virale lors de l'infection.

En tant que virus respiratoire, le SRAS-CoV-2 infecte premièrement les voies aériennes supérieures, provoquant des symptômes grippaux comme la toux (permettant sa transmission par voie aérienne) et pouvant aller jusqu'à une pneumonie et une détresse respiratoire mortelle dans les cas les plus graves. Cependant, le SRAS-CoV-2 semble avoir un tropisme très large, infectant un grand nombre d'organes et provoquant des symptômes inhabituels pour un virus respiratoire, comme des diarrhées, des vomissements ou la perte du goût et de l'odorat. De nombreuses études ont notamment signalé la forte expression d'ACE2 dans les testicules par rapport aux autres organes, y compris les poumons.

À l'autopsie, les testicules d'hommes décédés ayant souffert d'un COVID sévère présentent souvent des œdèmes interstitiels, des cellules germinales et somatiques dégénérées et des infiltrats inflammatoires. Les patients sévères vivants présentent notamment une altération des paramètres du sperme (diminution du nombre et de la motilité des spermatozoïdes jusqu'à 3 mois) et des paramètres hormonaux (diminution de la testostérone) en corrélation avec le niveau de fièvre et d'inflammation systémique, et avec la gravité de la maladie. Chez ces patients, de faibles niveaux de LH ont été observés, confirmant une défaillance systémique de l'axe HPG. Cependant, chez les patients légèrement à modérément infectés, la diminution de la testostérone semble être associée à des niveaux élevés de LH, suggérant plutôt une défaillance directe du testicule.

Sept études ont rapporté un total de 39/71 cas de détection de l'ARN ou de protéines virales dans les testicules humains lors d'autopsies mais les rapports d'excrétion dans le sperme sont



contradictoires. En effet, sur les centaines d'échantillons de sperme étudiés, moins de 20 se sont révélés positifs par RT-qPCR, principalement pendant la phase aiguë de l'infection.

Dans l'ensemble, seules des preuves éparses suggèrent que le SRAS-CoV-2 pourrait infecter directement le testicule et être excrété dans le sperme de patients légèrement infectés. Cependant, les méthodes d'échantillonnage et de détection sont controversées et des contaminations (par une excrétion urinaire résiduelle dans le sperme notamment) sont également suggérées. En outre, l'impact direct de l'infection du testicule (au-delà de l'inflammation systémique) sur les paramètres spermatiques et hormonaux des patients est incertain. L'étude de l'infection testiculaire humaine par le SRAS-CoV-2 permettrait donc de mieux comprendre les conséquences de l'infection sur la santé et les fonctions reproductrices des patients.

## OBJECTIFS

Les virus à ARN présentent un processus d'adaptation et d'évolution leur permettent d'étendre leur tropisme d'hôte et d'organes, et d'échapper à la reconnaissance et aux réponses immunitaires. Ces capacités peuvent conduire à l'utilisation de voies de transmission plus efficaces et à l'établissement d'une persistance dans les organes cibles. La compréhension et la prévision des multiples modes de transmission de ces virus émergents sont essentielles pour contrôler leur propagation.

La transmission sexuelle des virus par le sperme est une préoccupation majeure pour la santé publique, comme l'illustre la propagation hors des zones endémiques du ZIKV ou le déclenchement de la résurgence épidémique de l'EBOV. L'étude de cette voie de transmission constitue donc un défi important pour les épidémies futures. Notamment, les infections virales d'un organe immunoprivilégié tel que le testicule ont un grand potentiel pour constituer une source de virus persistants.

Mon projet de thèse a donc étudié la capacité du testicule humain à être une cible privilégiée pour l'infection et la persistance des virus à ARN émergents ; ainsi que les potentielles conséquences d'une telle infection en termes histologique, immunologique, hormonal et d'excrétion dans le sperme. Ainsi, ma thèse a couvert trois étapes du processus d'infection, chacune correspondant à un chapitre de mes résultats :

- La persistance virale du ZIKV dans le testicule.
- La réplication virale du SRAS-CoV-2 dans le testicule
- Les facteurs d'entrée virale des virus ARN émergents dans le testicule.

## RESULTATS

### I- La persistance virale du ZIKV dans le testicule

Dans cette correspondance publiée dans la revue du Lancet Infectious Diseases, nous rapportons la caractérisation des cellules séminales non spermatiques infectées par le virus Zika chez trois patients d'une cohorte guadeloupéenne de l'épidémie de 2016, pour lesquels nous avons collecté des frottis de sperme longitudinaux jusqu'à 160 jours après le début des symptômes.

Grâce à l'analyse de marqueurs cellulaires (DDX4, EPCAM, CD14, CD68 et MPO) et des antigènes du virus Zika (l'enveloppe E et la protéine non structurale NSP1) par microscopie à immunofluorescence, nous avons constaté une excrétion persistante de cellules germinales testiculaires (DDX4+) infectées par ZIKV dans le sperme des trois patients. Dans l'ensemble, ces résultats montrent que les cellules germinales testiculaires humaines constituent un réservoir cellulaire persistant majeur dans lequel le virus Zika se réplique activement jusqu'à 60 jours post-symptômes (jps) et dont l'ARN est détecté jusqu'à 160 jps – les cellules germinales représentant alors 65,5% de toutes les cellules infectées. Dans une moindre mesure, des neutrophiles (MPO+) furent également retrouvés infectés jusqu'à 60 jps ; ainsi que des cellules épithéliales (EPCAM+) et des monocytes (macrophages notamment – CD14/CD68+) jusqu'à 90 jps ; représentant respectivement 20,3% et 5,7% de toutes les cellules infectées à ce temps.

Ces dernières cellules peuvent provenir de plusieurs organes du TGM (tels que l'épididyme, les canaux déférents, les vésicules séminales, la prostate ou l'urètre) qui peuvent ainsi contribuer à l'excrétion virale dans le sperme. Cependant, l'infection prolongée des cellules germinales du sperme indique que le testicule humain est bien un « réservoir viral » de ZIKV, foyer de l'infection persistante à l'origine de l'excrétion étendue du virus dans le sperme.

Dans le but d'explorer la persistance virale dans les cellules germinales testiculaires, notamment au niveau des facteurs favorisant et limitant l'infection, ainsi que des réponses immunitaires innées mises en place lors de l'infection, des expériences de séquençage ARN à l'échelle de la cellule unique (scRNA-seq) sont envisagées.

## II- La réplication virale du SRAS-CoV-2 dans le testicule

Au cours de ces travaux soumis au Journal of Clinical Investigation, nous avons réalisé des cultures organotypiques *ex vivo* de testicules humains provenant de donneurs sains, que nous avons découpés en fragments et que nous avons infectés pendant la nuit avec  $10^5$  TCID<sub>50</sub> de SRAS-CoV-2, avant de les cultiver pour un total de 9 jours. Tous les 3 jours, nous avons changé et collecté le milieu de culture ainsi que certains des explants pour réaliser une série de tests.

Ainsi, nous avons premièrement évalué la réplication du SRAS-CoV-2 grâce à la mesure de l'ARN viral par RT-qPCR, et de la libération *de novo* de virions infectieux dans les surnageants de culture par la technique des plages de lyse. Nous avons alors constaté que le SRAS-CoV-2 était capable de répliquer son génome dans le testicule humain, avec un pic de production de virions infectieux ( $1,44 \cdot 10^4$  PFU/mL) à 6 jours post-infection (jpi), déclinant ensuite autour de  $8,6 \cdot 10^2$  PFU/mL à 9 jours.

Afin d'étudier la localisation cellulaire du SRAS-CoV-2 dans les explants infectés et au cours de la culture, nous avons effectué des hybridation *in situ* (HIS) par RNAscope à l'aide de sonde spécifiques de l'ARN viral génomique et réplicatif. Nous avons ainsi confirmé une infection progressive au fil du temps en termes d'intensité et de localisation, passant du compartiment interstitiel au jour 3, au compartiment intratubulaire à 9 jpi. De manière intéressante, et entre autres analyses, le couplage de l'HIS à une immunohistochimie (IHC) fluorescente contre des marqueurs cellulaires spécifiques et le récepteur ACE2 nous a permis de corrélérer l'infection avec l'expression de ACE2 dans les cellules de Leydig et de Sertoli, se révélant être les cibles cellulaires principales du virus dans le testicule humain. L'infection des cellules de Sertoli fut notamment confirmée *in vivo* par l'analyse par microscopie fluorescente des cellules infectées dans le sperme d'un patient français de 2020 à 5 jps.

Nous avons ensuite étudié la réponse immunitaire innée du testicule humain à l'infection *ex vivo* par le SRAS-CoV-2, en mesurant par RT-qPCR l'expression d'un panel de senseurs et d'effecteurs immunitaires innés. Dans l'ensemble, les senseurs ARN du SRAS-CoV-2 (MDA5, RIGI) ainsi qu'une série de gènes effecteurs antiviraux (OAS1, MX1, RSAD2, IFIT1, ISG15) étaient surexprimés à 6 jpi, correspondant au pic de réplication virale. En revanche, seul CXCL10 parmi tous les médiateurs pro-inflammatoires testés (IFN $\beta$ , IL1 $\beta$ , IL18, IL-6, TNF $\alpha$ , CCL5 et CXCL18) fut surexprimé sur l'ensemble de la culture.

Ensuite, afin d'évaluer l'impact de l'infection sur la morphologie du testicule, nous avons utilisé un panel de techniques d'histologie et de dosage hormonal afin d'explorer la viabilité cellulaire, la structure tissulaire, et l'intégrité de la barrière hémato-testiculaire. Nous n'avons ainsi révélé aucun

impact majeur de l'infection, et l'analyse par RTqPCR des marqueurs des cellules germinales et des cellules de Sertoli n'a révélé aucun changement significatif dans leur expression, révélant ainsi que ces cellules n'ont certainement pas été affectées par l'infection. En revanche, l'expression des enzymes stéroïdogènes STAR et CYP11A1 a été significativement réprimée à 9 jpi et nous avons pu observer une tendance à la baisse des autres marqueurs cellulaires des Leydig, ainsi que de la production de testostérone au même temps. Au contraire la sécrétion d'inhibine B par les cellules de Sertoli ne sembla pas impactée.

Grâce à l'analyse d'échantillons de sang de 7 patients atteints de SRAS-CoV-2, non obèses et sans comorbidités, nous avons pu évaluer, par dosages, l'impact *in vivo* de l'infection sur les paramètres hormonaux. Alors que les fonctions hormonales des cellules de Sertoli ne semblaient pas être impactées, les niveaux de testostérone des patients aux jours 1 à 3 post-hospitalisation (jph) étaient significativement inférieurs aux niveaux de références établis dans des cohortes européennes et nord-américaines. Néanmoins, ces niveaux ré-augmentaient significativement, à la limite des niveaux de référence, à 9 jph. Les taux de LH, tout en restant dans les limites de la normale, présentaient une tendance inverse à celle de la testostérone, diminuant au cours du temps. Le rapport Testostérone/LH était ainsi significativement plus faible à 1-3 jph qu'à 9 jph, suggérant que cette dérégulation résultait d'une défaillance primaire du testicule plutôt que de l'axe HPG.

En conclusion, ce travail a principalement montré pour la première fois que le SRAS-CoV-2 infecte le testicule humain et se réplique principalement dans les cellules de Leydig et de Sertoli *ex vivo* ; que l'infection se produit probablement via le récepteur ACE2 (largement exprimé dans ces deux types cellulaires) et qu'elle altère la fonction stéroïdogénique des cellules de Leydig, bien qu'étant rapidement contrôlée par les réponses antivirales innées du testicule. L'infection de cet organe a notamment été confirmée *in vivo* par l'excrétion de cellules de Sertoli infectées dans le sperme de patients, pendant la phase aiguë de l'infection. Aussi, l'infection par SRAS-CoV-2 semble entraîner un hypogonadisme primaire transitoire chez les patients modérément infectés.

Cela dit, l'altération des fonctions testiculaires et le contrôle de l'infection n'ont pas pu être vérifiés à des temps plus tardifs que 9 jpi. En particulier, la relation causale entre la tendance à la dérégulation de la stéroïdogénèse *ex vivo* et l'hypogonadisme primaire chez les patients *in vivo* ne peut être qu'hypothétique, de même que la relation entre la réponse antivirale et le déclin de la production de virions infectieux. Par ailleurs, il a été démontré que le SRAS-CoV-2 est capable de se répliquer de façon cyclique et prolongée dans un modèle épithélial bronchique humain. Notre modèle

bénéficierait donc d'une durée de culture allongée, afin d'évaluer l'effet à long terme de l'infection sur la morphologie du testicule, la stéroïdogénèse des cellules de Leydig et la persistance virale potentielle. Ceci serait notamment permis par l'utilisation d'un dispositif micro-fluidique que nous sommes en train d'optimiser au laboratoire, permettant la culture d'explants testiculaires jusqu'à 6 mois, et l'analyse approfondie de différents paramètres protéiques, hormonaux, métaboliques et immuns.

### III- Les facteurs d'entrée virale des virus ARN émergents dans le testicule

Pour la plupart des virus excrétés dans le sperme, leur tissu d'origine est encore indéterminé et leur effet sur les fonctions masculines mal caractérisé. La première étape de l'infection est l'entrée virale, qui consiste en la reconnaissance et la liaison d'un virus à son ou ses récepteurs cellulaires, et dans certains cas, implique le clivage de la protéine de fusion du virus par des protéases cellulaires spécifiques. Le tropisme viral, et par conséquent la sensibilité des cellules et des organes, dépendent donc en premier lieu de la disponibilité de ces facteurs d'entrée à la surface des cellules. La cartographie des facteurs d'entrée virale dans les cellules testiculaires peut donc donner un aperçu du potentiel des virus ARN émergents à infecter cet organe. A cette fin, nous nous sommes concentrés sur les virus retrouvés dans le testicule et le sperme humain et avons étendu nos recherches à plus de 40 virus d'importance médicale des mêmes genres viraux. Nous avons ensuite effectué une analyse *in silico* de l'expression de 89 protéines dont il a déjà été prouvé qu'elles pouvaient servir de médiateurs à l'entrée des virus sélectionnés.

Nous avons distingué les récepteurs d'attachement non spécifiques, concentrant les particules virales à la membrane cellulaire ; les récepteurs d'entrée spécifiques, permettant les changements de conformation des protéines virales pour la fusion ; et les protéases, qui dans certains cas clivent les protéines d'entrée virales pour révéler leur peptide de fusion. Nous avons reporté ces données, entre autres informations de tropisme, sur une seule « carte thermique en fausses couleurs » afin d'avoir un aperçu global de l'utilisation des facteurs d'entrée par les différents virus.

Ensuite, pour effectuer la prédiction *in silico* de la susceptibilité du testicule humain, nous avons utilisé un ensemble de données de scRNA-seq déjà publiées, pour les différentes cellules testiculaires de 9 donneurs avec une spermatogénèse normale. Nous avons représenté une matrice d'expression sous forme de graphique en points (dotplot), sur lequel, pour chaque facteur d'entrée, la taille du point fait référence à la proportion de cellules l'exprimant, et la couleur du point correspond au niveau d'expression moyen du facteur d'entrée dans un type cellulaire.

Dans l'ensemble, cette étude vise à créer un atlas des facteurs d'entrée des virus émergents dans le testicule, et sert deux objectifs principaux : (i) dans le cas de virus déjà connus pour infecter le testicule humain, elle permet de visualiser facilement les facteurs d'entrée candidats dans les différentes populations de cellules testiculaires; et (ii) pour les virus émergents non encore identifiés dans le testicule, elle aide à prédire le potentiel d'infection des différentes populations de cellules testiculaires.

Un exemple d'interprétation suggère que le WNV pourrait infecter les cellules de Sertoli (démonstré *in vivo*) en se liant à AXL, Tyro3 et TIMD4 entre autres récepteurs : une hypothèse qui devra être vérifiée par diverses techniques *in vitro* telles que des expériences de gain et de perte de fonction. L'expression d'AXL et de TIMD4 dans les macrophages suggère également que ces cellules pourraient être susceptible à l'infection au WNV. De même, sur la base de la transcription de leurs récepteurs d'entrée virale, le testicule humain représente une cible potentielle pour les virus DENV et le CHIKV dont l'ARN a déjà été retrouvé dans le sperme de patients. Puisque les cellules cibles putatives comprennent les cellules du compartiment intratubulaire, le testicule pourrait alors représenter une des sources d'excrétion de ces virus dans le sperme.

En ce qui concerne les virus apparentés à ceux ayant été détectés dans l'appareil génital masculin, le récepteur CD55 du virus Sin Nombre (SNV) ; le récepteur BSG du virus de la rivière Ross (RRV) et du virus de l'encéphalite équine de l'Ouest (WEEV) ; les récepteurs LAMP1 et TRFC des Mammarenavirus du Nouveau Monde (JUNV) sont tous largement exprimés dans les différents types de cellules testiculaires. Ainsi, le testicule pourrait être sensible à l'infection par ces virus peu étudiés, qui n'ont de fait, encore jamais été signalés dans le TGM ou le sperme humain.

Dans l'ensemble, les données présentées dans cette analyse devront être renforcées par l'analyse de données scRNA-seq supplémentaires et par la validation de l'expression des récepteurs d'intérêt par des expériences de RT-qPCR, IHC ou cytométrie de flux. Des analyses complémentaires de l'équipement en facteurs de restriction virale des cellules testiculaires contribueront à appréhender la susceptibilité du testicule humain à une série de virus, et à prédire l'infection de cet organe. Le testicule humain étant une source potentielle de persistance virale et d'excrétion séminale, ces connaissances sont importantes pour anticiper le risque (ou l'absence) de persistance et de transmission sexuelle des virus à ARN émergents.

## DISCUSSION GENERALE

L'objectif de mon doctorat était de caractériser l'infection du sperme et du testicule humain par deux virus émergents, le ZIKV et le SARS-CoV-2, et de mettre en œuvre une analyse de l'expression dans le testicule des récepteurs d'entrée virale putatifs pour une gamme de virus à ARN émergents. Pour cela, j'ai utilisé un ensemble d'approches, allant de la culture organotypique et de cellules (approches expérimentales) à la recherche bibliographique et à l'exploration de données de séquençage ARN (approches *in silico*). Dans cette discussion, je présente les avantages et les limites de ces approches, ainsi que les perspectives qu'elles offrent quant aux projets présentés dans la partie Résultats de ce manuscrit, mais aussi quant aux différents projets annexes entamés au cours de ma thèse.

Ainsi, je présente et compare les approches expérimentales de culture de cellules immortalisées et de cellules primaires isolées avec la culture organotypique, l'expérimentation animale et les études cliniques. Dans l'ensemble, nos approches complémentaires *ex vivo et in vivo* ont révélé le tropisme testiculaire du ZIKV et du SARS-CoV-2. Nous avons également confirmé l'excrétion prolongée du ZIKV dans le sperme et caractérisé la nature des cellules infectées ainsi que la durée de leur infection chez les patients. En ce qui concerne le SARS-CoV-2, nous avons dévoilé ses principales cellules cibles testiculaires ainsi que le récepteur probablement impliqué dans leur infection. Nous avons évalué l'impact direct de l'infection sur la fonction des cellules de Leydig et l'avons relié à l'altération de la stéroïdogénèse chez les patients. Comme détaillé dans cette discussion, d'autres approches expérimentales permettront d'affiner les mécanismes impliqués dans l'infection du testicule et leurs conséquences sur l'homéostasie de l'organe.

Notamment, afin de déchiffrer les mécanismes sous-tendant l'infection des principales cellules cibles du ZIKV dans le testicule, j'ai cherché à déterminer la disponibilité des récepteurs d'entrée au ZIKV dans ces cellules. En utilisant la cytométrie en flux sur des cellules dissociées provenant d'explants de testicules humains, j'ai exploré l'expression des principaux récepteurs du ZIKV, à savoir les familles de lectines « TAM » et « C-type ». Alors que les protéines AXL, MERTK et DC-SIGN étaient détectées dans les macrophages testiculaires, aucune ne l'était dans les cellules germinales. Dans ces cellules, notre approche expérimentale initiale n'a donc pas permis de dévoiler les facteurs d'entrée.

Dans ce contexte, nous avons cherché à découvrir des récepteurs candidats pour le ZIKV dans les cellules germinales et à prédire de nouveaux récepteurs potentiellement impliqués dans l'infection par d'autres virus, en utilisant des approches *in silico*. Pour étendre les avantages de cette approche,

nous l'avons utilisée comme outil pour prédire la susceptibilité potentielle du testicule aux virus émergents. Cette approche vise à donner une image globale des facteurs d'entrée putatifs exprimés par les différentes populations de cellules testiculaires, pour un large éventail de virus de familles distinctes. Nous avons ainsi découvert plusieurs récepteurs potentiellement impliqués dans l'infection du testicule par une variété de virus ARN simple brin.

Cependant, cette approche, tout en étant une grande source d'information pour des recherches ultérieures, présente également plusieurs lacunes et défauts. En effet, la restriction de nos recherches aux virus médicalement importants laisse de côté les virus animaux, dont plus de 30 sont déjà connus pour infecter le tractus génital de leur hôte. Sachant que 75% des virus émergents sont zoonotiques, certains d'entre eux pourraient bientôt franchir la barrière d'espèce et potentiellement infecter le testicule humain. Aussi, travailler sur les virus émergents implique qu'ils sont, pour la plupart, peu étudiés. Ainsi, les informations sur leur tropisme, l'utilisation de récepteurs et leur mécanisme d'infection sont souvent incomplètes ou mal comprises. De plus, nous avons cherché à intégrer à cette étude uniquement les facteurs d'entrée dont l'utilisation a été prouvée par des expériences de gain et de perte de fonction, entraînant une augmentation ou une diminution de l'entrée virale dans les cellules cibles. Cette méthode exclut donc les facteurs d'entrée dont on a seulement prouvé qu'ils n'avaient que des capacités de liaison à des protéines virales ou qu'ils appartenaient à la famille de protéines impliquées dans l'entrée du virus d'intérêt. Tout en cherchant à être précise, cette méthode pourrait être considérée comme trop stricte pour une approche initiale, nécessitant fatalement des confirmations supplémentaires de l'utilisation du facteur d'entrée. De plus, certaines informations publiées ont pu être manquées lors de notre revue de la littérature. Enfin, l'étude de la transcription des facteurs d'entrée est basée sur un seul ensemble de données. Bien que cet ensemble ait déjà été utilisé pour décrire l'expression des facteurs d'entrée ACE2 dans les cellules de Sertoli par exemple, certaines divergences avec d'autres ensembles de données publiés existent et une analyse plus objective et robuste nécessiterait l'intégration de plusieurs jeux de données de séquençage. Par conséquent, cette approche bénéficierait grandement d'un mécanisme participatif afin d'être continuellement mise à jour par la communauté scientifique, à mesure que l'utilisation de nouveaux facteurs d'entrée est confirmée et que de nouvelles données de scRNA-seq deviennent disponibles.



Si cette approche permet de prédire la sensibilité d'une cellule en fonction de l'expression des récepteurs d'entrée dans ce type cellulaire donné, des études ont montré que certains facteurs d'entrée pouvaient agir "en trans" d'une cellule à une autre. Ainsi, dans un tissu où des interactions étroites entre les cellules existent, comme dans le testicule, il faut se rappeler que la susceptibilité d'une cellule peut ne pas dépendre uniquement de son expression des facteurs d'entrée, mais aussi de celle des cellules environnantes.

En conclusion, cette analyse *in silico* n'est qu'une première étape dans la caractérisation de la susceptibilité du testicule, qui nécessite plusieurs expériences complémentaires : de confirmation de l'expression protéique des facteurs d'entrée d'intérêt et de leur utilisation pour l'entrée virale, et d'investigation des facteurs de réplication et de restriction virale exprimés au sein des cellules cibles.

## CONCLUSION

En conclusion, les travaux entrepris au cours de mon doctorat ont contribué à caractériser l'excrétion prolongée du ZIKV dans le sperme, et le tropisme du SRAS-CoV-2 pour le testicule humain. Nous avons ainsi démontré que le testicule est un réservoir de ZIKV dans le sperme. Il est également important de noter que nous avons détecté une excrétion prolongée de cellules épithéliales et de leucocytes infectés, indiquant que d'autres organes du tractus génital masculin supportent une infection prolongée par ZIKV. La ou les sources exactes de ces cellules doivent encore être déterminées.

À l'aide d'une culture *ex vivo* d'explants de testicules humains, nous avons révélé la réplication du SRAS-CoV-2 dans cet organe, principalement dans les cellules de Leydig et de Sertoli. Bien que l'infection semble avoir un impact sur la stéroïdogénèse des cellules de Leydig, la réplication virale diminue au fil du temps, suggérant que l'infection testiculaire pourrait ne pas être persistante. Les patients COVID-19 analysés dans cette étude présentaient un hypogonadisme primaire transitoire, étant en faveur d'un impact direct de l'infection par le SRAS-CoV-2 sur le testicule. De plus, des cellules de Sertoli infectées furent détectées dans le sperme d'un patient, confirmant *in vivo* le tropisme du SRAS-CoV-2 pour le testicule humain.

Par ailleurs, nous avons commencé l'analyse de l'expression des facteurs d'entrée de virus ARN émergents dans le testicule. En utilisant une approche de séquençage de l'ARN à l'échelle de la cellule unique, nous avons cherché à prédire la susceptibilité des différentes cellules testiculaires à une série de virus. Des analyses complémentaires sont nécessaires pour valider la pertinence de ces résultats

et déterminer l'expression des facteurs facilitant et limitant l'infection. Néanmoins, ce travail constitue un premier pas vers la caractérisation du tropisme testiculaire des virus ARN émergents, et une base de données informative pour guider de futures recherches.

Dans l'ensemble, ce travail a contribué à une meilleure compréhension de l'infection du testicule humain et de sa susceptibilité aux virus émergents. Alors que les preuves s'accumulent sur leur capacité à être excrété dans le sperme et à être transmis sexuellement, la prédiction et la caractérisation de la source d'excrétion sont essentielles pour contrôler leur propagation. Le testicule, en tant qu'organe immunoprivilégié, apparaît comme un site important pour une infection et une excrétion virale prolongée. La compréhension des mécanismes qui sous-tendent l'entrée virale et l'infection persistante du testicule est cruciale pour prévenir et anticiper les conséquences des virus émergents sur la santé reproductive des hommes et sur leur propagation par voie sexuelle.

# Introduction

## I. Emerging Infections

### A. Generalities

Emerging infections are defined as “infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range” <sup>1</sup>, and “which cause public health problems, either locally or internationally” <sup>2</sup>.

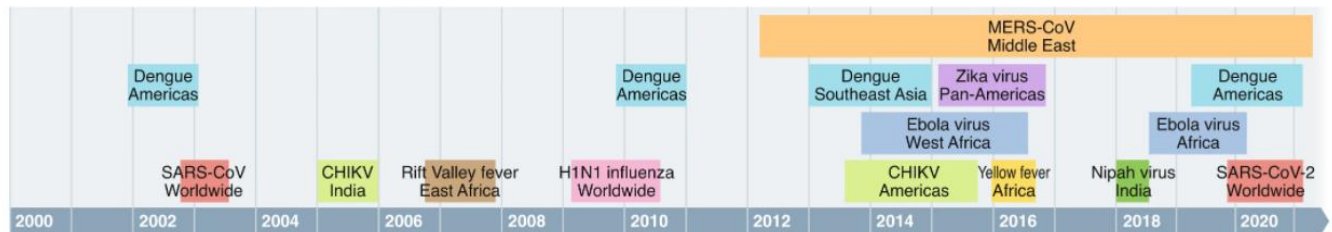
Since the 1970’s, more than 1400 new pathogens have been identified, 13% of which are considered as emerging or reemerging <sup>3</sup>. Among these emerging pathogens, about 75% have originated from animals since the 1980’s <sup>4</sup>; the majority of which are viruses <sup>5,6</sup> (**Figure 1**).

In total, nine viruses were indexed in the last priority public health risk list updated by the Research and Development (R&D) Blueprint initiative of WHO in February 2018 <sup>7</sup>:

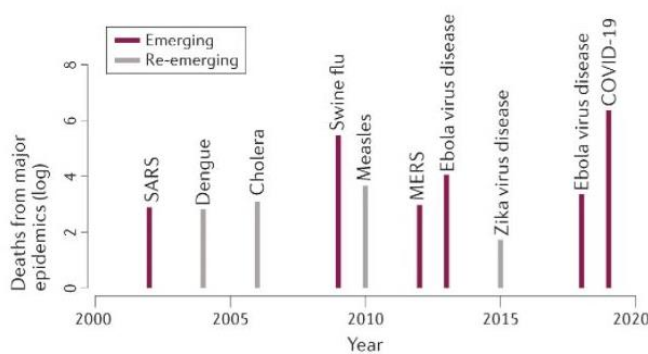
- Arenaviral hemorrhagic fevers (including Lassa fever)
- Chikungunya (CHIKV)
- Crimean-Congo hemorrhagic fever (CCHFV)
- Filoviral diseases (including Ebola (EBOV) and Marburg (MARV) viruses)
- Middle East Respiratory Syndrome coronavirus (MERS-CoV)
- Severe Acute Respiratory Syndrome (SARS) and other highly pathogenic coronaviral diseases
- Nipah (NiV) and related henipaviral diseases
- Emerging non-polio enteroviruses
- Rift Valley fever (RVFV)
- Severe Fever with Thrombocytopenia Syndrome (SFTS)
- West Nile virus (WNV) disease
- Zika virus (ZIKV) disease

- « Disease X » which “represent the knowledge that a serious international epidemic could be caused by a pathogen currently unknown to cause human disease” and which seeks R&D preparedness

A



B



**Figure 1: Overview of the 21<sup>st</sup> century’s emerging infections burden.**

(A) Timeline of the 21<sup>st</sup> century viral outbreaks and geographical spread <sup>8</sup>. (B) Log deaths from major epidemics of the 21<sup>st</sup> century <sup>9</sup>.

## B. Emergence processes

Emerging infections can originate from three different events: (i) a newly evolved strain of pathogens; (ii) an existing pathogen infecting the human population for the first time; (iii) a known pathogen sporadically infecting humans but which incidence suddenly increased at a given location <sup>5</sup>. The emergence process occurs through 3 major steps <sup>6,10</sup>:

**The introduction** of a pathogen to a new host species, which depends on the ability of the pathogen to bind to different cellular receptors, determining the host range.

**The establishment** of the pathogen in the host, which depends on its ability to complete its reproduction cycle and evade immune responses for efficient replication.

**The dissemination** of the pathogen among the host population. This depends on its ability to replicate at an efficient viral load in key tissues, determining the transmission route, in order to found a reservoir population in which the pathogen continuously circulates.

## C. Transmission routes

Transmission routes play a key role in efficient dissemination and can occur via different modes, classified under the vertical versus horizontal dichotomy <sup>11</sup>. The vertical mode corresponds to the parent-to-offspring transmission. It can be subdivided into cellular transmission via the gametes; transplacental transmission; perinatal at birth or neonatal transmission via breast feeding. The horizontal mode can be divided into sexual or non-sexual transmission. Non-sexual transmission then consists in airborne mode, via direct contact with lesions (infected blood, body fluids) or via indirect contact (contaminated food and water, dust, contaminated objects designed as “fomites” or vector-borne) (**Table 1**). The transmission is dependent on the pathogen, but also on the host biology and ecology since the density of population, the frequency of contacts, the movement of individuals will also be determining factors. Most pathogens can be transmitted via multiple routes, but adaptation to each might involve evolutionary trade-offs and the selection of unique genetic variants. Some arthropods-borne “arboviruses”, primarily only infecting insects, adapted to new ways of transmission for dissemination in the vertebrate population. As an example, the Rift Valley Fever Virus (RVFV) is maintained in the mosquito population by transovarial vertical transmission, infects livestock through mosquito bites and disseminates to human by direct contact with infected animals or the consumption of their milk.

Modes		Routes		Viruses	References	
Vertical		Transplacental		HIV, ZIKV	12	
		Perinatal		HIV, Enterov	13	
		Neonatal (breast feeding)		HIV, WNV, EBOV	14,15	
Horizontal	Sexual	Genital-genital		HIV, ZIKV, EBOV	16,17	
	Non-sexual	Airborne		Beta-CoV	18	
		Direct contact		EBOV, MARV, NIV	19,20	
		Indirect contact	Environmental	Contaminated food and water	Beta-CoV, NIV	21,22–24
				Fecal-oral	Enterov	25
		Fomites		Contaminated surfaces-hands	Airborne & direct contact-borne viruses	21,26,27
				Needle-blood	HIV, EBOV, LASV	28,29
		Vector-borne		Cutaneous penetration (bite)	FlaviV, AlphaV, PhleboV	30
				Skin-to-skin	NiV, EBOV	27,31
				Fecal-oral	HantaV	32

**Table 1:** Classification of human transmission modes illustrated with examples of emergent viruses including HIV and SARS-CoV-2. V stands for “viruses”. (Modified from Antonovics et al., 2017<sup>11</sup>).

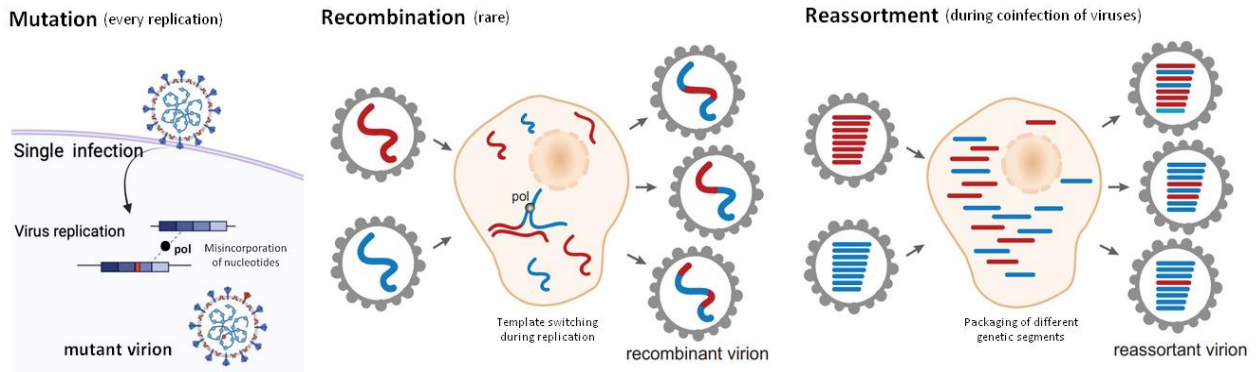
## D. Factors of viral emergence

For a microorganism to arise in the human population, several factors are implicated at each stage of emergence and imply rapid evolution of the pathogen to adapt to the selective pressure of a new host or environment. In that respect, RNA viruses are of special concern and represent up to 44% of the 21<sup>st</sup> century’s emerging diseases, due to their faster evolutionary rate of all living organisms<sup>33</sup>.

Nowadays, over 200 species of RNA virus are able of infecting humans, and two or three new species are discovered every year <sup>34</sup>.

The factors of emergence are grouped into 4 categories:

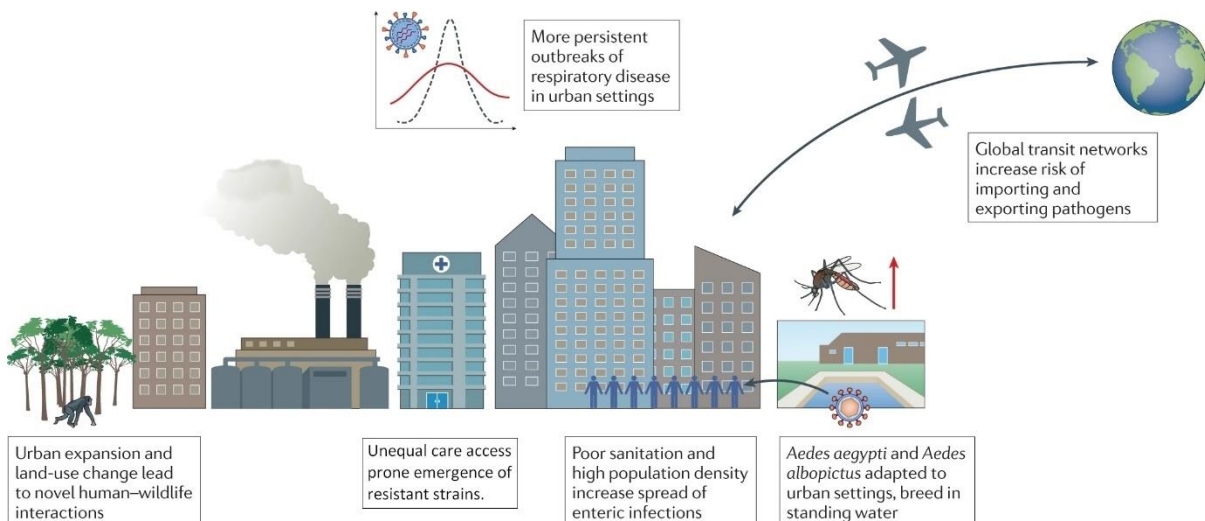
i) Viral evolution. To infect a new host, the virus needs to be able to bind to the host cellular receptors to enter cells. Binding to new receptors often imply changes in the structural proteins of the virus. The evolution of RNA viruses can occur through 3 types of evolutionary mechanisms allowing protein changes, namely mutations, recombination, or reassortment (**Figure 2**). These mechanisms allow RNA viruses to infect a large range of cells and hosts, expanding their reservoir and transmission scope; ii) Host permissiveness. Once entry is achieved, the virus needs to replicate in this cell, which is then characterized as permissive. The sex, age, physiological and genetic parameters of the host are involved in the modulation of the innate and adaptive immune responses. The equilibrium between virulence and immune escaping determine the permissiveness and the ability of the virus to persist in its host <sup>35,36</sup>; iii) Environment. The climate influences a virus survival in its environment, its seasonality as well as its vectors distribution <sup>9,37</sup>. Natural disasters cause sudden changes in local ecology that may influence reservoir populations density and migration in proximity to human communities, enhancing the chance of species-jump and viral emergence <sup>38</sup>; iv) Human activities and behavior. Several activities can modify the exposition to viral threats and influence the emergence process, they are resumed in **Figure 3**. <sup>9</sup>. Lately, Carlson et al. estimated that more than 10.000 virus species have the capacity to infect humans, but at present are circulating silently in wild animals. According to their model, climate change as well as land-use expansion are responsible for creating new opportunities for cross-species transmission and would result in more than 15.000 viral spillover by 2070 <sup>39,40</sup>. All these factors are intimately interconnected and influenced the recent emergence of ZIKV and SARS-CoV-2.



**Figure 2: Mechanisms of RNA viruses' evolution.**

(adapted from Laura Herrero's creation for The Conversation, Lam et al., 2010 and Webby et al., 2004<sup>41-43</sup>).

**Mutation** is driven by the virus RNA-dependent RNA polymerase (RdRp) which generally lacks proof-reading activity. This way, mis-incorporated nucleotides during replication are not corrected and RNA viruses present a mutation rate of  $10^{-6}$  to  $10^{-4}$  mutations per nucleotide per cycle<sup>44</sup>. **Recombination** is driven by the RdRp template switching during replication. Genetic fragments can thus be exchanged between two RNA strands of the same origin or between different viruses infecting the same cell<sup>33</sup>. **Reassortment**, occurs during viral packaging of segmented viruses infecting the same cell. Exchange of genomic segments lead to the generation of reassortant virions which, in the case of influenza viruses, were at the origin of multi-species jumps leading to the 2009 H1N1 pandemic and "antigenic shift"<sup>45</sup>.



**Figure 3: Examples of impacts of the human activity on emerging infections.**

(Modified from Baker et al., 2022<sup>9</sup>).



## II. The male genital tract

The Male Genital Tract (MGT) is composed of a series of organs and ducts which ensure the male gamete production, storage, and transport. The testes endorse the dual role of gametes (spermatogenesis) and hormonal production, the latter responsible for the maintenance of the male body phenotype and of reproductive functions <sup>16,46</sup>.

### A. The Testis

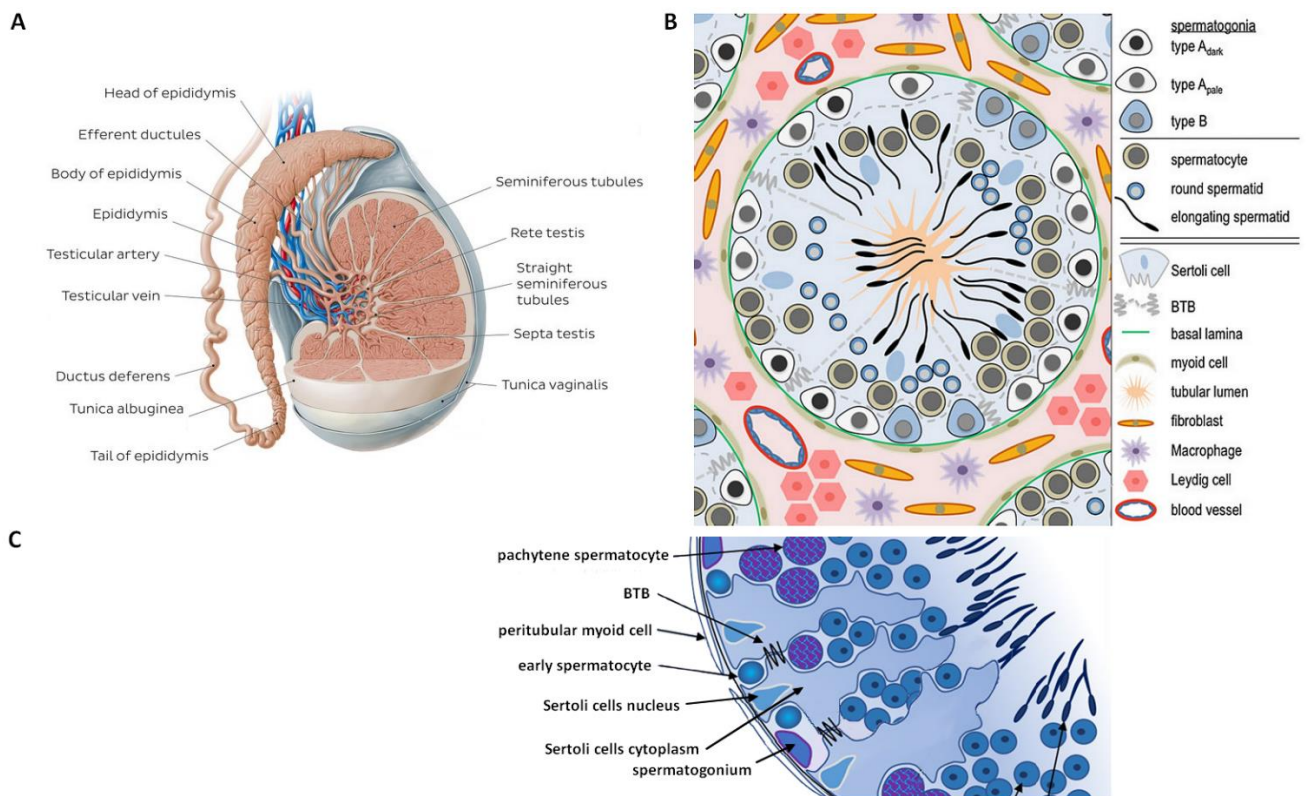
#### A.1. Morphology and function

Each testis is located in the scrotum and surrounded by a thick envelope of conjunctive tissue called the tunica albuginea. From the albuginea radiate connective tissue walls called septa, separating the testis parenchyma into several hundreds of lobules. These lobules converge to the upper part of the tunica, in a thicker compartment called the mediastinum testis. Each lobule contains several tubules (where spermatogenesis takes place) surrounded by a loose conjunctive interstitial tissue. The seminiferous tubules of all lobules join in a network of canaliculi called the straight tubules, which in turn form the collecting system called the rete testis, located inside the mediastinum testis. From the mediastinum leave the efferent ducts that rejoin the epididymis (**Figure 4, A**) <sup>47,48</sup>.

The testis is morphologically divided into two compartments (**Figure 4, B, C**):

The interstitial tissue contains blood and lymphatic vessels and various cell types including the testosterone producing Leydig cells and macrophages, the latter being the most numerous immune cell type present in the testis. These cells play a key role for the testicular homeostasis.

The seminiferous tubules are surrounded by the peritubular myoid cells. They encompass the polarized Sertoli cells, which support the germ cells, from spermatogonial stem cells localized at the basal membrane of the tubule, up to the spermatozoa towards the lumen. Their secretive function provides a nursing role for the developing germ cells, while their tight junctions constitute the physical part of the Blood-Testis-Barrier (BTB), providing a protective role against systemic aggressions.



**Figure 4: The testicular morphology and histology**

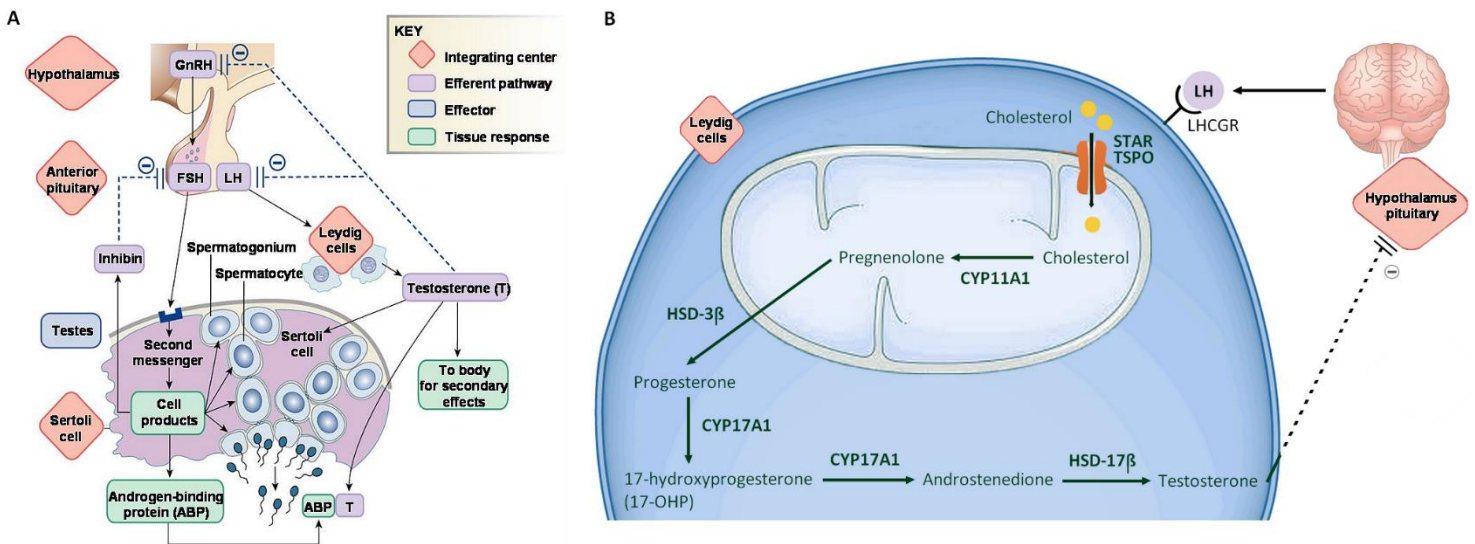
**(A) The testicular and epididymis morphology** (adapted from Paul Kim’s creation on Kenhub).

**(B) Illustration of testicular histology**<sup>49</sup>. The BTB separates the basal compartment from the immune-privileged central “adluminal compartment”.

**(C) Illustration of the seminiferous tubule** depicting more precisely the morphology of Sertoli cells (modified from Loveland et al., 2017<sup>50</sup>).

The production of the male gametes is controlled by an endocrine hormonal system that involves several organs composing the hypothalamic-pituitary-gonadal (HPG) axis. The Gonadotropin-Releasing Hormone (GnRH) is produced and released in a pulsatile manner by the hypothalamus to activate the anterior pituitary gland. In turn, the pituitary produces the Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), which are secreted in the circulation. The LH activates Leydig cells to initiate the synthesis of testosterone. The FSH potentiates spermatogenesis by regulating the gene expression of Sertoli cells<sup>51</sup> (**Figure 5, A**). The GnRH secretion is kept at a physiological level through different testicular feedbacks:

(i) A negative feedback is exerted on by testosterone, which inhibits the LH secretion; and by Sertoli-secreted inhibin B, which inhibits FSH production, directly from the pituitary gland<sup>52</sup>. (ii) A positive feedback is exerted by Sertoli-secreted activin A, which stimulates the FSH production from the pituitary<sup>53</sup>.



**Figure 5: The hypothalamic-pituitary-testicular endocrine system, simplified.**

**(A)** From the Reproductive system course of Austin Community College <sup>54</sup>

The ABP (androgen binding protein) secreted by Sertoli cells enhances testosterone action.

**(B)** The steroidogenesis of testosterone in Leydig cells, simplified. (Data from Chakraborty et al., 2021 <sup>56</sup>)

Interstitial and intratubular cells thus collectively orchestrate the testicular functions of gametogenesis, steroidogenesis and immune privilege, allowing the promotion and maintenance of male fertility <sup>55</sup>.

## A.2. The interstitial tissue

### A.2.a. The Leydig cells

Leydig cells are the most abundant cells of the interstitium. Their primary function is to synthesize and secrete androgens necessary for spermatogenesis to proceed and male fertility to be maintained. They are either located in clusters near blood vessels or found close to the seminiferous tubules. They are notably found in regions where Spermatogenic Stem Cells (SSC) are preferentially located, thus mirroring their endocrine and paracrine function <sup>55</sup>.

Upon binding to its receptor (LHCGR), LH activates cholesterol translocation into the mitochondria via the steroidogenic acute regulatory (STAR), and the translocator (TSPO) proteins. There, cholesterol is converted to pregnenolone by the Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1) enzyme. Pregnenolone is converted to progesterone by the Hydroxysteroid dehydrogenase-3 $\beta$  (HSD-3 $\beta$ ) followed by its conversion to 17-hydroxyprogesterone (17-OHP)

by CYP17A1. 17-OHP is further converted into androstenedione by CYP17A1. The HSD-17 $\beta$  dehydrogenase finally converts the androstenedione into testosterone (**Figure 5, B**)<sup>56,57</sup>.

Testosterone diffuses into the seminiferous tubules and blood vessels of the interstitial tissue. Upon binding to its Androgen Receptor (AR) on Sertoli cells, testosterone modulates their surface protein expression (e.g. the connexins), strengthening their connection to the germ cell they nurture. It thus promotes their proliferation as well as their entry into meiosis and spermiogenesis. This is done via the modulation of the BTB, which opens to allow their differentiative migration toward the lumen of the seminiferous tubules<sup>51,58</sup>.

By binding to the peritubular myoid cells, testosterone induces the production of vasopressin, oxytocin and prostaglandins, which modulate their contractility. The peritubular cell contractibility is required for the effective detachment of the spermatozoa into the lumen of the seminiferous tubules, called spermiation, and their migration towards the straight tubules<sup>55</sup>.

Testosterone is also metabolized by the 5  $\alpha$ -reductase into the more potent Dihydrotestosterone (DHT), which has a higher affinity for the AR. It can also be transformed by aromatase in estradiol, which might be implicated in germ cell survival and acrosome formation<sup>49,59</sup>.

Finally, testosterone exerts an immunosuppressing role. It balances the numbers of macrophages and promotes the expansion of immunotolerant lymphocytes (Treg). In this regard, Leydig cells also produce the Growth arrest-specific Gene 6 (Gas6) and Protein S, which inhibits innate responses in macrophages, Sertoli and dendritic cells<sup>60</sup>.

Among other specific molecules (<sup>55,58</sup> for review), Leydig cells produce the insulin like factor 3 (INSL3), which in the adults plays a role in the inhibition of the germ cells apoptosis, and is a marker of Leydig cells integrity<sup>58</sup>.

### **A.2.b. The Macrophages**

In addition to the BTB, testicular macrophages primary role is to maintain the immunologic microenvironment of the testis in order to protect the germ cells and in turn fertility. They present a dual role of immunoregulation between the maintenance of the organ immune privilege and its homeostasis. Indeed, puberty-risen germ cells are recognized as foreign by the immune system and testicular macrophages play a role in the regulation of the induced auto-immune responses. Macrophages are also involved in the phagocytosis of infecting pathogens and apoptotic cells<sup>61</sup>.

In mice, testicular macrophages have been reported to arise from 3 distinct waves of hematopoiesis, with embryonic yolk sac macrophages replaced by either fetal liver monocytes or bone marrow-derived monocytes<sup>61</sup>. They represent the most abundant resident immune population of the testis and have been divided in two main populations of different origins, localizations and functions<sup>61-63</sup>:

**Interstitial Testicular Macrophages** (iTm), are believed to be embryonic-derived and present at birth. They are round cells, scattered in the interstitial tissue<sup>63</sup>. In rats, they are intimately associated to Leydig cells of which they are believed to promote proliferation and support the spermatogenesis regulation. Indeed, they secrete 25-hydroxycholesterol which is also cleaved by HSD-17 $\beta$  to produce testosterone. Besides, iTm present a CD206<sup>+</sup>, CD64<sup>high</sup>, MHC-II<sup>low</sup> profile and are thought to contribute to the anti-inflammatory environment by inducing testosterone production and by secreting IL-10 cytokine and TGF $\beta$ <sup>61</sup>.

**Peritubular Testicular Macrophages** (pTm) origin remains disputed. Mossadegh et al., using a mice model, hypothesized that they arise from bone marrow-derived blood monocytes after birth<sup>64</sup>. However, other authors showed that this population could maintain itself without depending on the bone marrow for replenishment<sup>65,66</sup>. Nonetheless, studies agree on their localization, lining the seminiferous tubules and in close contact to peritubular myoid cells. They present a tolerizing CD206<sup>-</sup>, CD64<sup>low</sup>, MHC-II<sup>high</sup> profile, and are thought to participate in the phagocytosis of degenerating germ cells and processing of their antigens. By presenting them to naive T cells, in rats, they promote their differentiation into anti-inflammatory regulatory T cells (Treg)<sup>61</sup>.

Both populations also express the colony stimulating factor 1 (CSF1) and key enzymes in the retinoic acid synthesis which stimulate SSC proliferation and differentiation, respectively<sup>61,63</sup>. However, the existence and role of these macrophages subpopulation in the human testis is still unknown and awaits further characterization.

The characteristic immunosuppressive phenotype of TMs is believed to be governed by the local testicular microenvironment, which is full of immunomodulatory molecules such as testosterone, prostaglandins, corticosterone and activin. However, little is known regarding the capacity of this microenvironment to influence the phenotype of infiltrating blood-derived monocytes upon testis infection and inflammation. These inflammatory cells notably release cytokines that can alter Sertoli

cells tight junctions and induce breakdown of the BTB. This facilitates the access inside the seminiferous tubules, to germ cells and ultimately to semen.

Importantly, the tolerogen phenotype of TMs makes them primary targets for viral infections (HIV-1, ZIKV, Ebola). Furthermore, macrophages cell-to-cell transfer of HIV-1 is well characterized and TM are suspected to disseminate viruses through their privileged contacts with Leydig and peritubular myoid cells. Accordingly, iTM were demonstrated to be required for the dissemination of ZIKV into the seminiferous tubules of mice *in vivo* <sup>61</sup>.

### **A.2.c. Other interstitial cell populations**

Besides macrophages, the testicular interstitium contains dendritic cells, T cells and mast cells as part of the immune population, as well as vascular and lymphatic endothelial cells, fibroblasts, and the newly discovered telocytes.

**T lymphocytes** are the only population of lymphocytes found in the human testis. Different subsets have been described, with a majority of CD8+ cytotoxic T cell (CTL) and regulatory T cell (Treg) as well as a few Th1 and Th17 cells <sup>67</sup>. In rat testes, CTL express proinflammatory mediators such as TNF $\alpha$ , INF $\gamma$  or FasL, which intrinsically promote cell death. However, in normal condition they tend to be inhibited by the local immunosuppressive factors (IL10, TGF $\beta$ , activin A) secreted by the surrounding cells. Additionally, Treg cells, upon germ cell antigens activation, produce TGF $\beta$ , which also promotes autoreactive CTL's elimination. However, upon Sendai virus infection, rats macrophages, Sertoli and Leydig cells were shown to produce large amounts of type I IFN, ISG and proinflammatory cytokines <sup>16</sup>. Added to a disturbed testosterone production by Leydig cells, this pro-inflammatory microenvironment was found to trigger systemic T helper cell infiltrates in the testis. These cells produce Th1 and Th17 cytokines that are able to potentiate the proliferation of the resident inflammatory T cells and to inhibit Treg, thus altering the anergic state of the lymphocyte population and threatening germ cells survival. <sup>62,67</sup>.

**Dendritic cells**, which are the main antigen-presenting cells of the adaptive immune system, are very rare in the testis. In rats, they play a role in the tolerance induction of T cells by releasing IL10 and indoleamine 2.3-dioxygenase (IDO) <sup>16</sup>.

**Mast cells** pre-synthesize cytotoxic substances (such as histamine, tryptase and TNF $\alpha$ ) in granules they release upon their activation by autoreactive T cells. Of note, the tryptase also enhances the

proliferation of testicular fibroblasts and their collagen synthesis leading to the fibrosis of the inflamed tissue <sup>62</sup>.

**Endothelial cells** constitute the epithelium of lymphatic and blood vessels. The latter supply the testis in oxygen and metabolites and allow the endocrine secretion of hormones maintaining the secondary sex characteristics. Blood vessels thus play a role in the tissue homeostasis and organization, and Leydig cells preferentially establish clusters in proximity to these vessels <sup>55</sup>.

**Telocytes** is a new cell population, recently identified in rodent and human's testis <sup>68,69</sup>. They can be defined as "stromal cells with telopodes" which are interconnected and establish a complex reticular network along the interstitium. They were found in close contact with endothelial and peritubular myoid cells. While their function is currently unknown, they are thought to be engaged in juxtacrine and paracrine intercellular signaling, allowing the coordination of the tissue homeostasis <sup>69,70</sup>.

### A.3. The seminiferous tubules

#### A.3.a. The Peritubular Myoid Cells

In human, Peritubular Myoid Cells (PMC) are elongated smooth muscle-like cells physically delimiting the inter- and intratubular compartments by 5 to 7 permeable layers. On the contrary, rodents present a one-layer of cell joined by junctional complexes <sup>71</sup>. They cooperate with the Sertoli cells to form the basal lamina of seminiferous tubules and are thought, in mice, to regulate the formation of the BTB by the production of Leukemia Inhibitory Factor (LIF) <sup>58</sup>. In human, PMC produce the anti-angiogenic Pigment Epithelium Derived Factor (PEDF / SERPINF1) to prevent the tubules vascularization, critical for the BTB and spermatogenesis maintenance <sup>55</sup>. Under testosterone binding to its androgen receptor, mice PMC produce and secrete the Glial cell line-Derived Neurotrophic Factor (GDNF) as well as the Colony-Stimulating Factor 1 (CSF1), promoting the SSC self-renewal proliferation. In human, both molecules are expressed by PMC but only GDNF role on SSC was confirmed <sup>72,73</sup>. Testosterone also promotes the prostaglandin-driven PMC contraction allowing the migration of immobile sperm cells along the seminiferous tubules towards the epididymis <sup>55,72</sup>.

#### A.3.b. The Germ cells

The germ cells define all cells at any stage of the spermatogenesis leading to the formation of the male gamete: the spermatozoa. Spermatogenesis takes 74 days to complete in the human's

Seminiferous Epithelium (SE) <sup>49</sup>. Each step of the spermatogenesis has a defined constant duration and assure a nonsynchronous but continuous sperm formation to sustain male fertility. The SE is thus in constant turnover as “generations” of SSC start differentiating on the basal lamina. Differentiated spermatozoa end up at the apical lumen of the seminiferous tubule, where they will be released to join the straight tubules <sup>49,74</sup>.

Spermatogenic Stem Cells (SSC), also called type A<sub>dark</sub> spermatogonia (SPG), lie on the basal membrane of the ST. The control of PMC and Sertoli-derived GDNF maintain them in a self-renewing state allowing them to proliferate actively. They thus serve as a pool of cells able to repopulate the SE in case of cytotoxic stress and apoptosis of the differentiating germ cells. Upon action of retinoic acid, A<sub>dark</sub> SPG differentiate into A<sub>pale</sub> SPG, exhibiting altered histone modifications preparing them for mitosis. Spermatogenesis can be divided into three phases (**Figure 6, A**):

**The mitotic phase** starts with the first division of A<sub>pale</sub> SPG into an A<sub>pale</sub> progenitor SPG (responsible for their self-renewal) and a type B SPG, committed to differentiation. Follows the second division of B SPG into two “preleptotene” primary spermatocytes (SPC) which detach from the basal membrane, transit through the BTB and enter the adluminal compartment of the SE, engulfed by Sertoli Cells (SC) <sup>49,51</sup>.

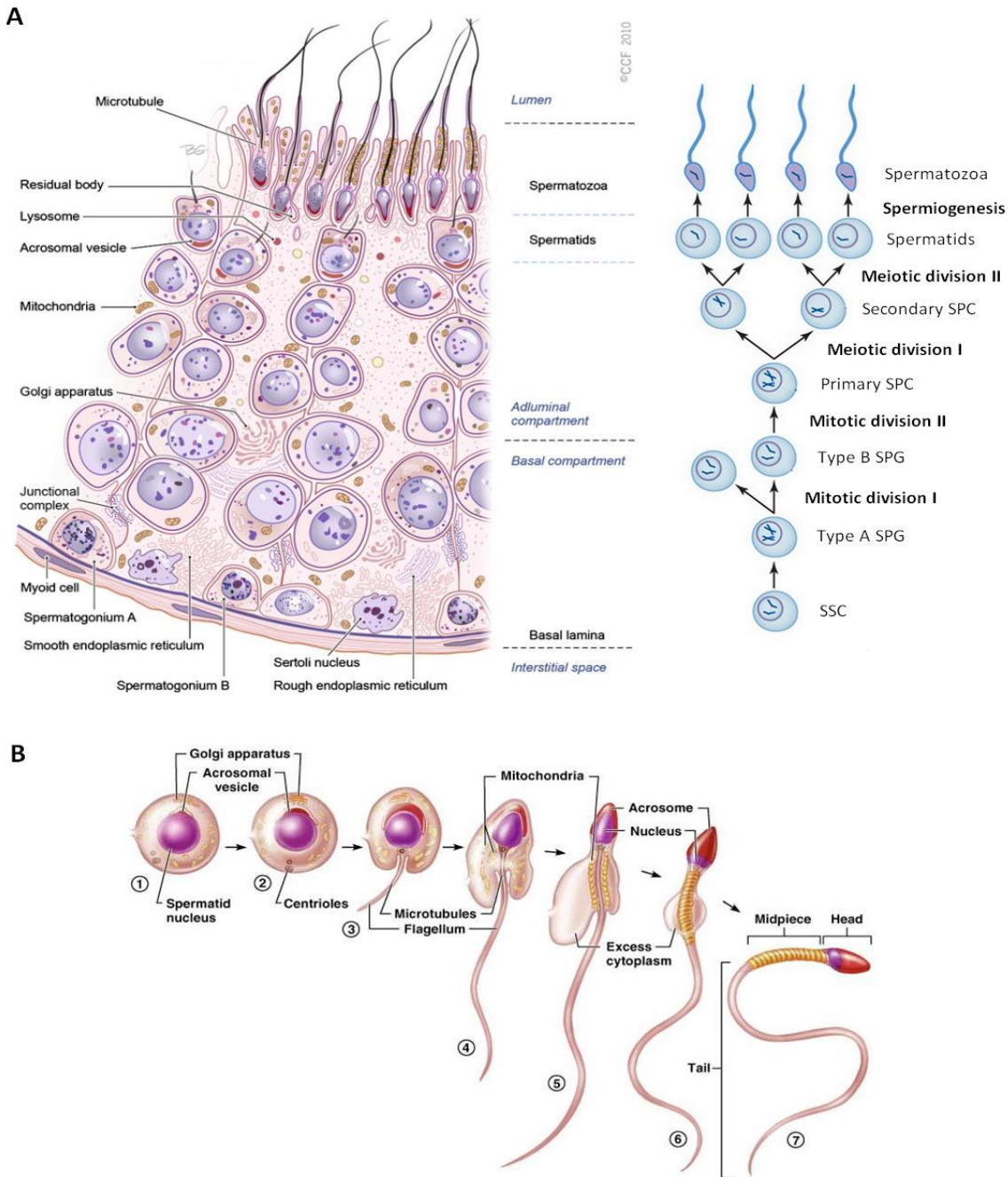
**The meiotic phase** proceeds with the entry into prophase I of diploid preleptotene SPC which itself can be subdivided into 5 stages of SPC differentiation. Retinoic acid-stimulated STRA8 allows the chromosomal condensation of the leptotene SPC that goes through the different stages: the zygotene (pairing of homologous chromosomes), pachytene (crossing over of chromatids and exchange of genetic information) and diplotene (repulsion of chromosomes) stages until the “diakinesis” breakdown of the nuclear membrane. Metaphase, anaphase and telophase follow quickly to form two new haploid secondary SPC. These cells then undergo a second meiotic division to produce four round haploid spermatids, in which sister chromatids of chromosomes are segregated <sup>49,75</sup>.

**The spermiogenic phase** corresponds to the spermatids (SPT) maturation and is characterized by drastic changes of the cell morphology. The SPT condense their chromatin to form the head of spermatozoa, bringing the cessation of transcription. They develop a posterior flagellum for motility



as well as an anterior acrosome for cell fusion with the ovum. They finally remove the bulk of their cytoplasm, which will become residual bodies phagocytosed by Sertoli cells <sup>49,75</sup> (**Figure 6, B**).

This phase ends with the spermiation, i.e. the disengagement of mature SPT, called spermatozoa, off the apical part of Sertoli cells (SC) into the seminiferous tubule lumen. This whole process being prone to errors, apoptosis is a common feature of the spermatogenesis and degenerative germ cells are also phagocytosed by SC. The spermatozoa are then propelled along the seminiferous tubules by the peritubular cell's contraction up to efferent duct and the epididymis where they will further mature to acquire motility.



**Figure 6: The spermatogenesis**

**(A)** Section of the seminiferous epithelium and representation of the different germ cell categories (Adapted from the Encyclopaedia Britannica and Sharma et al., 2011 <sup>76,77</sup>)

**(B)** Spermiogenesis (From the Reproductive system course of Austin Community College <sup>78</sup>)

### A.3.c. The Sertoli cells

Sertoli cells are the somatic component of the seminiferous epithelium. They present a morphological and secretive plasticity that create a specific microenvironment with nutrient supply and paracrine signals. This environment allows testicular germ cells (TGC) to survive, replicate and differentiate along the seminiferous epithelium<sup>49</sup>. Of note, the number of Sertoli cells is proportional to the sperm production capacity, as they only can engulf and nurture a fixed number of TGC per cell. Through the expression of diverse proteases (CTSL and CTSB among others) and protease inhibitors, Sertoli cells change structure during the spermatogenesis cycle. Extending their cytoplasm from the basal membrane to the lumen of the seminiferous tubules (ST), they make and break multiple highly specialized junctions with each other and TGC to form and deform the Blood-Testis-Barrier (BTB). This mechanism allows the transit of developing germ cells and the active release of mature spermatozoa into the tubule's lumen<sup>62,74</sup>.

Upon FSH and testosterone binding to their receptor, Sertoli cells produce cAMP that activates the synthesis of prostaglandins, transferrin, and GDNF among a hundred of genes<sup>49,74</sup>. Transferrin, besides its immunoregulatory role in semen, is a tight junction iron transporter, allowing salt's distribution to TGC for efficient meiosis, mitosis and maturation of spermatids.

Sertoli cells also synthesize and secrete retinoic acid (RA) which upregulates the Stem Cell Factor (SCF) and STRA8 expression while downregulating GDNF. This cascade promotes the Spermatogonia Stem Cells (SSC) exit of their self-renewal state into differentiation. GDNF expression is regulated by FSH, testosterone and RA, allowing the balance between proliferation and differentiation of SSC. Besides, SCF is also thought to play a paracrine role in Leydig cells for their proliferation and regulation of the steroidogenesis, adding to the complex constant spermatogenesis retro-control by somatic cells<sup>49</sup>.

Finally, Sertoli cells, under TAM receptor regulation, have a phagocytosis role towards spermatids residual bodies and apoptotic cells. They can subsequently transfer TGC antigens through their basal part to peritubular antigen-presenting cells (i.e. pTM and dendritic cells) of which they promote immunotolerance by conjointly secreting TGF $\beta$ ,IDO and activin A. Presenting Fas ligand (FasL) on their surface, they additionally induce apoptosis of CD8+ T cell upon binding to their FasR receptor<sup>16,74</sup>. Sertoli cells are therefore key actors of the testis immune privilege.

## A.4. The blood-testis barrier

The BTB is a physical barrier that assembles at the onset of meiosis to separate the TGC from the immune cells. Indeed, TGC are formed at puberty, after the immunological self-tolerance has been established. They thus express novel antigens that would result in autoimmune responses, cell destruction and infertility if detected by the immune system <sup>74,79</sup>. The BTB also protects the most differentiated TGC against pathogen infection and aggression coming from the blood circulation. While the BTB commonly refers to the Sertoli Cell Barrier (SCB), it can also include the semi-windowed Vascular and Lymphatic Endothelium (VLE). However, in contrast to the blood-brain-barrier, the testis VLE provide evidence of high degree of permeability <sup>80</sup>. The BTB can be defined as encompassing two components: a physical barrier that protects germ cells against acquired immunity, and a molecular barrier that filtrates chemical components:

### A.4.a. The physical barrier

The physical barrier describes the cellular and protein components allowing the formation of the barrier and defines the “fence function” of the BTB. It prevents leukocytes and antibodies to cross inside the seminiferous tubule <sup>79,81</sup>. It can be subdivided into the Sertoli Cell Barrier and the vascular and lymphatic endothelium. The Sertoli Cell Barrier is composed of a layer of Sertoli cells linked together by 4 types of junctions (**Figure 7**):

Tight junctions are mainly composed of claudins (3, 5, 11), Junction Adhesion Molecules (JAM-A & JAM-B) as well as Zonula occludens (ZO-1,2,3) scaffolding proteins that anchor claudins to the cell cytoskeleton <sup>79</sup>. In the SCB, they form a continuous anastomosis of fibrils surrounding the cells and sealing the paracellular space to create an impermeable barrier <sup>81</sup>.

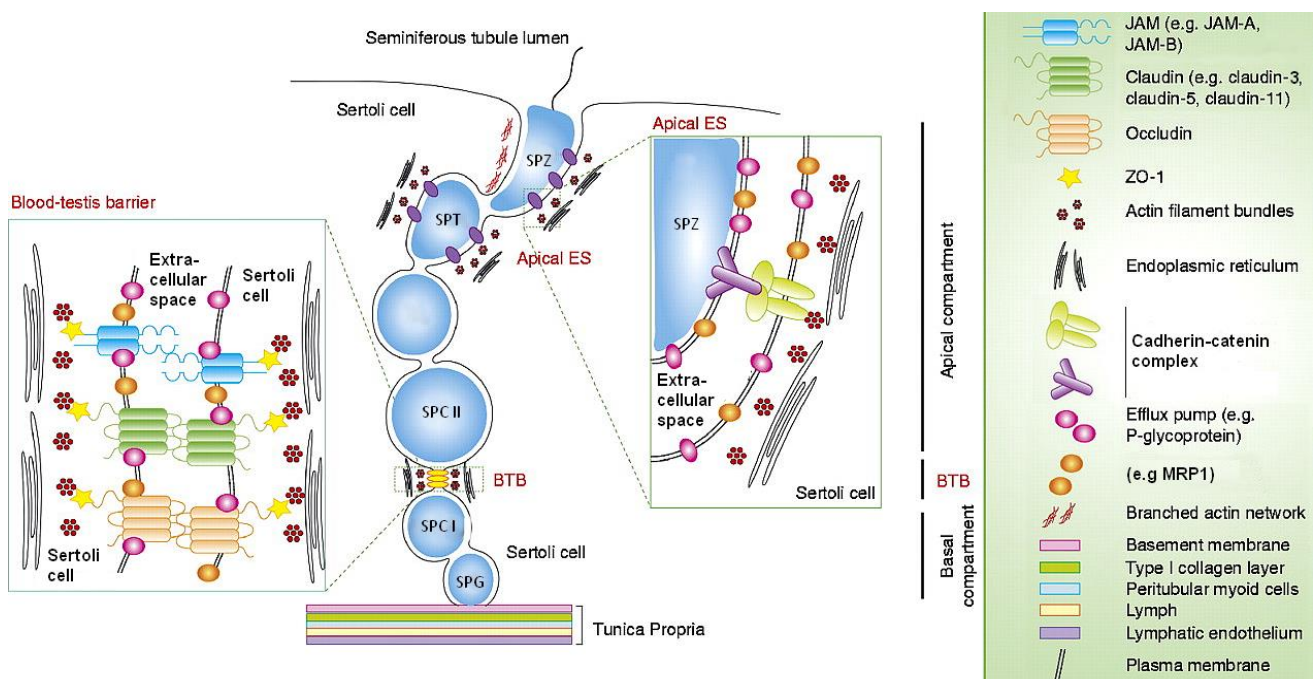
Desmosomes are cell-cell junctions mediating a robust adhesion of adjacent membranes.

Gap junctions are cell-cell channels allowing the diffusion of metabolites, second messengers, salts and small molecules.

Ectoplasmic Specialization (ES) are mainly composed of cadherin-catenin complexes and serves as adhesion junctions between Sertoli but also with spermatids at the apical part of Sertoli cells <sup>81,82</sup>.

Theoretically, only pathological conditions (direct infection causing Sertoli cells apoptosis or cytokine imbalance, fever causing heat stress, and inflammation) can disrupt the SCB, making meiotic TGC accessible to the elements of the blood compartment <sup>81,83</sup>.

In fact, the SCB separates the seminiferous epithelium in a basal compartment containing the non-protected spermatogonia and preleptotene SPC on one side; and an abluminal compartment containing the meiotic and post-meiotic TGC on the other. The TGC maturation implies their migration towards the lumen of the seminiferous tubule and thus the opening and closing of tight-junctions (TJ) to allow the passage of leptotene SPC. This phenomenon involves the transient creation of an intermediary compartment. This compartment results of a testosterone-driven TJ assembly behind the migrating preleptotene; coordinated with the TJ disassembly ahead of it <sup>81,84</sup>.



**Figure 7:** Illustration of the relative distribution of junction proteins and transporters.

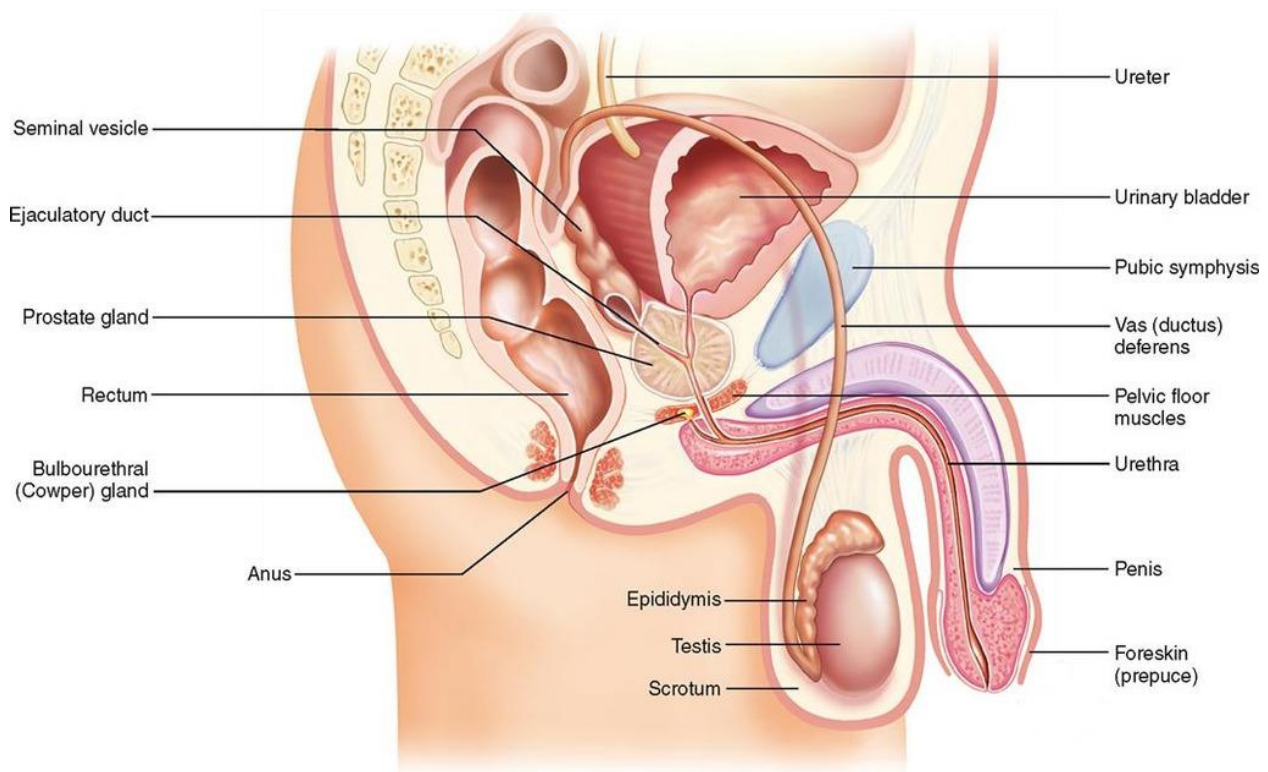
(Adapted from Su et al., 2011 <sup>82</sup>)

#### A.4.b. The molecular barrier

Sertoli cells express various transporters allowing the controlled passage of molecules across the SCB, responsible for the “gate function” of the BTB. This function enables the maintenance of the seminiferous epithelium homeostasis <sup>79,81</sup>. Among these transporters are the P-glycoprotein (P-gp), expressed in the human vascular endothelium, in peritubular myoid cells and in Sertoli cells <sup>85–87</sup>; as well as the Multidrug Resistance Protein 1 (MRC1) also expressed in human Leydig cells <sup>88</sup>. Both these proteins are efflux pumps actively promoting the outflow of substances off the seminiferous epithelium and off the testis parenchyma <sup>89</sup>. Also, the proto-oncogene tyrosine-protein kinase SRC (expressed in Sertoli cells) promotes clathrin-caveolin mediated endocytosis and transcytosis of cargo across the polarized SCB <sup>81</sup>. Although the primary role of these transporters is defending the seminiferous epithelium and TGC integrity, their detoxification function may also impair access to some antiviral drugs <sup>90</sup>. In addition to the selective permeability of the SCB, these transporters participate in making the immune-privileged testis also a pharmacological sanctuary, prone to viral persistence.

## B. The semen

Semen is composed of an acellular fraction, the seminal plasma, mostly produced by sex accessory glands; and a cellular fraction, mostly composed of spermatozoa coming from the testis. The main function of semen is to mediate fertilization during sexual reproduction <sup>91</sup>. During ejaculation, spermatozoa stored in the epididymis cauda upon their maturation in the caput, are mixed with epididymal secretions and travel through the vas deferens to the seminal vesicles. Seminal vesicles secretions are added to this mix, which further progresses through the ejaculatory ducts to the prostate. The prostate discharges motility-enhancing fluids allowing the progression of spermatozoa through the urethra, where the bulbourethral glands secrete the pre-ejaculatory fluid. Ejaculation of spermatozoa and other cells shed along the MGT occurs at the penis meatus <sup>91</sup> (**Figure 8**).



**Figure 8: The male genital tract**

Littre glands are scattered along the urethra. (Original creation by “generalpeach” from the Quizlet website).

## B.1. Cell composition, function and origin

The cellular fraction of semen is mainly composed of spermatozoa (above 39 million in normal ejaculates), which represent 85% of all cells<sup>92</sup>. Of the remaining 15% of Non-Sperm Cells (NSC), Smith et al.<sup>93</sup> reported that the majority (84%) were immature germinal elements, exfoliated from the testis as part of the natural spermatogenesis process<sup>93,94</sup>. Among them were anucleate bodies (59%) deriving from degenerated shed germ cells from which only the cytoplasm remains<sup>92,93</sup>. Spermatids at different stages of development were also found in semen (22.2%) as well as few spermatocytes (2.8%)<sup>93</sup> (**Figure 9, A**). In this study, leukocytes were the second most represented population (around 13%). They encompassed neutrophils (representing 12% of the total NSC and 50-60% of all leukocytes); monocytes and macrophages (0.9% of the total NSC / 20-30% of leukocytes) as well as CD4 and CD8 T lymphocytes (0.1% of NSC / 5% of leukocytes)<sup>93,95</sup>.

The primary function of neutrophils and macrophages in semen is believed to be the phagocytosis of the degenerated bodies of mis-formed spermatozoa and bacteria<sup>92,93</sup>. While the presence of some leukocytes in semen is common, a number exceeding 1 million/mL leukocyte usually indicates MGT infection or global inflammation and is called “leukocytospermia”<sup>96</sup>. The association between the

number of seminal leucocytes and semen quality is still debated. Leukocytes have been suspected to negatively impact sperm quality through the production of deleterious Reactive Oxygen Species (ROS). However, 11% of a fertile men cohort presented elevated numbers of leukocytes in semen<sup>97</sup> and leukocytospermia did not hinder assisted reproductive techniques<sup>98</sup>.

Male accessory gland infections (MAGI) impacting the seminal vesicles, prostates and bulbourethral gland are often associated with leukocytospermia. In post-vasectomy samples, the number of leukocytes in semen decreases by 85 to 90%, indicating that a majority of these cells originate from the testis or epididymis<sup>96</sup>. Several comparative histological analyses of leukocytes in semen and in various organs of the MGT were undertaken. They tend to agree on the epididymis and rete testis as the main origin of lymphocytes and macrophages in semen, while prostate is believed to mostly contribute granulocytes<sup>99</sup>. Some studies undertook a characterization of seminal leukocytes and reported that seminal CD4 lymphocytes harbored CD103 ( $\alpha 4\beta 7$ ), a marker expressed almost exclusively by T cells found in the epithelium and lamina propria of the mucosae, and which is present on T cells within penile urethra<sup>100,101</sup>. The expression of this marker on T lymphocytes present in the other MGT organs is unknown. In order to determine the tissue origin of HIV in semen, our team used single genome amplification to sequence and compare SIV strains retrieved from macaques' seminal plasma, infected seminal cells and MGT organs. Phylogenetic analysis revealed high genetic similarities between strains in semen and strains retrieved from seminal vesicles, epididymis and vas deferens, indicating that these organs contribute to virions and infected leukocytes in semen<sup>102</sup>.

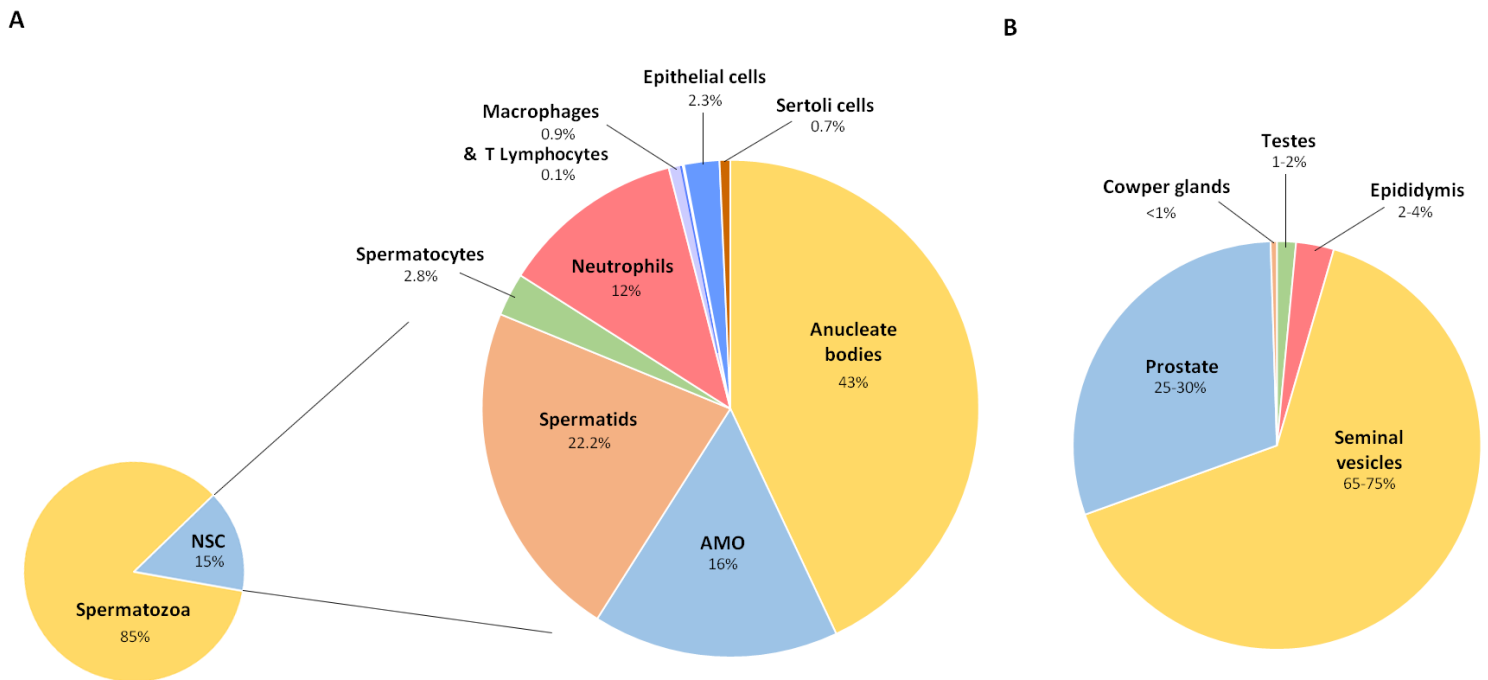
In the study from Smith et al. on 20 fertile men, epithelial cells represented 2.3% of all NSC in the semen<sup>93</sup>. Among them, comparative cytological staining allowed the characterization of transitional epithelial cells, originating from the bladder and urethra<sup>93</sup>, as well as squamous cells originating from the seminal vesicles, prostate and epididymis<sup>91,92,94</sup>. A refined molecular characterization of leukocytes and epithelial cells in semen and MGT organs, e.g. through scRNA-seq, is required to better determine their tissue origin.

Sertoli cells exfoliated from the testis appear a minor occurrence in semen (0.7%) and are thought to be shed from the terminal segment of the seminiferous tubules<sup>93</sup>.

The amount of each cell type in semen is interdependent and varies with MGT infection and inflammation, which can trigger epithelial cells desquamation, damage of the seminiferous tubules leading to the shedding of Sertoli and degenerating germ cells, in turn increasing the number of leukocytes recruited for "cleaning".



Overall, the origins of Non-Sperm Cells and especially infected NSC in semen is largely unknown and need further investigation. This characterization would help decipher the organs of the MGT implicated in the seminal excretion of some viruses and their role as viral reservoirs, besides the suspected immune-privileged testis.



**Figure 9: The semen composition**

**(A)** Composition of the cellular fraction of semen. AMO: Anucleate Mass with Organelles.

(Data from Fedder, 1996 and Smith et al., 1989<sup>92,93</sup>)

**(B)** Tissue origins of the secretions composing the seminal plasma. (Data from Rolland et al., 2013<sup>103</sup>)

## B.2. Seminal plasma origin, composition and function

The seminal fluid is mostly produced by the accessory glands, which emit fluids in a sequential manner. This fluid is composed of secretions from the seminal vesicles (around 70%), the prostate (25-30%), the testes (1-2%), the epididymis (2-4%), and the bulbourethral Cowper glands (<1%) **(Figure 9, B)**<sup>103</sup>.

The seminal fluid is particularly rich in molecules and proteins (over 2500 proteins have been described)<sup>103</sup>. These molecules support spermatozoa survival and transport, preserve their fertilizing abilities and modulate the immune responses, both in the male and in the female reproductive tracts

<sup>104</sup>. Of note, seminal proteins, hormones and cytokines prevents the rejection by the female immune system of foreign antigens through the inhibition of natural killer (NK) cells, anti-proliferative effects on lymphocytes and shift of T-cells towards immunotolerance <sup>91,104</sup>. However, while the immunotolerance induced is beneficial for sperm and embryo survival, it may facilitate the infection of the partner's genital tract by sexually transmitted pathogens. These infections are only partly controlled by the antimicrobial properties of certain molecules and proteins of the seminal fluid, such as defensins which are present in high levels and have mostly antibacterial activities <sup>16</sup>. As for viruses, a number of seminal molecules can influence their interaction with the genital mucosa of the partner <sup>16</sup>. For instance, seminal exosomes (prostasomes), which are physiologically involved in spermatozoa maturation and fertilization, were found to block the binding of ZIKV, DENV and WNV to their target cells. Besides, several glycoproteins (gp17, clusterin, mucin 6) have the ability to compete with viruses for their receptors <sup>16</sup>. In summary, the seminal fluid exerts a complex mix of inhibitory and enhancing effects onto the viral infection of the recipient's genital mucosa.

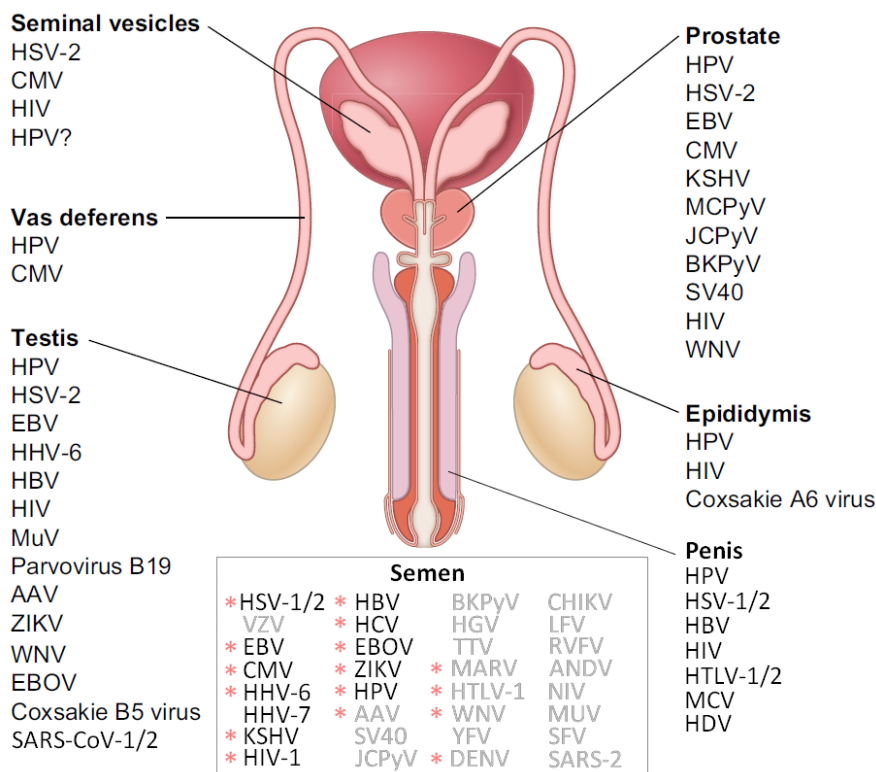
## C. Viral infections of the MGT and their consequences

### C.1. Public Health Importance

In 2021, over 1 million of Sexually Transmitted Infections (STI) were acquired everyday worldwide, representing 374 million new cases per year, of which the majority are asymptomatic. More than thirty different pathogens can be sexually transmitted, among which sixteen are viruses, and 4 are incurable (HIV, HSV, HBV, HPV)<sup>105</sup>. STI are spread via sexual contacts (vaginal, anal and oral) and have a direct impact on sexual and reproductive health, leading to infertility, cancers, and pregnancy complications. Some are also vertically transmitted and result in stillbirth or congenital abnormalities among others <sup>16,105</sup>. Condoms are the most effective methods of prevention, however, despite public health and sexual education campaigns, 1.5 million people still acquired HIV in 2020, most through unprotected intercourses <sup>106</sup>. Infertility affects 8 to 12% of couples <sup>107</sup> and male infertility is responsible for 20 to 50% of cases. Among them, infectious events are considered important contributors, representing 6 to 15% of all causes <sup>61</sup>.

## C.2. Overview of the viruses detected in the MGT

The first identification of viruses in human semen and male organs date from the late 1960's with the detection of Herpes Simplex Virus (HSV) and Cytomegalovirus (CMV) viruses in semen. Concerns about the sexual transmission of viruses increased with the beginning of the HIV pandemic in the 1980's. These viruses establish chronic infection in their host and their persistence in the MGT and semen is therefore not unexpected. However, the sexual transmission of Zika and Ebola viruses by cured men during recent outbreaks came as a surprise and revealed that the MGT also constitutes a reservoir for acute viruses, in principle cleared from the peripheral circulation. Evidences are mounting that a number of other emerging viruses may infect the human MGT and be shed in semen. Nowadays, 32 viruses have been detected in human semen (**Figure 10**)<sup>16</sup>. However, several are isolated case reports that need confirmation in greater numbers of patients and more rigorous identification. Importantly however, some of them were retrieved many days after systemic clearance (e.g. ANDV 278 days post symptoms onset<sup>108</sup>, RVFV 4 months post symptoms onset<sup>109</sup>) and have been implicated in sexual transmission cases (DENV<sup>110</sup>).



**Figure 10: Viruses detected in the human MGT and semen in vivo.**

(Adapted from Le Tortorec et al., 2020 and Teixeira et al., 2021<sup>16,111</sup>)

Viruses in black were detected in human biopsies or secretions of the internal genitalia. Viruses in grey were detected in case reports and their excretion in semen awaits confirmation in a greater number of individuals. Viruses bearing a (\*) mark have been at least once reported as being transmitted sexually.

### C.3. Viral dissemination into the MGT

In men, the penile mucosal epithelia (foreskin, meatus, urethra) are considered the main entry doors for sexually acquired viruses (e.g. HSV, HPV, HIV) which either infect epithelial cells of the mucosa or cross the penile epithelial barrier. They may lead to localized genital infections (e.g. HSV, HPV) or spread to the general circulation. Conversely, viruses responsible for systemic infection (e.g. ZIKV, EBOV), inducing a sufficiently high viremia (eg HIV and ZIKV) or infecting the vasculature (eg SARS-CoV-2), are believed to disseminate into the MGT via the hematogenous route. Entry in the testis interstitium for instance is thought to occur through the diffusion of virions and infected cells from the blood vessels. Moreover, many viruses reported in the human MGT are neurotropic and viral trafficking between the central nervous system and the testis has already been described for pseudorabies viruses<sup>112</sup>. Therefore, cross-seeding of these two immune-privileged organs could occur via nerves and participate in viral persistence<sup>16</sup>.

Some viruses are able to enter the seminiferous tubules (e.g. ZIKV), which might involve different mechanisms depending on the pathogen, such as the infection or transcytosis across the Sertoli cells constituting the BTB; the disruption of the Sertoli cell barrier by inflammatory factor; or the infection of unprotected SSC, which may carry the virus along the seminiferous epithelium during their maturation. Infection of the epididymis and downstream ducts and glands might occur via connected vasculature, as well as through the excurrent flow and migration of germ cells and macrophages during ejaculation<sup>16</sup>.

### C.4. Consequences of MGT infection

The infection of the MGT by viruses may affect the patients' reproductive function and health through various mechanisms. These affections can occur either via direct alteration of the tissue, or through the systemic or local reactions to the infection (e.g. inflammation, fever, oxidative stress). In addition to their impact on the infected individual, the infection of the MGT may also lead to dramatic consequences at the offspring and population levels (**Figure 11**).

#### **C.4.a. Alteration of the hormonal and sperm parameters**

Upon viral infection, sperm parameters (e.g. count, motility, morphology...) and semen parameters (e.g. volume, pH, viscosity of the seminal plasma...) can be altered to various degrees and results in azoospermia (absence of spermatozoa in semen), oligospermia (low sperm count per ejaculation), asthenospermia (number of motile spermatozoa under 32% of all sperms), teratospermia (number of morphologically normal spermatozoa under 4%). These alterations are often transient and may result from different mechanisms:

**Direct infection** of cells, altering their integrity and function. As an example, Mumps virus infects human Leydig cells *in vitro*, and inhibits their testosterone production, which could be involved in the impaired spermatogenesis leading to transient – and occasionally definitive - infertility in patients <sup>113</sup>.

**Local inflammation** of the testis (i.e. orchitis) upon infection is a well know feature of Mumps infection in men <sup>16</sup> and has been described in ZIKV-infected mice <sup>114</sup>. In the latter, it was characterized by the production of pro-inflammatory cytokines and chemokines (TNF $\alpha$ , IL6, CXCL10) from Leydig and Sertoli cells, leading to leukocytes infiltration, germ cell degeneration and overall testis atrophy. However, orchitis has not been described as a common occurrence in ZIKV-infected men, questioning the relevance of immunodeficient mouse models. In most COVID-19 patients, semen analyses revealed sperm parameters alterations (e.g. concentration, morphology, motility) along with high inflammatory cytokines (IL6, TNF $\alpha$ ) levels <sup>115</sup>. Besides, in mild to moderate patients, low levels of blood testosterone were detected while LH tended to increase <sup>116,117</sup>. These observations suggest that SARS-CoV-2 might trigger local inflammation in the testis leading to primary hypogonadism.

**Systemic reactions** to infection can also impact reproductive functions. Thus febrile episodes can cause elevation of the testis temperature above the 34°C needed for normal spermatogenesis <sup>118</sup>. This event was notably involved in spermatogenesis impairment upon influenza infection <sup>119</sup>. It is also believed to be associated with transient sperm alteration upon infection by various viruses, including SARS-CoV-2 infection <sup>120</sup>. Elevated systemic inflammation can also alter the hypothalamic-pituitary-gonadal axis centrally, dysregulating the secretion of LH and FSH and in turn testosterone and inhibin B secretions, leading to testicular and spermatogenesis impairment. It was notably observed in infections of the central nervous system provoking meningitis <sup>121</sup>, but also in systemic disease like the hemorrhagic fever with renal syndrome (HFRS) caused by the Puumala virus <sup>122</sup>. Of note, in COVID-19 severe patients, the hypothesis of secondary hypogonadism is privileged. Indeed, in multiple studies, low testosterone levels correlated with disease severity and were associated to

elevated markers of systemic inflammation (IL1 $\beta$ , IL6, IL4, TNF $\alpha$ )<sup>117,123,124</sup>. In severe patients, decreased LH levels were concomitant to decrease testosterone, rather suggesting an impact on the hypothalamic-pituitary-gonadal axis<sup>117,123,125</sup>.

Additionally, chronic infections like HBV also increase systemic levels of ROS which induce membranes and DNA damages to sperm cells<sup>126,127</sup>.

Besides effects on the testis, these systemic reactions also provoke epididymitis and prostatitis that can be deleterious for reproductive functions as they can alter semen parameters (of which they participate at 25-35% of the plasma) or causing irreversible ductal obstruction and fibrosis. These MGT affection account for up to 12% of infertility cases<sup>16</sup>.

#### **C.4.b. Cancers**

Viruses can initiate or favor carcinogenesis through complex interplay with the host genetics, immunity and inflammation and seven viruses are well recognized as having a transforming or tumorigenic activity in humans (e.g. EBV, KSHV, HPV, HBV). In particular, high-risk HPV (HPV-16, 18, 45 mainly) are established risk factors in squamous cell carcinoma of the penis. Testicular cancer is considered to primarily stems from developmental abnormalities during fetal life, with exposure to various factors during adolescence and adulthood potentially promoting its development. However, a significant link between testicular cancer and a history of pathogen-induced epididymo-orchitis was recently evidenced, reopening the question of the potential role of viral infection in the testis tumorigenesis<sup>16</sup>.

#### **C.4.c. Vertical transmission and endogenization**

Viruses shed in the semen - either as cell-free-virion, in infected cells or attached to cell surfaces - may contaminate the sexual partner but also the offspring, either indirectly via the mother infection during pregnancy or via direct infection of the embryo or fetus.

**Indirect transmission** through semen has been witnessed for HIV, HBV, ZIKV and CHIKV<sup>128</sup> for example. These viruses primarily infect the mother who, in case of high viral load, can infect her offspring via the hematogenous transplacental route. Infection would then either induce congenital defects at various time points during pregnancy (ZIKV) or induce predisposition for viral chronicity in the offspring (HIV, HBV). Findings in a mouse model suggested that the risk of in utero transmission of ZIKV is greater upon exposure of the mother to infected semen than upon mosquito bites<sup>129</sup>.

**Direct transmission** through sperm might occur in the case of viruses infecting or attached to the membrane of spermatozoa (HIV, HSV, HPV, HBV, ZIKV). These viruses could be transferred to the embryo upon fertilization, especially in the case of medically assisted fertilization that bypasses the zona pellucida physical barrier of the oocyte, as it was shown for HIV, HBV and HPV in a hamster model <sup>16</sup>. However, there are to date no direct evidence of sperm-mediated vertical transmission of these viruses in human.

In case of viruses that have managed to integrate their DNA in the genome of the gametes, the viral sequences may be transferred to the embryo genome and eventually become endogenous in the population, as occurred during our evolution for several retroviruses but also for other RNA viruses <sup>16</sup>. A recent study from our lab demonstrated that HIV has the ability to integrate its DNA into the genome of testicular germ cells in vitro, raising the possibility of endogenization in future generations <sup>130</sup>. Strikingly, non-retroviral DNA (HHV-6) and RNA (Filoviridae, Bornaviridae, Bunyaviridae, Alphavirus and Flaviviridae, Enterovirus) viruses' sequences were also discovered integrated in the genome of several vertebrates including humans, marsupials and bats. The mechanism of integration of such RNA viruses is not fully understood but is thought to proceed via the action of an endogenous reverse-transcriptase particularly active in germ cells. However, a sperm endonuclease cleaves foreign DNA and when robustly activated, degrades the host DNA to cell death, probably controlling these integration processes and explaining the inefficiency of HIV endogenization <sup>16</sup>.

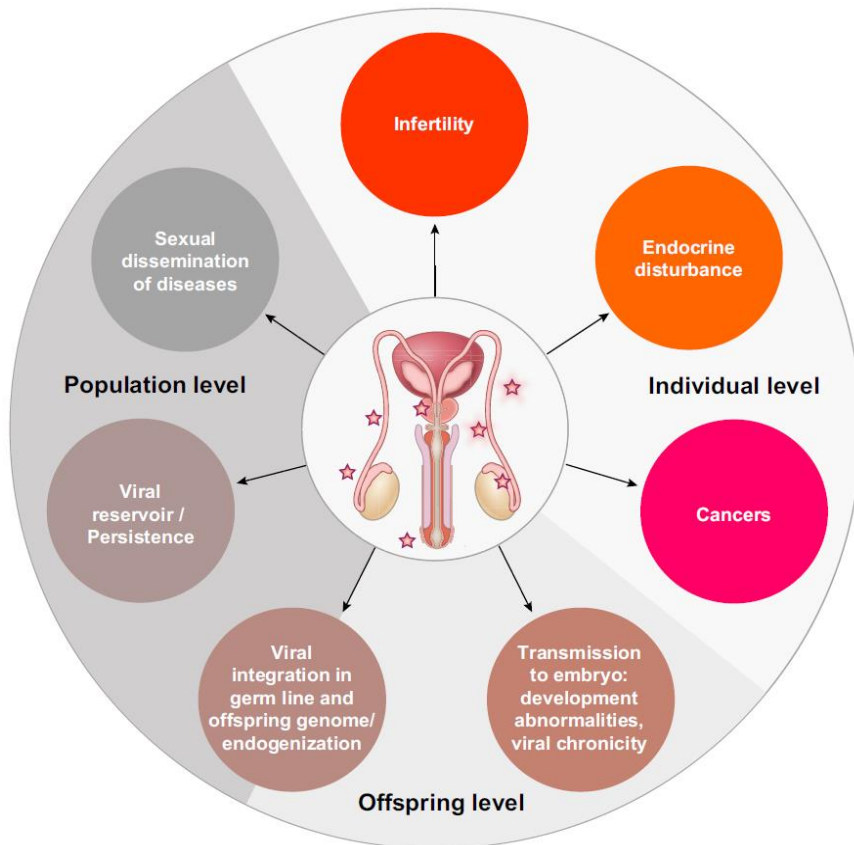
#### **C.4.d. Viral persistence in the MGT and sexual transmission**

Prolonged and intermittent seminal shedding of chronically infecting viruses (HIV, HSV, CMV) have been described since the 1980's. However, several case reports and cohorts' studies observed prolonged infection of the MGT by viruses that do not establish persistent infection in their host (e.g. EBOV, ZIKV, ANDV, RVFV etc.). Among them, emergent arboviruses and life-threatening viruses have been found excreted in semen for extended periods of time in the absence of viremia. Some of these viruses have been sexually transmitted by men who had recovered from the infection.

As with arboviruses, and apart from ZIKV which will be discussed later, case reports have described the seminal shedding of Yellow Fever (YFV), and Dengue (DENV) and Chikungunya (CHIKV), RNA up to 19, 37, 56 days post symptom onset (dps) respectively <sup>16,131</sup>. The Rift Valley Fever Virus (RVFV) RNA was retrieved 4 months after symptoms onset in the semen of an immunosuppressed patient, albeit his viremia was cleared after 2 months <sup>109</sup>. Two cases of sexually transmitted DENV were reported in 2019 <sup>110,132</sup> and WNV was suspected to be sexually transmitted from a man to his wife 30 dps in 2016

<sup>133</sup>. Importantly, life-threatening Filoviruses RNA were found to persist in semen from survivors up to 1178 days for Ebola virus (EBOV) <sup>111</sup> and up to 101 days for Marburg (MARV) virus <sup>134</sup>. Infectious particles were retrieved 61- and 81-days post recovery respectively. Sexual transmission of EBOV was proved until 470 days after recovery and MARV sexual transmission reported two months after recovery <sup>111</sup>. This prolonged viral excretion may impact the dynamic of EBOV re-emergence : phylogenetic analysis actually infer that the 2021 outbreak in Guinea resulted from the sexual transmission by a person infected during the 2014-2016 outbreak <sup>135</sup>. This hypothesis was already formulated regarding the 2014-2016 outbreak <sup>134</sup> demonstrating the strong impact of viral persistence in semen on emerging diseases. Nonetheless, as for ZIKV, Filoviruses persistence in the MGT is not lifelong and viral loads decline overtime to eventually disappear, within months in most cases. An association between seminal shedding of EBOV and eye inflammation was found, and the latter associated with higher viral load in the acute phase of the disease. These correlations suggest that high viral load at the onset of infection may allow the infection and persistence in the MGT. How is the infection eventually eradicated from the cellular and tissue reservoir is unknown, as are the sources responsible for the prolonged seminal shedding <sup>16</sup>. EBOV antigens were detected in the testicular interstitial and intratubular compartments with no histological lesions or inflammation during the autopsy of a human fatal case <sup>136</sup>. In acutely infected macaques, EBOV was localized in endothelial cells of the testis interstitium, epididymis, prostate and seminal vesicles, with no histological lesion. At later times, EBOV staining was associated with macrophages in the lumen of the epididymis and in intratubular cells resembling Sertoli <sup>137</sup>. In guinea pigs' models, EBOV infected epithelial cells, stromal macrophages and fibroblasts of the penis as well as the testicular interstitium <sup>16</sup>. EBOV notably persisted in the testes of mice engrafted with human immune cells <sup>138</sup> and was recently shown to productively infect mice Leydig and Sertoli cell lines (MLTC-1 and 15P-1). The latter was able to sustain infection until 28 dpi by balancing EBOV entry and replication (via regulation of the endocytosis and cellular replication mechanisms) with cell survival processes (via improved detoxification and non-standard metabolic pathways) <sup>139</sup>. Unlike EBOV, MARV infection induces testicular inflammation in infected individuals. In NHP models, MARV infection of the testis was shown to arise from blood vessels, primarily infect Leydig cells and peritubular cells then actively replicate in Sertoli cells where the virus persisted <sup>16</sup>.





**Figure 11:** Consequences of viral infections of the MGT <sup>16</sup>.

Overall, these findings indicate that the testis might be involved in the persistence of several viruses in the MGT. It is therefore crucial to understand the mechanisms that underlie the long-term infection of this organ. The ability of infectious agents to persist in a given organ is based on its ability to accommodate the host immune system recognition and responses. The immune privilege environment of the testis is therefore suspected to favor viral persistence. Beside the acquired immunity, the innate immunity represented by the cellular antiviral defenses is important to rapidly control infections. In the case of SARS-CoV-2, the antiviral state induced by type I interferons production in the cells is essential to the control of the infection *in vitro* and in patients <sup>140–142</sup>. Importantly, our laboratory has evidenced a poor innate immune response of the human testis to ZIKV *ex vivo*, unlike that of the mouse testis <sup>143</sup>. Similarly, in human primary Leydig cells, neither Mumps virus (MuV) infection nor a Poly(I:C) viral analog induced type I IFN production <sup>113,144</sup>, which again differed from mouse Leydig cells infected by MuV or Sendai virus <sup>145,146</sup>. Our team demonstrated that an over-expression of type I IFN in the testis induces germ cell apoptosis and sterility <sup>147</sup>. A dampened antiviral response in the human testis could therefore be required to

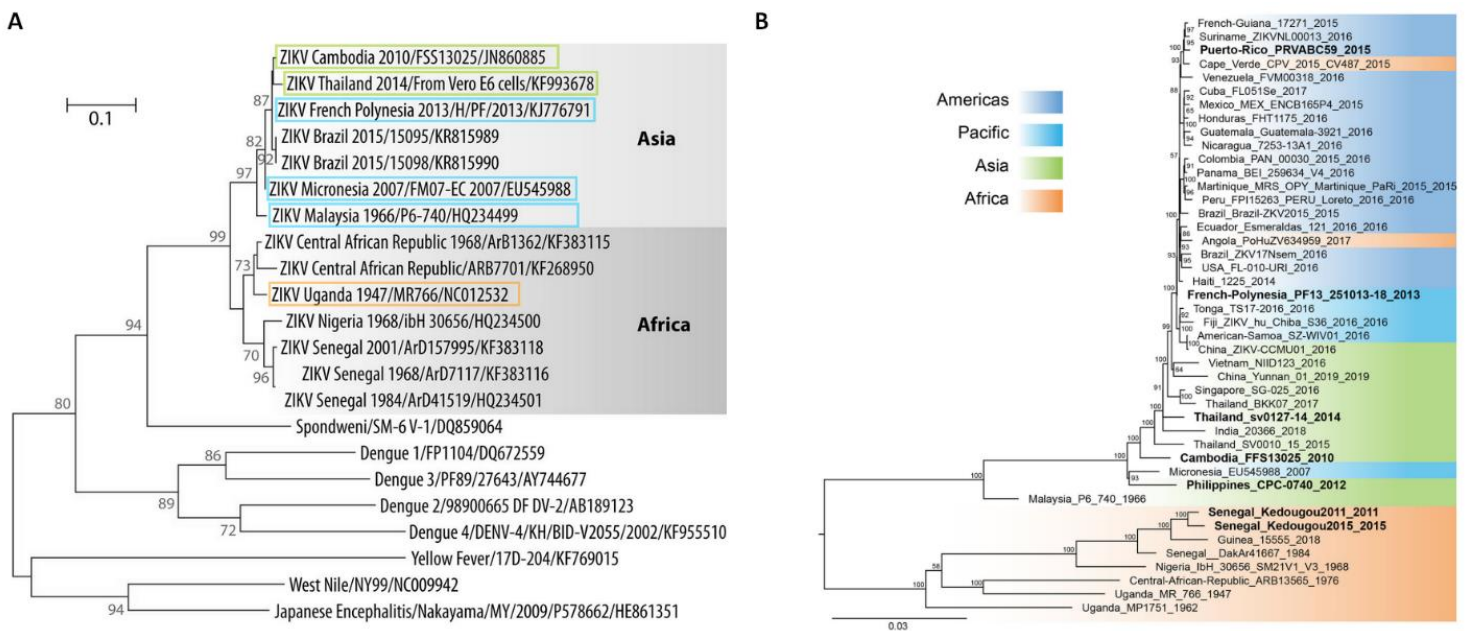
maintain the organ homeostasis. However, a poor innate response combined with an anergic acquired immunity may make the testis an ideal shelter for viruses. A better understanding of the role of the testis innate immune responses in the persistence of viruses is needed. In that respect, investigations are undergoing in the lab to decipher the cell and molecular pathways differentially activated by persistent (e.g. ZIKV) versus non-persistent (e.g. MuV) viruses.

For all these reasons, the human testis is suspected to be one of the main organs responsible for viral persistence in the MGT and prolonged seminal shedding. Nevertheless, the persistent shedding of ZIKV in semen of vasectomized patients suggests that distal MGT organs such as prostate, seminal vesicle or urethra constitute additional reservoirs<sup>16</sup>.

### III. Zika Virus

#### A. Taxonomy and emergence

Zika virus (ZIKV) belongs to the Amarillovirales order and the Flaviviridae family which contains the genres Hepacivirus (of which HCV is part), Pegivirus, Pestivirus and Flavivirus. ZIKV is part of the Flavivirus genus as are YFV, DENV, WNV and JEV, and can be classified in two main lineages that are geographically and temporally distinct: the African and Asian lineage (**Figure 12, A**). The Asian lineage can also be subdivided into three clusters: the historic Asian, the Pacific, and the American sub-lineages <sup>148,149</sup> (**Figure 12, B**).



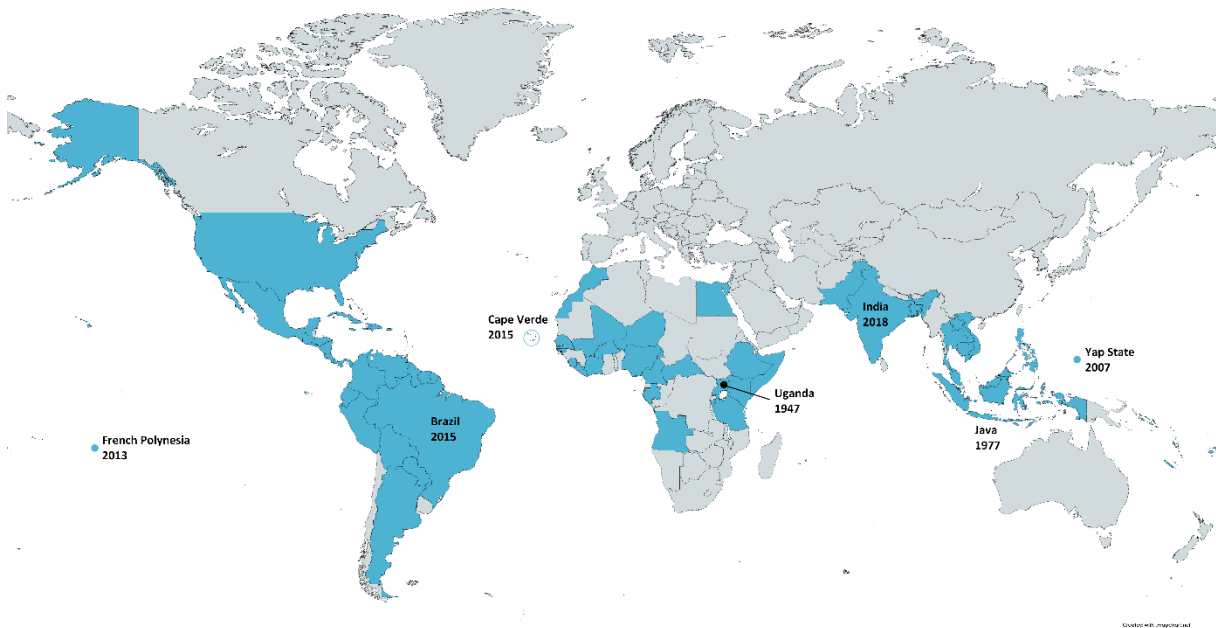
**Figure 12: Phylogenetic trees of the main ZIKV strains.**

**(A) African and Asian lineages main strains** in regard to clinically relevant Flaviviruses <sup>150</sup>.

**(B) African, Asian and sub-lineages main strains phylogenetic tree** <sup>151</sup>.

The colored background represents the geographic origin of strains. The backdrop ZIKV strains spanning the current viral genetic diversity are framed (A) or in bold (B).

ZIKV first discovered strain (MR766) was isolated in 1947 in a sentinel Rhesus monkey in Uganda, becoming the prototype of the African lineage. The virus was then isolated from humans for the first time in 1954 in Nigeria. Otherwise, the Asian strain prototype (P6-740) was isolated for the first time in *Aedes Aegypti* mosquitoes in 1966 in Malaysia while the first human case of the continent was reported in 1977 in Java, Indonesia. Since its discovery in Africa, only 14 cases of ZIKV infection were reported until the 2007 epidemic in Malaysia<sup>150,152</sup>. ZIKV then emerged on the Yap island State as an outbreak of “dengue-like illness” accompanied by unusual conjunctivitis. It contaminated 72.6% of the population whereas only 18.4% developed symptoms during the 3 months epidemic<sup>150</sup>. ZIKV then reemerged in 2013 in French Polynesia where the outbreak lasted approximately 5 months. It contaminated an estimated 50-60% of the population, 11.5% being symptomatic with the first cases of severe neurological complications. The virus then rapidly spread to many Pacific islands including New Caledonia (infecting 0.8% of the population in 7 months), Cook and Salomon Islands in 2015. The first cases in the Americas were reported in early 2015 in North Brazil<sup>150</sup> and spread to 33 countries<sup>153</sup>, contaminating over 750,000 people and reporting the first cases of congenital transmissions<sup>154</sup>. This led to the declaration by WHO of ZIKV being a Public Health Emergency of International Concern. In March 2017, WHO last report stated that a total of 61 countries had ongoing ZIKV transmission with an estimated higher bar of 1.5 million cases<sup>153</sup>. Of note, the virus reemerged in 2015 in Africa (Cape Verde) where infections were only sporadically described since its discovery, and several imported cases were reported in Europe between 2013 and 2015<sup>150</sup>. Since then, the number of cases declined but ZIKV is thought to be silently circulating in many areas as evidenced by an occasional outbreak in India in 2018<sup>155</sup>.



**Figure 13:** Temporal and geographical distribution of ZIKV (1947-2018).

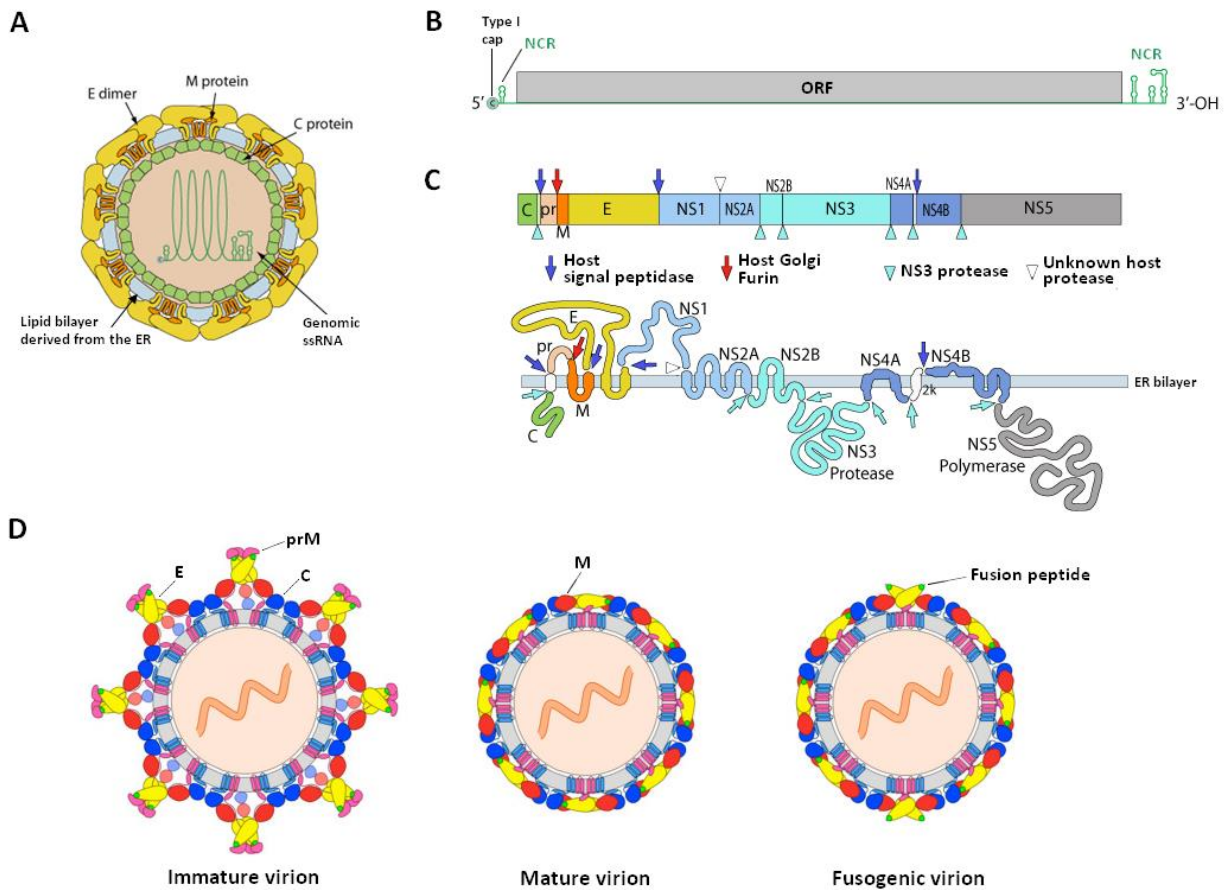
(Created with mapchart.net with data from the CDC and ECDC <sup>156,157</sup>)

## B. Molecular biology and evolution

### B.1. Structural and genomic organization

ZIKV is an icosahedral enveloped virus with a diameter of about 50 nm. The viral particle is composed of a capsid protein (C) protecting the viral genome to form the nucleocapsid. The latter is encased in a lipid bilayer membrane deriving from the endoplasmic reticulum (ER) in which are anchored the membrane (M) and envelop (E) proteins (**Figure 14, A**).

ZIKV genome is a linear non-segmented, single-strand positive RNA (ssRNA) of around 10.8 kilobases (kb). It presents a 5' type I methylated cap ( $m^7GpppAmG$ ) allowing the RNA protection from degradation and the initiation of replication <sup>158,159</sup>. This cap is followed on the genome by a non-coding region (NCR), a single open reading frame (ORF) coding for a single polyprotein, a 3' NCR and a 3' end with no poly-A tail (**Figure 14, B**). The two NCR are partly complementary and present several secondary and tertiary structures allowing the modulation of viral transcription and replication. They notably promote the genome circularization and binding of the replication complex. Also, they counteract host restrictions by serving as a substrate for exoribonuclease in the case of the 3' NCR



**Figure 14: Zika virus organization.**

ZIKV particle **(A)** and genomic **(B)** structures. **(C)** Illustration of the polyprotein arrangement in the ER bilayer and protease cleavage sites (Modified from Viralzone <sup>160</sup>, with data from Yun and Lee, 2017 <sup>149</sup>).

**(D)** Illustration of the different virion conformations (Modified from Wilken and Rimmelzwaan, 2020 <sup>161</sup>, with data from Hasan et al., 2018 <sup>162</sup>).

After translation, ZIKV polyprotein is cleaved, co- or post-translationally, by cellular and viral proteins into 10 major proteins: the 3 structural proteins C, M and E; and 7 non-structural proteins (NSP) essential for viral replication, maturation, assembly, and virulence <sup>149</sup> **(Figure 14, C)**.

The C protein is present in the particle as homodimers and displays two main domains: a RNA binding domain allowing the formation of the nucleocapsid (NC), and a hydrophobic domain allowing its binding to cell membranes and virion assembly <sup>162,163</sup>.

The M protein is translated as a prM precursor on the ER membrane, serving as a chaperone for E proteins assembly. It is later cleaved by Furin in the Trans Golgi Network (TGN) and released upon budding at the plasma membrane of the mature viral particle <sup>162</sup>.

The E protein is associated with prM and protrudes an elongated ectodomain presenting a hydrophobic fusion loop as well as region implicated in receptor binding and antibody neutralization <sup>149</sup>.

The nonstructural proteins roles are listed in **Table 2**.

Protein	Localisation	Role	References
NS1	RE lumen  Cytosol Secreted	ER membrane reorganization Modulation of viral replication Inhibition of TBK1 (IE) Inhibition of complement (IE)	164–166
NS2A	RE membrane	Modulation of viral replication Inhibition of RIG-I and MDA5 (IE)	167,168
NS2B	RE membrane	NS3 recruiter and cofactor Degradation of STING (IE)	169,170
NS3	Cytosol	Polyprotein protease RNA helicase and triphosphatase Modulation of viral replication Degradation of STING (IE)	167,169,171
NS4A	RE membrane  Cytosol	ER membrane reorganization (IE) Modulation of NS3 activity in the RC Sequestration of MAVS (IE)	172–174
NS4B	RE membrane	Modulation of NS3 activity in the RC	173
NS5	Cytosol  Nucleus	RNA-dependent RNA polymerase Methyltransferase and guanylyl transferase (IE) Broad inhibition of the type I IFN response (IE)	159,171,175,176

**Table 2:** Roles of ZIKV nonstructural proteins.

Functions implicated in the immune escaping are marked with a “(IE)”. RC is the Replication Complex.

As an immature virion, ZIKV presents Phosphatidyl Serines (PS) derived from the ER membrane and surface spikes composed of a trimer of prM-E heterodimers hiding E fusion peptide. Upon viral maturation in the TGN and budding, prM is cleaved and E proteins rearrange as antiparallel homodimers forming a smooth surface, thus hiding antigenic components and exposing the receptor-binding domain (RBD). After receptor binding and endocytosis of the viral particle into the host cell, the acidic environment of the endolysosome triggers the transformation of the virion into its fusogenic form: the E dimers dissociate to form trimers exposing the fusion peptide <sup>162</sup> (**Figure 14, D**).

Regarding the difference of pathogenicity observed between the two ZIKV lineages, several mutations on Asian ZIKV strains have been proposed to be responsible for the changes. Indeed, a 4 Amino Acid (AA) addition in the E proteins of the 2007 Micronesia and 2015 Martinique strains correspond to a glycosylation motif associated with Flaviviruses virulence <sup>177,178</sup>. A single mutation (S139N) in the prM protein of a 2013 strain, stably maintained in the Asian lineage, would have contributed to their increased neurovirulence and the incidence of microcephaly <sup>179</sup>. Additionally, a 2013 old fixed mutation (A188V) in the NS1 conferred the ability of inhibiting INF $\beta$  induction and thus evading the human host immune system <sup>166</sup>. Finally, several studies agree on a cell-specific attenuation of infection with Asian strains. This attenuation notably allows a long-term infection of immune and placental cells associated with enhanced neurotoxicity. While African strains tend to result in unnoticed early fetal loss, Asian strains thus led to congenital malformation of fetuses <sup>151,180-182</sup>.

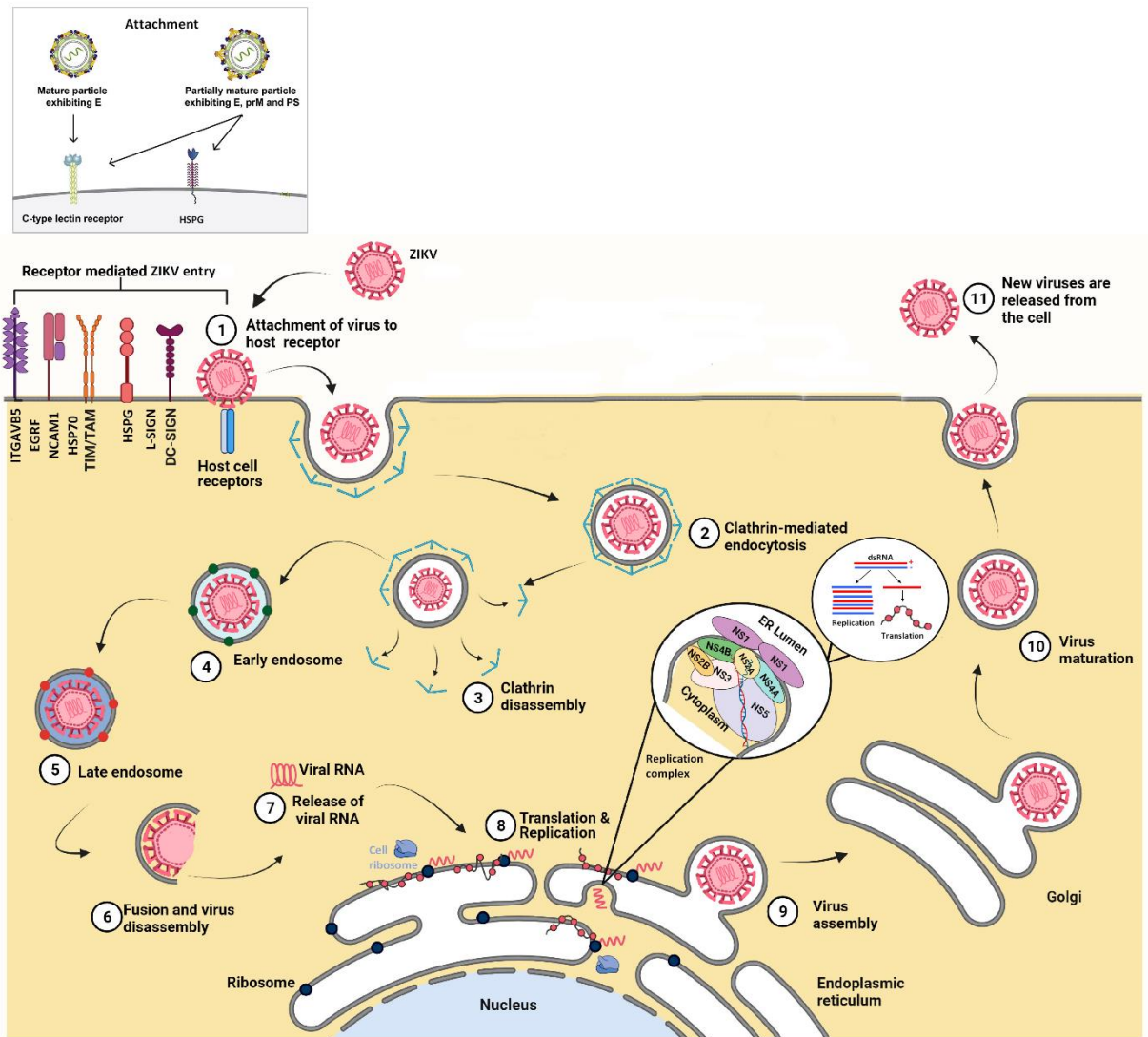
## B.2. Replication cycle

Zika virions first concentrate at the host cell membrane through attachment to non-specific attachment receptors. These receptors can bind ER-derived phosphatidyl-series on the viral particle, the E protein's glycosylation or, in the case of partially mature virions, prM <sup>162,183</sup>. They are then recognized by more specific entry receptors promoting their clathrin-mediated endocytosis into early endosomes <sup>183</sup> (**Figure 15**). As the endosome evolves into late endosome, the vesicular acidification triggers the irreversible trimerization of E proteins, resulting in the merging (fusion) of viral and endosomal cellular membranes <sup>149,162</sup>. The positive-sense genomic RNA (gRNA) is thus released in the cytoplasm and serves as a messenger RNA (mRNA). Host cell translation complex is then recruited at the RNA methylated cap and 5' NCR region to translate ZIKV polyprotein at the ER membrane <sup>149,184</sup>.



The polyprotein is then cleaved to produce nonstructural (NSP) and structural proteins which are embedded in the ER membrane where viral RNA replication takes place <sup>149,185</sup>.

The gRNA serves as a template for the NS5 RNA-dependent RNA polymerase (RdRp) to synthesize the complementary negative-sense replicative RNA (rRNA) strand. This forms a transitory double-stranded RNA (dsRNA) <sup>149,186</sup>. The two strands of the dsRNA will then be separated and dephosphorylated by the helicase and triphosphatase actions of NS3. The NS5 methyltransferase and guanylyl transferase activities will then synthesize new caps to the neosynthesized gRNA <sup>159,171</sup>. C proteins then bind gRNA to form nucleocapsids which interact with the RE lipidic membrane. The heterodimerization of prM and E proteins then triggers the budding inside the ER lumen of the neo-immature viral particles. The latter transit through the Trans-Golgi-Network (TGN) secretory pathway, where E proteins undergo a pH-driven positional reorganization and glycosylation. prM is then cleaved by furin for the particle maturation and mature virions are released by exocytosis at the cell surface <sup>149,162</sup>.



**Figure 15: ZIKV replication cycle.**

(Modified from Agrelli et al., 2019 and Nanaware et al., 2021<sup>183,187</sup>)

### B.3. Cellular receptors involved in viral entry

ZIKV infects an extensive set of cell types and studies have shown that its receptor usage can be cell as well as species-dependent and liable to the degree of the virion maturation<sup>183</sup>.

ZIKV viral entry is initiated by the binding and concentration of viral particles to attachment receptors. In that matter, Heparan Sulfate Proteoglycans (HSPG)<sup>188–190</sup> and C-type lectins (DC-SIGN, L-SIGN) were shown to bind to the N154 glycosylation of the E protein to mediate ZIKV attachment<sup>191,192</sup>.

**The TAM receptor family** (AXL, TYRO3, MERKT) are considered the main receptors involved in ZIKV entry of human skin cells <sup>191</sup>, endometrial cells <sup>193</sup>, endothelial cells <sup>194,195</sup>, neural cells <sup>196,197</sup> and Sertoli cells <sup>198,199</sup>. They were shown to bind the ER-derived phosphatidylserine on the virion membrane via Gas6 and Protein S adaptive molecules, and to trigger endocytosis in a process called “apoptotic mimicry” <sup>183,200</sup>. However, the infection of human neural progenitor cells (important for pathogenesis) <sup>201</sup>, Leydig cells <sup>198</sup> as well as several mouse organs <sup>202,203</sup>, including testis <sup>202,204</sup>, seems independent of the TAM family receptors. In that respect, TAM receptors were shown to rather promote infection via the modulation of the host cells' innate immune responses, by antagonizing type I IFN signaling <sup>196,205</sup>. Using the same “apoptotic mimicry”, ZIKV was shown to enter placental cells <sup>206</sup> via the binding of phosphatidylserine (PS) to the TIM-1 receptor <sup>200</sup> and to cooperate with AXL for the infection of skin cells <sup>191</sup>.

In regard of this cell receptor usage discrepancy, several other molecules have been proposed as alternative receptors. Among them, the **integrin (ITG)  $\alpha$ V $\beta$ 5** was identified by a CRISPR-Cas9 screening in human glioblastoma stem cells and was shown to trigger ZIKV infection in neural stem cells by binding to the virus PS. ITG  $\alpha$ V $\beta$ 5 blocking by neutralizing antibody (NAb) and molecular inhibitors notably inhibited ZIKV internalization and alleviated its neurotoxicity in a mouse model <sup>207</sup>.

The **Heat Shock Protein (HSP) 70**, also known as HSPA4, was shown as implicated in the infection by ZIKV of HEK293T, Huh7.5 and Vero cells. In these cells, the protein overexpression as well as inhibition by NAb, protein competitor and chemical inhibitor modulated the viral entry, replication and egress <sup>208</sup>. Particularly, apart from viral entry, HSP70 was shown to take part in the replication complex formation, as well as the capsid biogenesis and particle assembly in human neural stem cells and placental trophoblasts <sup>209</sup>.

While tracking for interacting cellular factors required for the early-stage entry of ZIKV, Srivastava et al. described the **Neural Cell Adhesion Molecule (NCAM1)** as a putative ZIKV receptor, which coimmunoprecipitates with the virus E protein. Its role in the virus entry was then confirmed via knockout (KO), competition and neutralization by NAb which inhibited ZIKV attachment and internalization in Vero and U-251 MG cell lines <sup>210</sup>.

The **Epidermal Growth Factor Receptor (EGFR)** was also suggested to participate in viral entry as A549 cells infection by ZIKV led to a rapid co-internalization of EGFR to the endosomes. In addition, EGFR-negative CHO cells are not susceptible to ZIKV but overexpression of EGFR in these cells was sufficient to allow ZIKV entry. Finally, the inhibition of EGFR internalization by Erlotinib impaired the virus internalization by endocytosis <sup>211</sup>.

Moreover, Flavivirus fusion loop is responsible for a broad antibody cross-reactivity and its antigenic feature contributes to viral infection in a process called “Antibody-Dependent Enhancement” (ADE) <sup>183</sup>. Well known in the case of the different DENV serotypes, ZIKV infection through ADE was also described in U937 and K562 human cells as well as in mice. In addition, antibodies (Ab) against DENV can cross-reacted with different ZIKV strains and allow their internalization in myeloid cells through the Fragment crystallizable region of Ab-receptor (FcγR) <sup>212–214</sup>.

## C. Transmission modes

As an arbovirus, the main mode for ZIKV transmission is vector-borne. However, the virus also uses alternative routes for human-to-human transmission. This includes vertical transmission, from mothers to their offspring, and sexual transmission <sup>153</sup> (**Figure 16**).

### C.1. Vector-borne transmission

Vector-borne transmission is characterized by the mammalian host infection from a female mosquito bite during blood feeding. Different species of *Aedes* mosquitoes are responsible for ZIKV transmission among humans although the genus *Aedes aegypti* and *Aedes albopictus* are the main vectors. Of note, their competence for transmission are geographic, demographic and ZIKV strain-dependent <sup>150,153</sup>.

### C.2. Vertical transmission

Perinatal transmission was first reported in 2013 in French Polynesia as ZIKV RNA was detected in both mothers and infants’ serum at delivery. Transplacental transmission was later confirmed during the Brazilian outbreak as viral RNA was detected in the amniotic fluids of infected pregnant women, placenta, and in microcephalic brains of products of miscarriages. A persistent maternal viraemia was uncovered as viral RNA was detected in pregnant women blood up to 107 days post symptoms

onset (dps)<sup>150,215</sup>. Additionally, ZIKV RNA and infectious particles were retrieved from breast milk up to 32 dps<sup>216</sup> but only one case report of a Venezuelan mother in 2016 could confirm breast feeding transmission<sup>217</sup>.

### C.3. Sexual transmission

Sexual transmission was first suspected in 2008 when a convalescent scientist infected in Senegal transmitted the virus to his wife after returning to USA<sup>218</sup>. In 2013, infectious ZIKV particles were isolated from the semen of a French Polynesian patient<sup>150,215,219</sup>. Several cases of sexual transmission have later been documented as people living in non-endemic areas became infected after sexual intercourse with partners coming from endemic areas<sup>219</sup>.

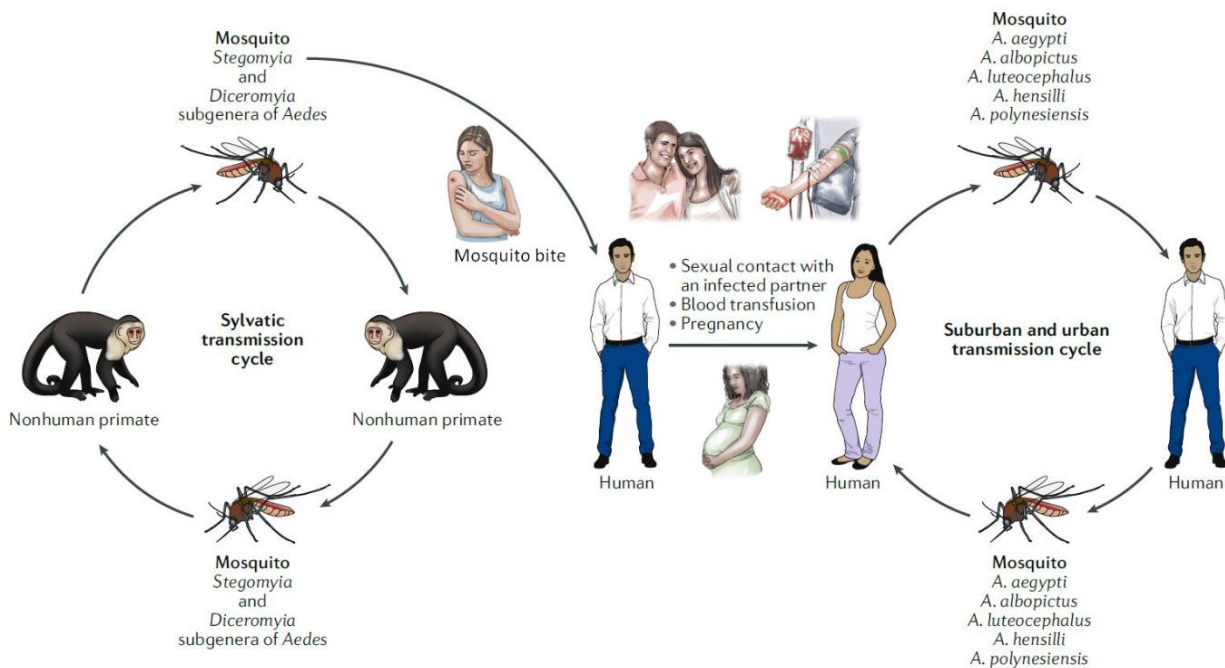
During the last outbreak (2015), viral dissemination through sexual transmission was reported in over 14 countries outside the vector endemic area<sup>220</sup>. Sexual transmission was described by genital, oral and anal intercourse, but mainly from male to female contact, up to 44 days post symptoms (dps)<sup>219</sup>. In non-human primates (NHP), sexual transmission was also reported after vaginal exposition of baboons to infected semen<sup>221</sup>. Importantly, viral dissemination in female genital tract and transmission to fetus was enhanced through semen in a mouse model<sup>129</sup>. Modeling estimates of the last Zika epidemic assessed sexual transmission contributed to a range of 9 to 46% of cases<sup>219,222</sup>.

In the female genital tract and secretions, ZIKV RNA was shown to persist up to 180 dps and up to 2 dps as infectious particles<sup>215,219</sup>. In men, high viral loads in semen and high percentage of men with virus in semen, either symptomatic or asymptomatic were found. In total, around 65% of studies having tried to isolate infectious ZIKV from semen reported positive results representing 20 to 73% of the men tested<sup>223</sup>. Prolonged shedding of viral RNA (vRNA) and infectious virus (up to 414 days and 69 days, respectively) was observed in the absence of viremia<sup>219,224</sup> indicating ZIKV tropism and persistence in the male genital tract. Worryingly, the persistence of virus in semen as well as sexual transmission occurred even in asymptomatic patients<sup>225</sup>.

## C.4. Auxiliary transmission

Transmission via blood-transfusion was first feared when in French Polynesia, ZIKV RNA was detected in 2.8% of asymptomatic blood donors tested in 2014. Lasting viremia was later confirmed in 2016 in 1.1% of a blood donor cohort in Puerto Rico and in 2017 when ZIKV RNA was detected from asymptomatic donors in the USA <sup>150,215</sup>. Indeed, ZIKV RNA was measured up to 49 dps in patients' blood <sup>226</sup> and the first transfusion-transmitted infections was later confirmed in late 2015 in Brazil. However, ZIKV transmission has never been reported via organ transplantation <sup>150,215</sup>.

Additionally, ZIKV RNA and infectious particles have been detected in saliva up to 91 and 6 dps respectively. RNA was also retrieved up to 30 and 25 dps in urine <sup>227,228</sup> and sweat respectively <sup>229</sup>. However, the risk posed by these fluids with regard of Zika transmission are not defined.



**Figure 16: Transmission cycle of ZIKV.**

(Adapted from Kurscheidt et., 2019 with sketches from the CDC <sup>223,230</sup>)

## D. Physiopathology of the infection

### D.1. Generalities

After inoculation by the vector saliva into the host skin, the initial round of viral replication is thought to take place in fibroblasts, keratinocytes, Langerhans DC cells and microvascular endothelial cells for an incubation period of 4/5 days. The virus then migrates via infected cells to regional lymph nodes and systemically spreads through the blood and lymphatic circulation <sup>150,183,231</sup>. ZIKV has been detected in eyes, brain, uterus, placenta, as well as in vagina, testis and several body fluids including tears, saliva, cervical mucus and urine in addition to semen <sup>153,183</sup>. Because of 80% of asymptomatic infections and an overall non-specific pathogenesis, ZIKV diseases are often not detected or misdiagnosed and descriptions are thus scarce. However, a French Polynesian retrospective survey unveiled differences in symptoms based on the ZIKV strain. They also showed a 50% versus 30% ratio of symptomatic infections in adults versus children <sup>215</sup>.

### D.2. Complications in adults and newborns

ZIKV being barely neuro-invasive in adults, severe symptoms were never reported in patients in Africa. Similarly, during Micronesia's outbreak only common infection symptoms were reported like fever, rash, arthritis and arthralgia, myalgia, conjunctivitis and fatigue; probably related to the limited number of cases in this small island. However, the French Polynesian outbreak led to the first description of severe neurological complications with an unexpectedly high number of Guillain-Barré Syndrome (GBS) cases presenting a 20-fold higher incidence than usual <sup>150</sup>. These unexpected symptoms were later confirmed by a 10-fold higher incidence of GBS in Venezuela <sup>215</sup>. GBS is an autoimmune disease associated with aberrant inflammation and autoimmune response from antibodies raised against ZIKV and targeting peripheral nerves glycolipids. This leads to demyelinating polyneuropathy, muscle weakness and paralysis <sup>153</sup>. As of March 2017, 23 countries had reported increased incidence of GBS <sup>215</sup>, leading to 16 cases of fatal outcomes <sup>232</sup>.

Neonatal complications of ZIKV infections were first described during the Americas outbreak in 2015-2016. ZIKV was shown to particularly infects neural progenitor cells and thus impairs the development of the fetal brain via cell death and neurogenesis dysregulation. In a Rio de Janeiro cohort in Brazil, 46% of infected pregnant women delivered fetuses with congenital neural abnormalities, regardless of the pregnancy trimester <sup>153,215</sup>. On the contrary, the US Zika Pregnancy

Registry reported 6% of brain abnormalities in fetuses delivered from infected mothers, all of which infections occurred during the first trimester of pregnancy<sup>232</sup>. While microcephaly was observed in an average 20% of cases<sup>215</sup>, a 26-fold higher incidence in a 3 months period (*October 2015 – January 2016*) in Brazil, not mentioning fetal demises, led the WHO to declare ZIKV a global health emergency in February 2016<sup>150</sup>. As of March 2017, the WHO registered more than 2600 congenital syndromes associated with ZIKV infections in 31 countries<sup>215</sup>.

### D.3. Zika virus in the Male Genital Tract

ZIKV infection in men was associated to decreased sperm count and increased sperm abnormalities up to 90 dps, with fully restored semen parameters after 4 months<sup>224,233</sup>. Relatively mild endocrine perturbations (lower inhibin B concentration) were observed in the early days post-symptoms onset (dps), without any significant modification of testosterone levels<sup>233</sup>. Infectious virus was found associated to spermatozoa, along with viral antigen detected in one patient 56 days after symptom onset<sup>233,234</sup>. The impact of the infection reported on sperm parameters, along with the association of ZIKV with ejaculated spermatozoa thus suggested the infection of the testis and/or epididymis.

Using an *ex-vivo* model of infection and organotypic culture of testicular explants, our team revealed that African and Asian lineage ZIKV efficiently replicates in human explants. Replication occurs in a wide range of testicular somatic cells including resident macrophages, peritubular cells and to a lesser extent Leydig cells and Sertoli cells. ZIKV also infected immature and mature germ cells which were additionally found exfoliated *in vivo* in the semen of ZIKV-positive men<sup>143</sup>. In our model, the general testis structure and function was not impacted. The overall cell viability, ZO-1 expression in Sertoli cells, as well as basal testosterone and inhibin B levels were unchanged by the infection<sup>143</sup>.

ZIKV, upon infection, activates innate and adaptive immune responses through the sensing of its gRNA and rRNA by Toll Like and RIG-I Like Receptors (TLR and RLR) (principally being TLR3 &7, RIG-I, MDA5). This sensing notably leads to the production of type I and type III interferon (IFN) and inflammatory cytokines (CCL5, CXCL10). These IFN activate the JAK/STAT pathway leading to the production of antiviral Interferon Stimulated Genes (ISG: ISG15, OAS2, MX1, IFIT...). In some cells, ZIKV is able to impair the IFN signaling by preventing STAT1 phosphorylation and promoting JAK1 and STAT2 degradation via the action of NSP2/NSP3/NSP5<sup>150,153</sup>.

*Ex vivo* infection of the human testis tissue did not trigger IFN induction, which is needed to sustain an efficient antiviral defense, and induced only minimal pro-inflammatory cytokine stimulation<sup>143</sup>.



While IFN production and signaling can be counteracted by the virus NSP, the lack of IFN upregulation following viral exposure could be a feature of the human testis that is not specific to ZIKV. Indeed, relatively weak endogenous antiviral abilities are observed in human testicular cells <sup>144</sup> (Kuassivi et al. in revision). Altogether, the survival of infected cells and weak innate immune response may represent favorable conditions for ZIKV persistence in the human testis.

Several mice models were used to study ZIKV infection of the testis. In immune-compromised mice, lacking type I IFN receptor, ZIKV infection severely affected reproductive organs which presented large inflammatory infiltrates, including the testis. A broad range of testicular cells was infected and high levels of ZIKV was detected up to 42 days post infection (dpi) in both testis and epididymis. Consequently, testicular atrophy, collapsed hormone levels, breakdown of the BTB, germ cell degeneration, and infertility were observed <sup>16,114,204,223</sup>. In immuno-competent mice, that are normally resistant to the virus, ZIKV infection was still observed in testicular germ cells, and persisted in the testis for up to 60 days <sup>235</sup>.

In non-human primate (NHP) models, the presence of ZIKV RNA in the testis was demonstrated up to 28 days after infection <sup>236</sup> and up to 21 dpi in semen <sup>223</sup>. However, ZIKV infections in NHP generally either spared the testis or led to moderate infection with no deleterious effects <sup>16,223</sup>.

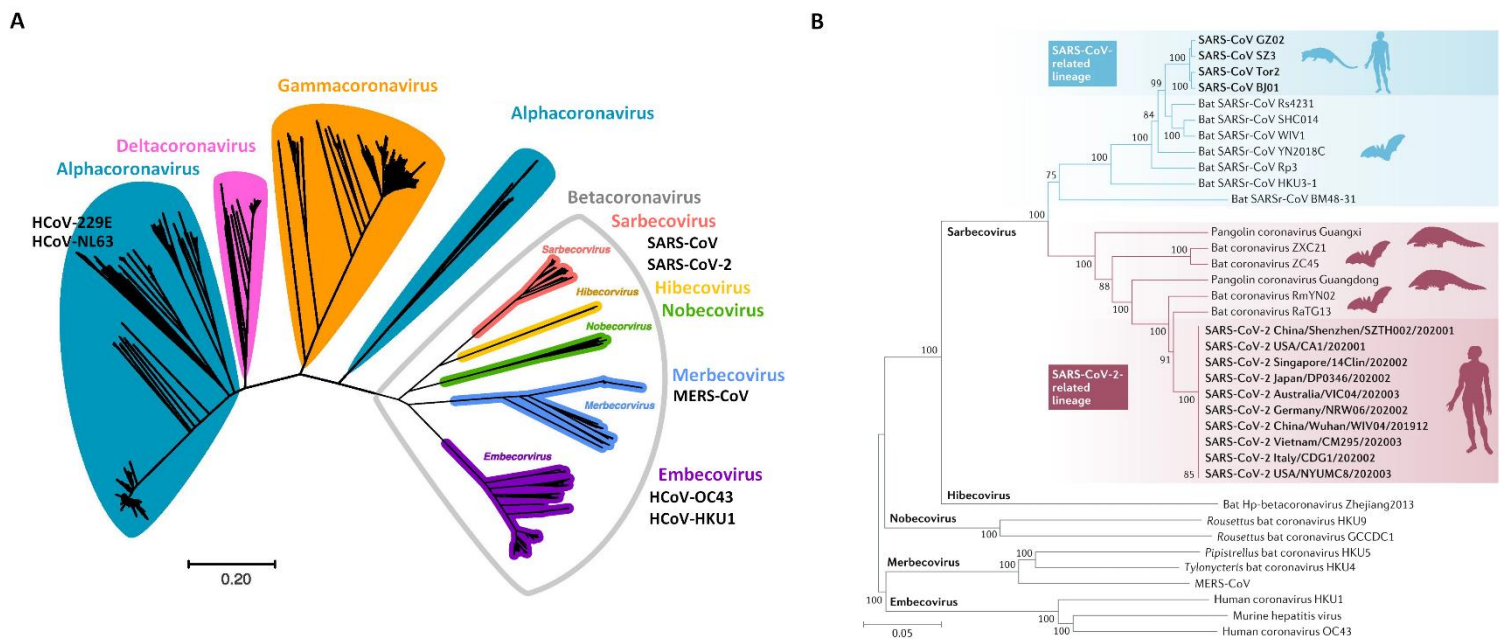
These observations suggest that the testis might be a reservoir for ZIKV and a source of viral persistence in semen. However, prolonged viral shedding (up to 281 dps) in the semen of a vasectomized men suggests that the prostate and seminal vesicle are probably also involved in the virus persistence <sup>16,237</sup>. Studies on immunocompromised mice described extended infection of the epididymis, considered as the main source of viral shedding in semen in this model <sup>238</sup>. In NHP model, acute and chronic prostatitis were reported as well as infection of the prostate and seminal vesicle, where scarcely infected cells were observed <sup>236,238</sup>. ZIKV was notably detected in the prostate and seminal vesicle of macaques up to 60 dpi <sup>239</sup>. *In vitro*, ZIKV tropism for human prostate was also suggested as human prostatic stromal 19I and epithelial LNCaP cell lines were infected in reconstituted organoids <sup>223</sup>.

Overall, the persistence of ZIKV in semen despite viral clearance in blood support the existence of a ZIKV reservoir within the MGT. However, evidence suggest that ZIKV might persist in several organs of the tract, which all could be involved in the virus long-term shedding. The characterization of the infected cells in semen would therefore help determining the exact source(s).

## IV. SARS-CoV-2 Virus

### A. Taxonomy and emergence

The Severe Acute Respiratory Syndrome-related Coronavirus “SARS-CoV-2” (SARS-CoV-2) belongs to the Nidovirales order, the Coronaviridae family and Orthocoronavirinae subfamily. The latter consists of 4 genera: Alpha, Beta, Gamma and Deltacoronavirus. SARS-CoV-2 belongs to the Betacoronavirus genus which contains 5 lineages: Embecovirus (previously Lineage A), Sarbecovirus (Lineage B), Merbecovirus (Lineage C), Nobecovirus (Lineage D), Hibecovirus (**Figure 17**). SARS-CoV-2 belongs to the Sarbecovirus lineage - as does the first SARS-CoV (SARS-1) -, and is the causative agent of the actual CoronaVirus Induced Disease (COVID-19) pandemic <sup>240,241</sup>.



**Figure 17: Phylogenetic trees of the main Orthocoronaviridae strains.**

**(A)** The Betacoronavirus genus in regard to all Orthocoronaviridae genera.

(Adapted from Wu and Zhao, 2020 <sup>242</sup>)

Among the hundreds of Orthocoronaviridae, only 9 were ever documented as infecting humans: the most common HCoV-229E, OC43, NL63 and HKU1 are seasonal viruses causing common colds and the SARS-CoV-1, MERS-CoV and SARS-CoV-2 are highly pathogenic <sup>243,244</sup>.

**(B)** SARS-CoV, SARS-CoV-2 and SARS-related main strains in regard to other Betacoronaviruses lineages

<sup>245</sup>. SARS-CoV-2 exhibits 79.6% whole genome sequence identity with SARS-1 and 50% with MERS-CoV.

In late December 2019, several health facilities in Wuhan, China reported clusters of patients with pneumonia of unknown cause. They presented symptoms of fever, cough, chest discomfort as well as dyspnea and bilateral lung infiltration in severe cases. Bronchoalveolar lavage fluid sampling and sequencing allowed the identification and characterization of the viral cause presented as a Betacoronavirus on January 9<sup>th</sup> 2020. While most of the first cases had an exposure history with the Huanan Seafood Wholesale Market of Wuhan, familial and nosocomial clusters of infection started to develop, unveiling the human-to-human transmission potential of the “nCoV 2019”. By the end of January, facilitated by the Chinese New Year movement of population, the virus had spread to all 34 provinces of China and occasioned sporadic international cases. This led to the declaration by WHO of a public health emergency of international concern on January 30<sup>th</sup>. The better characterized virus was then renamed SARS-CoV-2 and the COVID-19 disease was declared a global pandemic on March 11<sup>th</sup> 2020, as more than 200 countries on all continents were affected <sup>245</sup> and more than 35.000 cases and 1000 deaths were reported outside China <sup>246</sup>). As of April 13<sup>th</sup> 2022, more than 499 million cumulative cases and 6 million deaths were reported globally <sup>247</sup>.

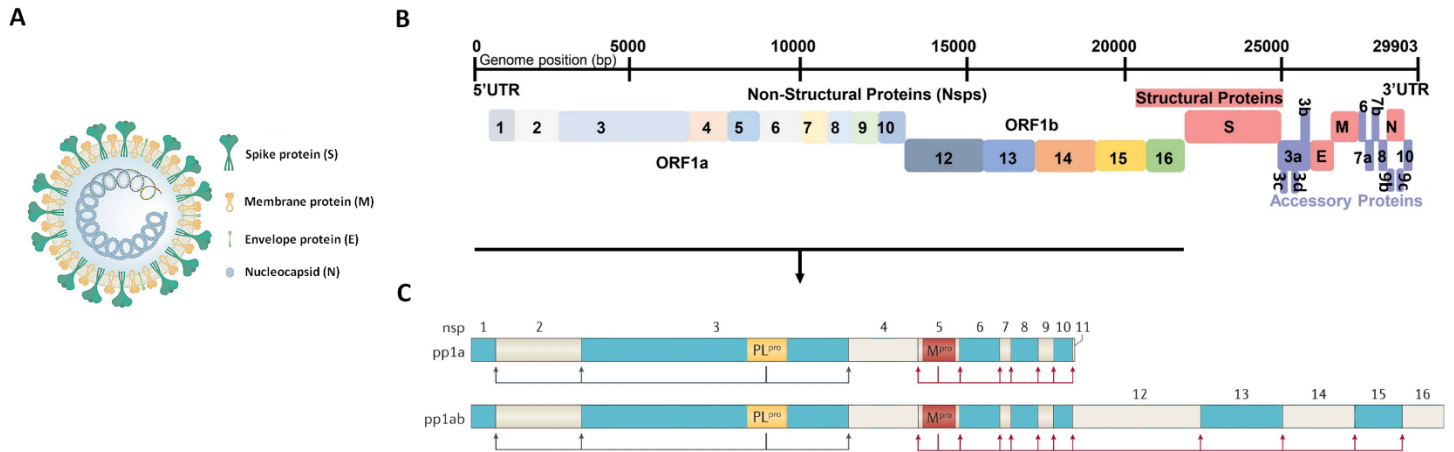
## B. Molecular biology and evolution

### B.1. Structural and genomic organization

SARS-CoV-2 is a spherical enveloped virus with a diameter of about 120 nm. The viral particle is composed of a nucleocapsid (N) protecting the viral genome to form the ribonucleoprotein. The latter is encased in a lipid bilayer membrane deriving from the Endoplasmic Reticulum (ER) in which are anchored the membrane (M), envelop (E) and spike (S) proteins <sup>(160,244)</sup> (**Figure 18, A**).

SARS-CoV-2 genome is a linear non-segmented, positive single-strand RNA of about 30 kb representing the longest known viral RNA genome <sup>241</sup>. It presents a 5' type I methylated cap, two 5' and 3' NTR containing secondary structures essential for RNA synthesis and a 3' polyadenylated tail. Two large ORF1a and ORF1b occupy 2/3<sup>rd</sup> of the genome and code for 16 NSP composing the Replication and Transcription Complex (RTC). In the last 3' third of the genome, 4 ORF code for the structural proteins and 8 interspersed ORF code for 11 accessory proteins involved in the modulation of the host immunity and the determination of pathogenicity <sup>244,248</sup> (**Figure 18, B**). Overall 31 proteins are encoded, involved in viral entry, uncoating, replication, transcription, assembly, release, immune escaping, etc... either individually, as cofactors or in complexes with host proteins.

After translation of the ORF1a and 1b upon entry, the polyproteins pp1a and pp1ab is cleaved by the cis-acting Papain-Like PLpro (NSP3) and Main (NSP5) proteases into the 16 NSP (1-16) constituting the RTC and which different roles are listed in the **Table 3**. It is only upon replication by the NSP12 that the 3' third of the genome is transcribed into subgenomic mRNAs coding for the structural and accessory (ORF3a, 3b, 3c, 3d, 6, 7a, 7b, 8, 9b, 9c and 10) proteins (**Figure 18, C**).



**Figure 18: SARS-CoV-2 virus organization.**

SARS-CoV-2 particle (from Enzo Life Sciences <sup>249</sup>) **(A)** and genomic <sup>250</sup> structures **(B)**.

**(C)** Illustration of the pp1a and pp1ab polyproteins organization and cleavage sites (Modified from V'kovski et al., 2021 with data from Jungreis et al., 2021 <sup>244,248</sup>)

The N protein is one the most abundant structural proteins found in infected cells. In parallel with the replication, it binds the neo-synthesized viral RNA (vRNA) to form what is called the ribonucleoprotein complex (RNC) which will later be enveloped during viral assembly. It also interferes with the IFN pathway, thus presenting a double-protective action against the host innate immune responses.

The M protein is the most abundant of all coronavirus proteins and the main component of the viral envelope. It maintains the shape of the particle and interacts via its C-terminal domain (CTD) with the N protein to promote viral packaging. Besides, M is also involved in the viral proteins processing and trafficking as well as in the immune escape, serving as an IFN antagonist.

The E protein, besides assisting viral assembly and budding, presents a transmembrane domain (TMD) structured as an ion channel and a CTD recognizing, binding and disrupting the human lung cell junction protein PALS1. This combined action causes leakage in the epithelial barrier and ultimately contributes to early viral dissemination <sup>250</sup>.

The S protein, organized as a protruding homotrimer on the viral envelope, recognizes the host receptor to mediate viral entry and is the main target of NAb. Upon maturation of the viral particle, S is cleaved by host proteases to separate its 2 subunits: the N-terminal receptor-binding fragment S1 and the C-terminal fusion fragment S2. Although, the 2 subunits remain bound by non-covalent interactions until viral entry. The Receptor Binding Domain (RBD) is composed of variable extended loops containing specific Receptor Binding Motif (RBM) responsible for receptor specificity. They are located in the CTD of the S1 fragments which are exposed on the apex of the spike trimer (Figure 21). The SARS-CoV-2 trimer constitutively lays on a closed/down “pre-fusion conformation” with the RBD nested inside the protein, as a strategy of immune evasion. However, some loops extend outwards of the structure as a result of the S1/S2 priming cleavage. The interaction of these loops with the cellular receptor switches the S protein conformation “up” for an efficient fusion with the cellular membrane <sup>241,250,251</sup>.

Accessory proteins result from the subgenomic RNA translation and ribosome leaky scanning. They have genus and species specificity among the coronaviruses which do not have the same numbers of proteins. They play essential roles in the regulation of the host innate immunity, apoptosis as well as viral proliferation and virulence <sup>250</sup>. The molecular function of several accessory proteins still needs characterization but the few recognized roles are listed in **Table 3**.

Protein	Localisation // ID with SARS-1	Role
<b>NSP1</b>	Cytosol	Bridge connection between host 40S ribosome and SARS-CoV-2 mRNA (translation hijacking) Promotes cleavage/degradation of host mRNA including type I IFN (IE)
<b>NSP2</b>	Endosome	Participates in viral replication, host immune regulation, mitochondrial biogenesis, endosomal transport but precise role is unknown
<b>NSP3 (PLpro)</b>	Transmembrane	Cysteine protease cleaving pp1a polyprotein to generate NSP1/2/3 Inhibition of the IFN and NFkB pathways (IE) Desubquitation of viral ssRNA (IE) Creation of DMV pores for vRNA transfer to the cytosol
<b>NSP4</b>	Transmembrane	ER membrane reorganization (IE)
<b>NSP5 (Mpro)</b>	Cytosol // Highly conserved	Cysteine protease cleaving pp1ab to generate NSP4-16 Inhibition of IFN signaling
<b>NSP6</b>	Transmembrane	Inhibition of hybrid pre-autophagosomal structures (IE) Sequestration of MAVS, TBK1, IRF3 (IE)
<b>NSP7 + NSP8</b>	DMV // Highly conserved	NTD binds viral RNA in the RTC Primase cofactors of NSP12 and NSP13
<b>NSP9</b>	DMV // 97% ID	Binds ssRNA and promote the formation of the RTC
<b>NSP10</b>	DMV // 99% ID	Cofactor of NSP14/16 methyltransferase (IE)
<b>NSP12</b>	DMV // Highly conserved	RNA-dependant RNA polymerase Possible guanylyltransferase ?
<b>NSP13</b>	DMV	RNA helicase and triphosphatase (IE) Cofactor of NSP14
<b>NSP14</b>	DMV // Highly conserved	3' exoribonuclease 5' methyltransferase (IE)
<b>NSP15 (EndoU)</b>	DMV // Highly conserved	Uridine-specific endoribonuclease cleaving polyU tail of rRNA to inhibit MDA5 recognition (IE)
<b>NSP16</b>	DMV	NSP10-dependant 2'-O-methyltransferase (IE)
<b>ORF3a</b>	Transmembrane // 72.7% ID	Viporin (ion channel) helping virions release Activates NFkB signaling and NLRP3 inflammasome (CS) Regulates host cell apoptosis and autophagy
<b>ORF3b</b>	Cytosol	Antagonist of IFN (IE)
<b>ORF6</b>	Cytosol	Antagonist of IFN (IE)
<b>ORF7a</b>	Transmembrane	Activates NFkB signaling and induces proinflammatory cytokines expression (CS) Inhibition of STAT2 phosphorylation (IE)
<b>ORF8</b>	Highly mutated	Promotes host cell apoptosis Binds MHC-I to mediate its degradation (IE) Antagonist of type I IFN (IE)
<b>ORF9b</b>	Cytosol // 40% ID (unique to SARS-CoVs)	Inhibits mitophagy and IFN signaling (IE)

**Table 3: Roles of SARS-CoV-2 nonstructural and accessory proteins.**

Functions implicated in the immune escape are marked with a "(IE)". Functions implicated in the cytokine storm are marked with a "(CS)". RTC stands for Replication Transcription Complex. ID stands for identity, vRNA stands for viral RNA. Among the 9 accessory proteins, only the characterized ones are described. The role of NSP11 is unknown.

(Data from Hu et al., 2021 and Yan et al., 2022<sup>245,250</sup>)

With millions of infections and the natural selection pressure imposed by host immunity and vaccination, SARS-CoV-2 is subject to frequent recombination and mutations, especially in the S protein<sup>241</sup>. Therefore, since its emergence, SARS-CoV-2 accumulated several mutations increasing its fitness towards the human population. First of all, SARS-CoV-2 presents a unique insertion of 4 Amino Acids (AA) residues (PRRA) at the junction between the S1 and S2 subunits of the S protein. Studies have recognized this feature essential for the zoonotic transfer to humans<sup>252</sup>. Indeed, this insertion generated a polybasic cleavage site (RRAR) enabling its cleavage by furin and other furin-like proteases. In parallel, this enhanced S1-S2 cleavage is thought to facilitate the conformational adaptation required to efficiently bind Angiotensin-Converting Enzyme (ACE2), the main receptor of SARS-CoV-2; but also, to have extended the specter of usable cell factors and thus cell tropism. Ultimately this insertion contributed to a broader infection and better replication of the virus, notably along the human upper airways, potentiating the interhuman transmission<sup>244,245</sup>. Interestingly, some S mutations now provide the capability to infect wild type mice and use mACE2, highlighting the ongoing spillover risk from human to mice during the continued evolution of SARS-CoV-2<sup>253</sup>.

To date, there are 5 globally recognized Variants Of Concern (VOC) for which a change in transmissibility and/or virulence, as well as a significant neutralizing antibody (NAb) escape and resistance to treatments was observed<sup>254</sup>. These VOC are Alpha, Beta, Gamma, Delta and Omicron (Table 3). Also, 8 Variants Of Interest (Epsilon, Zeta, Eta, Iota, Theta, Kappa) were identified. They present specific markers associated with changes to receptor binding, NAb escaping and reduced efficiency of treatments<sup>254</sup>. These variants emerged through mutations occurring principally in the RBD of the S protein and the ORF8 determinant of virulence<sup>250</sup>.

Variant	Mutation history	Main mutations	Effect
<b>Alpha</b> (UK, Sept 2020)	7 missense mutations, 3 deleted residues in S > Increased transmissibility, infectivity and disease severity	N501Y D614G P681H NTD deletions	Increase binding affinity of S to ACE2 by adding additional hydrogen bond Increased ability of RBD to shift to up position Increased S1/S2 cleavage ability Resistance to NTD-specific NAb
<b>Beta</b> (South Africa, May 2020)	77% of mutation in S including 7 missense mutations and 3 deleted residues > Increased immune escaping	N501Y K417N E484K	Increased binding affinity of S to ACE2 Resistance to NTD- and RBM-specific NAb
<b>Gamma</b> (Brazil, Nov 2020)	11 mutations in S, including 5 within NTD, 3 in RBD, 2 at the <b>FCS</b> , 1 in S2 > Slight increased immune escaping	N501Y K417N, E484K D614G, H655Y, T1027I	Increased binding affinity of S to ACE2 Resistance to NTD- and RBM-specific NAb
<b>Delta</b> (India, Oct 2020)	Numerous mutations in S, including 5 within NTD, 3 in RBD, 2 at FCS, 1 in S2 > 60% increase in transmissibility compared to Alpha	L452R T478K E484K	Increased infectivity and immune evasion Resistance to NTD- and RBD-specific NAb
<b>Omicron</b> (Globally, Nov 2021)	More than 50 mutations, mostly in S 50% mutations in RBD, 10 within RBM, 1 in NSP14, 1 in NSP5 > Increased transmissibility, immune escaping and reinfection rate	N501Y D614G P681H K417N T478K	Increase binding affinity of S to ACE2 Increased ability of RBD to shift to up position Increased S1/S2 cleavage ability Resistance to NTD- and RBM-specific NAb

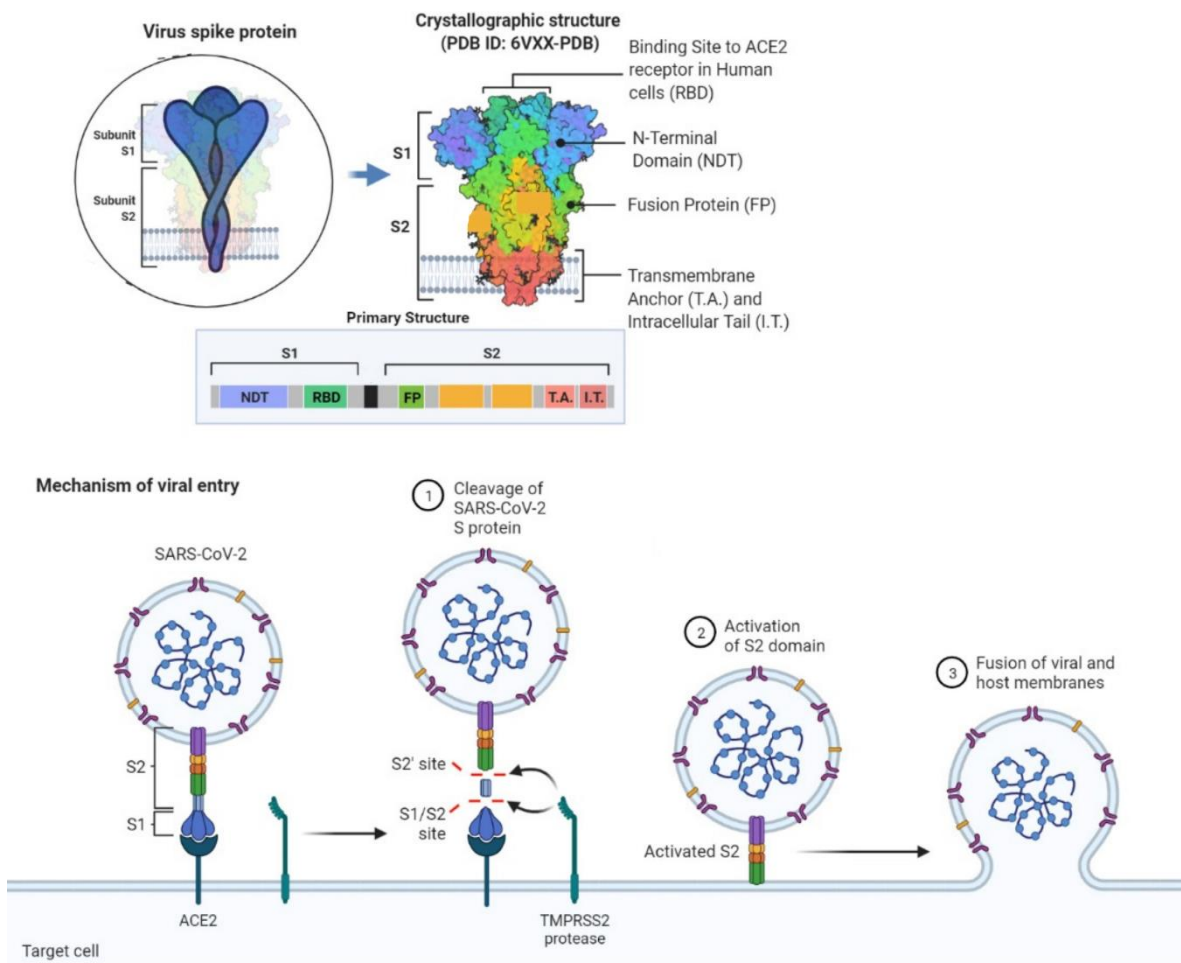
**Table 4:** Main mutations and adaptations of the SARS-CoV-2 variants of concern.

Some essential mutations (L452R; E484K/Q, N501Y, D614G and P684H/R) are also present in VOI variants such as Epsilon, Eta, Iota, Kappa, Zeta and Theta. For each variant, the earliest documented samples are indicated. FCS stands for Furin Cleavage Site. (Data from Yan et al., 2022 and Yang et al., 2022<sup>250,255</sup>)



## B.2. Replication cycle

SARS-CoV-2 cell infection is initiated by the binding of the spike protein to its receptor, via the RBD loops on the S1 subunit. This engages a conformational change allowing S2 cleavage by host protease revealing the S2' subunit fusion domain<sup>256–259</sup>. This fusion peptide is responsible for viral entry by anchoring into the cellular bilayer provoking the merging of viral and cellular membranes. This process can occur either at the cell surface directly, or after clathrin-mediated endocytosis into endosomes and ultimately ends with the viral RNA release into the cell cytoplasm<sup>260</sup> (**Figure 19**).

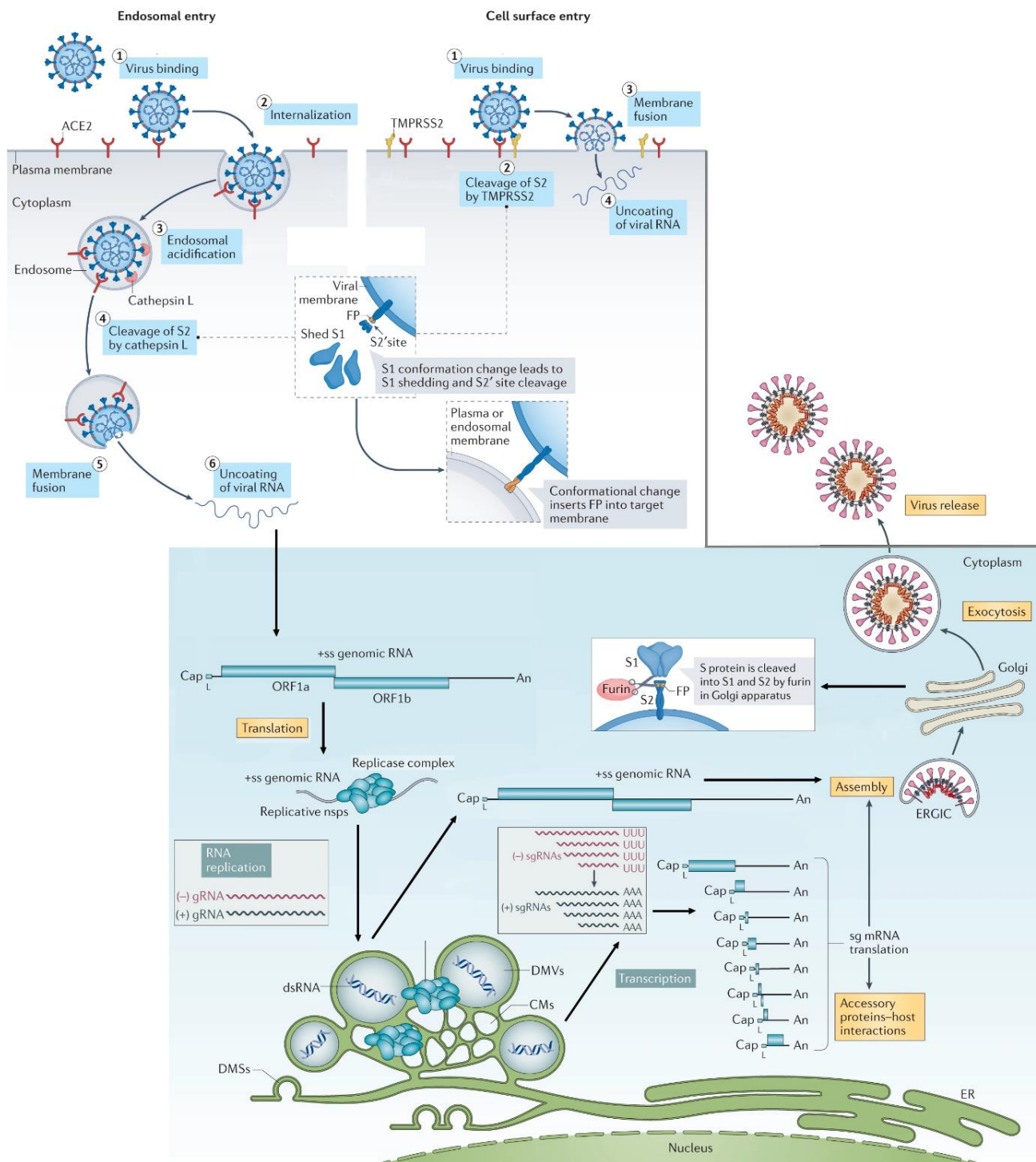


**Figure 19:** Schematic representation of SARS-CoV-2 spike protein structure and viral entry.

As discussed later, the TMPRSS2 is able to cleave both the S1/S2 and S2' sites.

(Adapted from Petrovszki et al., 2022<sup>261</sup>).

From the positive-sense 5' of the genome: ORF1a and ORF1b are then translated into the 2 polyproteins pp1a and pp1ab, that are cis-cleaved by PLpro to form the NSP1 to 3; and by Mpro to form the NSP 5 to 16 (**Figure 18, C**)<sup>244,250</sup>. The NSP12 RNA-dependent RNA polymerase (RdRp) and NSP7-8 cofactors perform the RNA replication. It is accompanied by NSP14 which provides a 3'-5' proofreading function. More precisely, NSP14 removes mis-incorporated nucleotides but also nucleotides analogs from the nascent RNA, thus participating in the resistance to treatments too<sup>250</sup>. The NPS10/13/14/16 complex then provides the methylated caps to the neosynthesized RNA<sup>244</sup>. The vRNA replication proceeds with the synthesis of full-length negative-sense rRNA strands, forming transitory dsRNA, along with sub-genomic RNAs (sgRNA). These sgRNA are transcribed as messaging sgRNA (msgRNA) and translated into the structural and accessory proteins. The N protein then takes over the neo-synthesized gRNA for protection and S/M/E are addressed to the ER membrane for viral budding into the ER-Golgi Intermediate Compartment (ERGIC)<sup>244</sup>. There, the S protein is primed-cleaved at the S1/S2 binding sites, essentially by Furin, and mature virions are addressed to the cell plasma membrane for exocytosis<sup>260</sup> (**Figure 20**).



**Figure 20: SARS-CoV-2 replication cycle.**

The main receptor (ACE2) and proteases (TMPRSS2 and Cathepsin L) implicated in endosomal and cell surface entry are displayed. (Modified from Harrison et al., 2020; Jackson et al., 2022 and V'kovski et al., 2021<sup>244,252,262</sup>)

### B.3. Cellular receptors involved in viral entry

**ACE2**<sup>263,264</sup> and **TMPRSS2**<sup>263,265,266</sup> are respectively considered to be the primary receptor and host protease for SARS-CoV-2 entry into cells. Although, several other proteins have been proposed as alternative entry factors.

Indeed, the polybasic site near the junction between S1 and S2 subunits (the S1/S2 site) can be recognized and cleaved by pro-protease convertase upon viral packaging<sup>267,268</sup>. This “priming cleavage” creates a “C-end rule” motif allowing the S1 subunit to have a greater affinity to ACE2 but also<sup>269,270,271</sup> to bind to new receptors including the Neuropilin 1 (NRP1)<sup>272</sup>.

**NRP1** was shown to enhance TMPRSS2 mediated entry by promoting the S2 protuberance and accessibility for proteolysis by TMPRSS2<sup>273</sup>. In HEK293T cells, the exogenous expression of NRP1 rendered the cells susceptible to SARS-CoV-2 infection and significantly potentiated viral entry upon co-expression with ACE2 and TMPRSS2. Additionally, a treatment with a NRP1 blocking antibody reduced infection by 40%<sup>274</sup>. SARS-CoV-2 infection was also reduced in Caco-2 cells upon NRP1 knock down with shRNA and treatment with a small-molecule antagonist<sup>272</sup>. Additionally, immunohistochemistry on human autopsy tissue of COVID-19 patients revealed high expression of NRP1 in infected olfactory epithelium where ACE2 was hardly detectable.

The **AXL** receptor tyrosine kinase was introduced as a candidate receptor by Wang et al., as its overexpression promoted SARS-CoV-2 infection as efficiently as ACE2 in HEK293T cells. Additionally, its downregulation in human primary lung epithelial cells significantly reduced it. Using pulled-down assays, they notably confirmed AXL direct interaction with the N-terminal domain of the SARS-CoV-S Spike protein<sup>275</sup>.

The **Basigin** (BSG / CD147) protein was also proposed as a putative receptor. Inhibition of the receptor by NAb significantly reduced SARS-CoV-2 infection of Vero E6 cells<sup>276</sup> and human iPS cell-derived kidney podocytes<sup>277</sup>. Additionally, an exogenous expression of BSG in non-permissive BHK-21 cells allowed viral entry<sup>276</sup>. However, its interaction with the RBD, shown by co-immunoprecipitation and ELISA assays is controversial. Indeed, Shilts et al.<sup>278</sup>, using highly sensitive binding assays, were not able to confirm direct interaction between recombinant spike protein and

the two most common isoforms of BSG. Also, they did not observe any impact on SARS-CoV-2 infection upon CRISPR-Cas9 extinction of BSG from CaLu-3 lung epithelial cells.

Finally, the T-cell immunoglobulin mucin domain 1 (**TIM-1** / HAVCR1) receptor was shown to promote SARS-CoV-2 Spike ectodomain virosomes uptake into human primary epithelial kidney organoids. As expected, the use of blocking antibodies in A549 cells decreased SARS-CoV-2 particles internalization<sup>279</sup>. However, while the high affinity interaction of the receptor with S protein RBD was confirmed, the expression of ACE2 in all models used for experiments questions the role of TIM-1 as an attachment receptor rather than a specific entry receptor.

Upon binding to its receptor, and depending on the cell type, the SARS-CoV-2 particle is meant to follow either an “early” or a “late” pathway of infection.

In **TMPRSS2** expressing target cells, the S1/S2 priming cleavage by viral producing cells seem to be essential for entry. It would allow the S protein to have the right conformation for TMPRSS2 to cleave the S2' site. The viral entry thus occurs at the plasma membrane representing the early, fast, pathway<sup>263,270,280,281</sup> to infect target cells.

The S1/S2 site, presenting a RXXR motif, was shown to be cleaved by **Furin**<sup>267,282</sup> and enhance infection<sup>270,283,284</sup>. However, furin inhibition cannot fully prevent the spike protein processing<sup>281</sup> suggesting that other proteases could be implicated. Using fluorogenic S1/S2 mimicking peptides, Jaimes et al. showed that the polybasic site can be cleaved by a variety of proteases including PC1 (PCSK1), Trypsin (PRSS1/2/3), Matriptase (ST14) and Cathepsin L or B. However, experiments on full length spike protein are still needed to prove their action on the protein native conformation<sup>285</sup>.

In TMPRSS2 negative target cells, SARS-CoV-2 S1/S2 priming does not seem to be essential<sup>263,281,284</sup>. Instead, SARS-CoV-2 binds to its receptor and is internalized via a clathrin-mediated endocytosis<sup>286</sup> to late endolysosomes where S2' is cleaved by **Cathepsins**<sup>263</sup>, CTSL being the most potent<sup>270,281</sup>. This cascade triggers the virus fusion with the endolysosome membrane, representing the late, slower, pathway for viral entry<sup>280</sup>.

While the TMPRSS2 cell surface expression seems to dictate the entry route taken by SARS-CoV-2 into host cells, some studies pointed the role of TMPRSS4<sup>287</sup> and TMPRSS13, as well as TMPRSS11D

(HAT), TMPRSS11E and F in processing the spike protein and enhancing infection, but with less efficiency<sup>266,288</sup>.

Besides free virion infection, SARS-CoV-2 spike-mediated cell-to-cell transmission seems to represent a critical feature as it can represent 50 to 90% of total viral spread in a 293T/293T-ACE2 co-cultures<sup>289</sup>. ACE2 and TMPRSS2 are still the primary entry factors implicated in this process<sup>284,290</sup>. However, treatment of cocultured cells with endosomal entry inhibitors impairs cell-to-cell transmission. This observation implicates the endosomal membrane fusion as an underlying mechanism. Conjointly, while Furin priming cleavage enhance cell fusion with TMPRSS2 expressing target cells<sup>291</sup>, donor cell's proteases TMPRSS2, CTSL or Trypsin can also cleave S2'<sup>280,292</sup>, during the protein biosynthesis or at the cell surface. It is assumed that fusion could thus occur in a receptor-independent way as it was demonstrated for other coronaviruses before<sup>267,293,294</sup>.

### C. Transmission modes

SARS-CoV-2 is an airborne virus which main mode of transmission are liquid droplets and aerosol particles. They are spread during patients' speeches, coughs or sneezes and inhaled by naive persons. Of note, SARS-CoV-2 may persist in closed environment's air at high concentration and stay viable for at least 3h<sup>295</sup>. The most recent strains (Delta or Omicron) are more resistant than ancestral Wuhan strain, contributing to their increased infectivity. SARS-CoV-2 characteristic resides in the fact that the virus quickly replicates in the upper respiratory tract and throat, leading to high viral loads as of the beginning of infection<sup>21,245,262</sup>. Asymptomatic cases thus play a crucial role in the virus transmission. Studies postulated that 79% of the documented positive cases would have originated from transmission by asymptomatic patients<sup>21,245</sup>.

Additionally, exhaled particles may contaminate surrounding surfaces and fomites thus represent a prolonged risk of infection by indirect contact as the virus can persist on stainless steel and plastic surfaces up to 72h. This mode of transmission may be increased for the more resistant Omicron compared to Wuhan ancestral strains. SARS-CoV-2 replicating in eye tissues, particles have been also detected in conjunctival secretions of some patients, and could be a source of auto- or cross-contamination<sup>295</sup>.

Besides, in a Chinese cohort, SARS-CoV-2 was recovered from feces of 29% of patients, up to 4 weeks after fever abatement whereas respiratory secretions were cleared within 2 weeks after symptoms

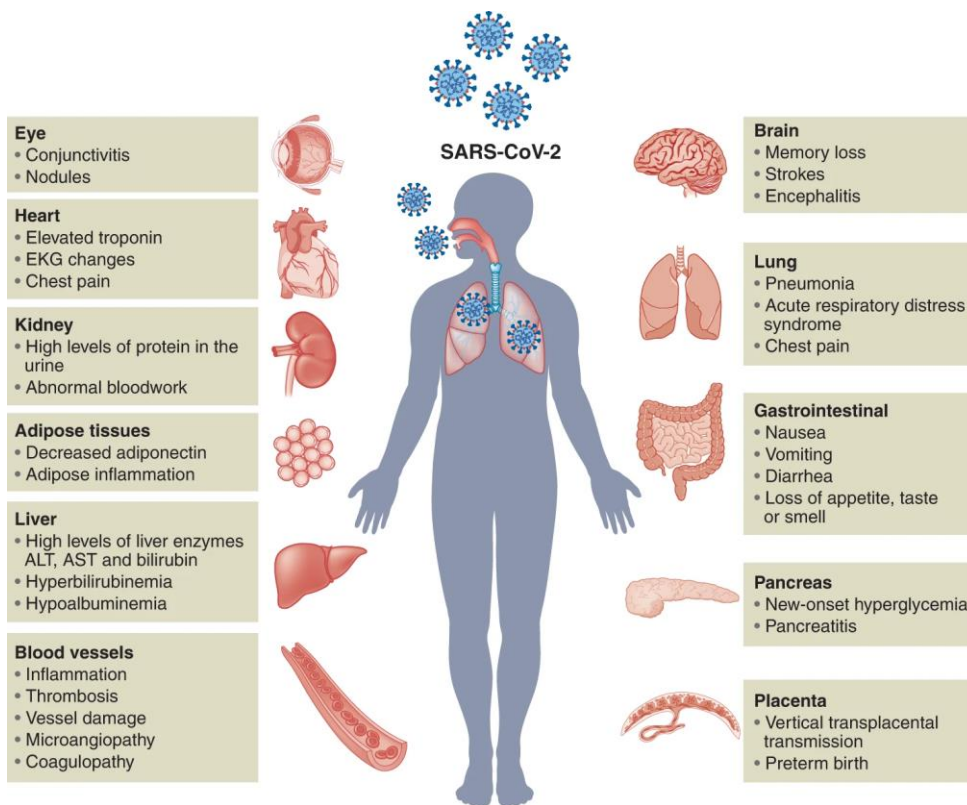
onset. Studies show that SARS-CoV-2 can resist many days in sewage. However, the fecal-oral transmission was never clearly demonstrated yet and its role in the pandemic is still unclear <sup>262,295</sup>. Finally, the high expression of ACE2 in the human maternal-fetal interface supposes that SARS-CoV-2 could be transmitted vertically. A first case was ever suspected in March 2020 as a neonate showed elevated blood concentration of IgM (witness of a recent infection) and cytokines only 2h after birth from an acutely infected mother <sup>296</sup> and other cases of neonates revealed positive shortly after birth <sup>297</sup>. However, the virus could not be retrieved from any of the amniotic fluid, cord blood or breast milk samples <sup>295</sup>.

## D. Physiopathology of the infection

### D.1. Generalities

Upon infection, ciliated cells of the nasal epithelium and pneumocytes of the lung are the primary targets of SARS-CoV-2 replication, followed by the infection of submucosal gland epithelium, endothelial cells and alveolar macrophages. Infection of the upper respiratory tract can progress to the lower airways and result in inflammation, further leading to systemic spread of the virus in extra-pulmonary organs. Eventually, infection can lead to fatal pneumonia and multi-organ failure <sup>245,262,298</sup>.

After an incubation period of 1 to 14 days (with a median at 5 days) depending on the variant, symptomatic patients thus generally present mild flu-like symptoms with fever, fatigue, dry cough, lymphopenia. In a report implying more than 72000 patients in China, 81% of cases were mild but 14% presented severe symptoms including headache, vomiting, diarrhea, dyspnea and pneumonia leading to Acute Respiratory Distress Symptoms (ARDS) that required hospitalization and ventilation (**Figure 21**) <sup>245,262,299</sup>. 5% of these patients, being predominantly old men with comorbidities, developed critical symptoms including septic shock and multiorgan failure. Of them, 2.3% patients died within 16 days. Recently, a literature review reported the high incidence of systemic symptoms: along the pandemic, gastrointestinal complications represented between 11 and 53% of cases, and liver injury 15 to 53%, correlating with the disease severity <sup>300</sup>. While the majority of patients recover within 2 weeks <sup>245</sup>, some present a prolongation of symptoms after disease resolution defined as “Long Covid Syndrome”. Extended lists of the common manifestations, complications and long covid symptoms can be found in Merad et al, 2022 <sup>301</sup> and Mehandru et al, 2022 <sup>302</sup>.



**Figure 21:** SARS-CoV-2 sex-independent tropism and associated complications. <sup>303</sup>

## D.2. Complications

Immune responses induced following infection are thought to drive pathogenesis and disease severity in COVID-19 patients. Chemokines and cytokines are released from infected lungs promoting leukocyte recruitment to the tissue and resulting in substantial inflammation in severe cases. Hallmarks of severe COVID-19 disease include high serum levels of IL-6, TNF- $\alpha$ , IP-10/CXCL10 and MCP-1, among others <sup>304,305</sup>. The host determinants and mechanisms decisive on whether COVID patients develop mild or severe disease still need better characterization, however, several observations and hypotheses have been made.

The pathogenesis of SARS-CoV-2-induced pneumonia likely occurs in two phases. First, the viral phase is characterized by viral replication resulting in direct virus-mediated tissue damage, the extent of which determines the pathogenesis of the secondary phase. This second phase is characterized by the recruitment of effector immune cells causing local and systemic inflammatory response that can persist even after viral clearance <sup>245,262,301</sup>.



Several risk factors for severe COVID-19 have been identified and suggest an inability to mount a timely antiviral response and to control SARS-CoV-2-driven inflammation.

Indeed, patients with mild symptoms seem to present a stronger line of defense within the alveolar macrophages. These cells, which survey the lumen of the respiratory tract, play an important sentinel role in the lungs by sensing and triggering potent antiviral immunity, through viral detection via TLR and RLR. They provide an adapted IFN and ISG-stimulated antiviral response, added to an adequate adaptive immunity involving CD4 and CD8 T cells.

On the contrary, alveolar macrophages are severely depleted in the lungs of patients with severe COVID-19, who display a delayed and imbalanced immune response leading to cytokine storm and multiorgan damages<sup>301</sup>. High IFN-I levels, devoid of ISG upregulation, can be observed in the lower airways of these patients<sup>306</sup>. This deficiency would lead to a rapid and uncontrolled viral replication during the initial steps of infection and a rapid systemic spread of the virus.

Additionally, SARS-CoV-2 induces ER stress, inflammasome and pyroptosis in specialized immune and infected cells, which enhances the production of proinflammatory cytokines (IL1 $\beta$ , IL6, IL8, IL18) and chemokines (CCL2)<sup>260,262,307</sup>. These molecules were shown to activate, recruit and sequester immune cells in the affected organs, therefore depleting their number in circulating blood as commonly observed in severe patients<sup>301</sup>. Added to the direct virus-mediated injury, inflammatory cytokines and infiltrating effector cells (Natural Killers) degranulation contribute to vascular permeability, hypercoagulation and tissue damages<sup>244,262,301</sup>.

Nonetheless, loss of function variants in loci that control TLR3- and IRF7-dependent type I IFN immunity have been identified in a small number of severe patients<sup>308</sup> and autoantibodies against type I IFN have also been identified in critically ill patients. These antibodies have been shown to contribute to the imbalanced adaptive immunity and delayed viral clearance<sup>309,310,311</sup>.

The exact contribution of viral loads in disease course is still unclear. Autopsies of deceased COVID-19 patients have revealed very little active viral infection and substantial accumulation of activated immune cells, suggesting that organ failure is unlikely to result from extensive viral-induced tissue damage but is instead caused by the over-activated immune system and vascular damage which are very common in severe COVID-19<sup>301</sup>. Autopsy of COVID-19 deceased patients thus revealed extensive immune infiltrates in patients' organs, leading to alveolar damages, desquamation of pneumocytes, fibrin deposit in the lung and bronchiolitis. These airways damages were frequently accompanied by acute cardiac, gastrointestinal, renal and hepatobiliary injuries<sup>245,252,301</sup>.

As said earlier, the determinants and mechanisms involved in many of these mechanisms are still unknown, but predisposing risks have been recorded. Age is thus by far the strongest bias, the risk of developing a severe, if not life-threatening, COVID-19 sharply increasing from 60 years old onward (Figure 22). Diabetes and hypertension as well as chronic kidney disease and smoking were also reported, as chronic inflammation lesions could enhance and perpetuate the inflammatory cycle induced by severe COVID. Metabolic syndrome in diabetic and obese patients might also lead to complications as they would generally present preceding vascular damages and impaired tissue repair<sup>252,301</sup>

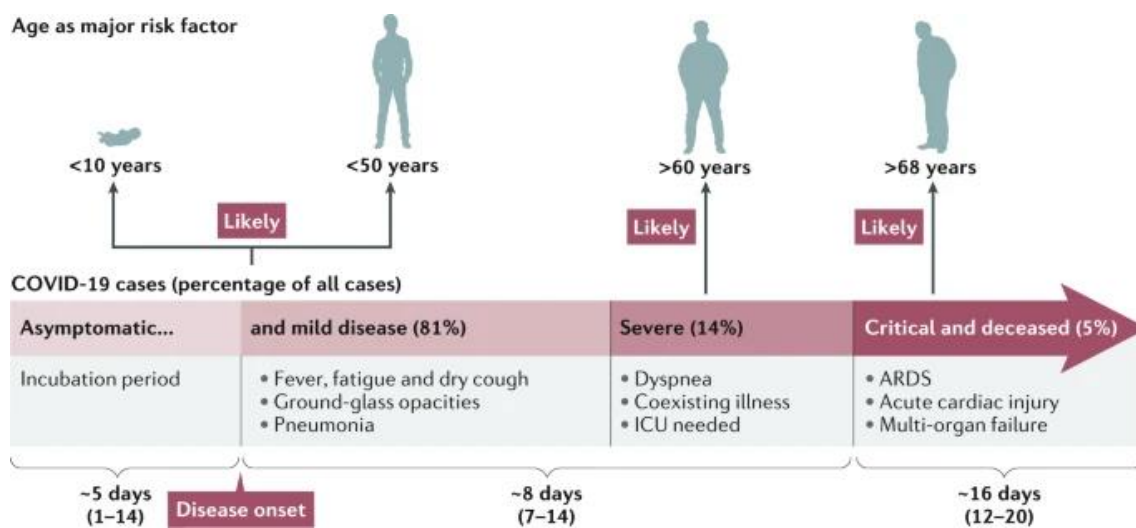


Figure 22: Age-related clinical features of COVID-19<sup>245</sup>

### D.3. The male predisposition and role of the renin-angiotensin system

Clinical manifestations also differ with sex, men being at greater risk to develop severe COVID with male patients representing 73% of deaths in an early study of 113 cases in China<sup>312</sup>. A gender-joint systematic review of 47 studies including 21.454 patients later concluded in an overall higher ICU admission (22 vs 12%), disease severity (25 vs 21%) and mortality (11% vs 4%) rates in men versus female patients<sup>313</sup>.

The Renin-Angiotensin System (RAS) is primarily implicated in the regulation of blood flow by constriction and dilation of many organs vessels. It involves the Angiotensin-Converting Enzyme (ACE) which converts Angiotensin I (AngI) in Ang II. When binding to its AT1R receptor, AngII promotes various signaling cascades including local vasoconstriction, inflammation and fibrosis, while it has the opposite effect when binding to its AT2R receptor. The homolog of ACE, ACE2, converts AngII in

Ang(1-7) which signaling attenuates the effects of inflammation and fibrosis through the Mas receptor (MasR) <sup>314,315</sup>. By binding ACE2, SARS-CoV-2 internalized the receptor by endocytosis, thus downregulating ACE2 expression at the cell surface. This phenomenon has been proposed to imbalance the RAS ACE/ACE2 ratio, promoting AngII-driven activation of the AT1R inflammatory pathway <sup>316-319</sup>. In the human testis, several components of the renin-angiotensin system have been described, including renin, ACE, MasR and AngII, which are expressed in Leydig, germ and sperm cells. However, their physiological role in the testis physiology remains elusive <sup>320</sup>.

Although studies do not all agree, ACE2 is suspected to be upregulated by androgens in organs of male patients, as it was shown in primary prostate and lung cells <sup>321,322,323</sup>, promoting the initial steps of infection. On the contrary estrogen/progesterone upregulation of ACE2 expression, are conjointly believed to allow the receptor replenishment and maintain the RAS equilibrium post-infection <sup>317,319,324</sup>. Additionally, testosterone was shown to inhibit innate immune responses <sup>325</sup> whereas estrogen is believed to stimulate a better humoral and cell-mediated immunity <sup>313,325</sup>.

#### D.4. SARS-CoV-2 in the Male Genital Tract

Early in the pandemic, numerous studies collectively reported that ACE2 RNA and protein were highly expressed in the human testis when compared to other human tissues, including lungs <sup>326,327</sup>. These observations suggested testes might therefore be at risk of infection. In particular, Sertoli and Leydig cells, and to a lesser extent spermatogonia, expressed the most ACE2 RNA in the testis <sup>328-330</sup>. Co-expression of ACE2 and TMPRSS2 was reported over single cell RNA-sequencing (scRNA-seq) data in Spermatogonial Stem Cell (SSC) and elongated spermatids in some studies <sup>329,331,332</sup>. However, others did not confirm this finding <sup>332,333</sup> and emphasized the notable discrepancies between mRNA and protein levels <sup>330</sup>.

Testis of deceased patients who suffered from severe COVID-19 presented damaged morphology including interstitial edema as well as inflammatory infiltrates (T cells and macrophages) and reduced Leydig cell numbers. The seminiferous tubules displayed impaired spermatogenesis, degenerated germ cells, Sertoli cell swelling and vacuolization <sup>115,334,335</sup>. Scrotal discomfort and orchitis were also reported in few studies during severe acute phases of the disease <sup>336-338</sup>. Contrariwise, a study following 253 patients with mild symptoms did not observe any signs or symptoms of orchitis <sup>339</sup>.

Regarding of SARS-CoV-2 general physiopathology and the immune infiltrates observed, the literature tends to attribute testes impairment to inflammatory and autoimmune reactions, which

would disrupt the BTB, allowing circulating leukocytes to enter the testicular parenchyma <sup>340</sup>. Indeed, inflammation of the testis (orchitis) can disrupt the blood-testicular barrier and break the immune-privilege, leading to autoimmunity against spermatogenic cells, as exemplified following infection by Mumps virus, a testis-tropic virus <sup>16</sup>. Supporting this hypothesis, some studies reported abundant IgG precipitation in the seminiferous epithelium of COVID patients <sup>115,335</sup>.

Nonetheless, 7 studies reported a total of 39/71 cases of SARS-CoV-2 RNA or protein detected in human testis samples of deceased patients, suggesting that this organ could be a direct target for SARS-CoV-2 infection <sup>115,116,334,335,340-343</sup>. The absence of detection of SARS-CoV-2 in the testes from approximately half of the other deceased patients may be due to a collection of samples too far from the acute infection and/or poor preservation of tissues and viral nucleic acids.

There are no proper longitudinal cohort studies and contradictory reports on SARS-CoV-2 shedding in semen. Out of hundreds of semen samples investigated <sup>340</sup>, about 10 tested positive in RT-qPCR during the acute phase of the disease <sup>337,344,345,346</sup> and 3 in recovered patients <sup>346,347</sup>. Of note, SARS-CoV-2 RNA was retrieved up to 81 dps in one convalescent man <sup>347</sup>. However, the few studies sampling semen after the early stages of infection mostly failed to detect the virus <sup>340</sup> and in case of positive results, some authors suggest the contribution of residual urine shedding. Indeed, the urinary and genital tracts join in the urethra and SARS-CoV-2 was frequently recovered from urine <sup>348</sup>.

In a large portion of cases, patients presented substandard semen quality, including decreased sperm concentration and total number per ejaculate, decreased motility and altered morphology <sup>337,349,350</sup> along with high leukocyte counts and inflammatory cytokines (IL6, TNF $\alpha$ ) <sup>115</sup>. Alterations of semen parameters were more pronounced in severe cases <sup>337,340,351</sup> although they have also been reported in patients with mild symptoms <sup>349,352,353</sup>. Several studies showing that the alterations of the semen parameters correlated with fever and disease severity suggest a systemic impairment <sup>120,337,340,351</sup>. Indeed, fever is the most common symptom of COVID-19 and an increased body temperature can hinder spermatogenesis and affect the quality of sperm up to 3 months <sup>119,354,355</sup> which correlates with long-term alteration of semen observed in one longitudinal-study <sup>356</sup>.

Multiple studies on COVID-19 patients have reported a decrease in blood testosterone levels after SARS-CoV-2 infection <sup>357</sup>. Low testosterone levels in patients correlated with disease severity and were associated to elevated markers of systemic inflammation (IL1 $\beta$ , IL6, IL4, TNF $\alpha$ ) during acute disease <sup>117,123,124</sup>. Elevated concentrations of LH were associated with low to normal testosterone

levels (the latter defined as compensated hypogonadism) in a subset of COVID-19 patients. Primary hypogonadism was thus evidenced in these mild to moderately infected patients, indicating a direct testicular failure<sup>116,117,340</sup>. However, 69 to 75% of severely infected patients from 3 studies presented concomitant low levels of LH<sup>117,123,125</sup> suggesting a secondary hypogonadism resulting from a dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis. The association between elevated markers of systemic inflammation and low levels of testosterone levels concomitantly to low levels of LH further point at an indirect effect of the infection onto the testis in these patients<sup>117,334,358</sup>. In large cohorts of men with moderate to severe COVID-19, FSH levels globally remained within normal values during the acute stage of infection, and inhibin B was unchanged in most patients<sup>125,359</sup>, suggesting that Sertoli cells functions were not affected.

Although prostate express high level of TMPRSS2 along with concomitant low expression of ACE2 in hillock and club cells<sup>328,360</sup>, all prostatic fluid tested until January 2021 were negative<sup>340</sup>.

In animal models, hACE2-expressing transgenic mice presented infected Sertoli cells until 6 dpi<sup>361</sup> and SARS RNA was detected in Syrian hamster testis up to 28 dpi without major histopathological impacts<sup>362</sup>. However, another study on Syrian hamsters described a drastic drop in sperm count and testosterone levels shortly after infection, accompanied by immune infiltration. These symptoms were not correlated to an extensive infection of testicular cells but triggered long-term testis damages, including Sertoli and Leydig cells necrosis. These led to reduced testosterone and inhibin B serum levels up to 120 dpi<sup>363</sup>. In a non-human primate model, SARS-CoV-2 was detected by PET/CT scan imaging in macaques' seminiferous tubules up to 2 weeks post-infection, along with epididymis and prostate infection, as well as immune infiltrates<sup>364</sup>.

Viral replication was evidenced over 72h post-infection of hamster testis ex vivo cultures, and human primary Sertoli cells supported low levels of viral replication in vitro<sup>362</sup>. These two examples represent the only reports of SARS-CoV-2 replication in testicular cells.

Overall, only sparse and controversial evidences suggest that SARS-CoV-2 could directly infect the testis and be shed in semen of mildly infected patients. Also, the direct impact of the testis infection (over systemic inflammation) on sperm and hormonal parameters of patients are uncertain. The investigation of the testicular infection in human by SARS-CoV-2 would therefore help understanding the consequences of infection on the patients' reproductive health and functions.

# Objectives

Emerging diseases are defined as “infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range”. Among these, up to 44% are due to RNA viruses<sup>33,365</sup>. Their adaptation and evolution processes allow them to extend their host and organs tropism, and escape immune recognition and responses. These capacities can lead in the use of more efficient transmission routes and in the establishment of persistence in target organs<sup>33</sup>.

Understanding and predicting the multiple modes of transmission of emerging viruses is key to control their spreading. Sexual transmission through semen, is particularly challenging as exemplified by the HIV pandemic still ongoing, and a concern for future epidemics. Nowadays, over 30 contemporary and emerging viruses have been reported in semen. A subset persists in this fluid for extended duration despite systemic clearance. The sexual transmission of viruses is a major concern for public health, as illustrated by the spreading outside endemic areas of ZIKV or the triggering of epidemic resurgence of EBOV. Viral infections of the immune-privileged testis have great potential to constitute a source of persistent virus. In addition, it can trigger a number of deleterious consequences on the reproductive and endocrine health of infected men, and impact not only partner but also their offspring. However, for most viruses excreted in semen, their tissue of origin is still undetermined and their effect on male functions poorly characterized<sup>366</sup>.

Two emerging viruses from distinct single strand RNA viral families have recently re-emerged (ZIKV) or emerged (SARS-CoV-2):

In 2015-2016, Zika virus, a flavivirus first isolated in 1947 in Uganda, was responsible for a major outbreak in the Americas. Although it is primarily transmitted by mosquito bites and originally provoked self-limiting febrile illness, the virus was unexpectedly found to be transmitted vertically as well as through sexual contacts. ZIKV was found to be shed in semen for over a year post-symptoms onset<sup>367</sup> and our lab demonstrated that ZIKV replicates in the human testis<sup>368</sup>. The organ and cellular reservoir responsible for the prolonged seminal excretion of ZIKV were unknown. A goal of my thesis was to decipher the role of the testis in this persistence.

In December 2019, a new beta coronavirus, SARS-CoV-2 rapidly spread around the world, causing the COVID-19 pandemic. Although it is primarily a respiratory virus, SARS-CoV-2 was found to infect several organs beyond the lungs. Many studies reported the high expression of its receptor, ACE2, in the testis when compared to other organs. In COVID-19 patients, alteration of semen and hormonal parameters have been reported in a number of studies, along with damaged testicular morphology observed at autopsy. Although SARS-CoV-2 RNA and proteins were detected in the testes of a subset of deceased patients and in some animal models <sup>340,369,370</sup>, there has been no formal evidence that SARS-CoV-2 replicates in the human testis and directly alters its functions and morphology. In this context, I was involved in a project aiming at determining the infection of the human testis and semen by SARS-CoV-2.

The first step of viral infection is the viral entry, which consists in the recognition and binding of a virus to its cell receptor(s), and in some cases, involves the cleavage of the virus fusion protein by specific cellular proteases. Viral tropism, and in turn cell and organ susceptibility, are thus firstly driven by the availability of such entry factors at cell surface <sup>371</sup>. The mapping of viral entry receptors in testicular cells can therefore give an insight into the potential for a given virus to infect this organ. To this end, I conducted an *in-silico* analysis of the expression of receptors proven to mediate the entry of several emerging viruses.

Altogether, my thesis covered 3 stages of the infection process, each corresponding to a chapter of my results:

**I. Viral persistence of ZIKV in the testis**

Article 1: Long-term Zika virus infection of non-sperm cells in semen

**II. Viral replication of SARS-CoV-2 in the testis**

Article 2: SARS-CoV-2 replicates in the human testis ex vivo and infects Leydig and Sertoli cells

**III. Viral entry factors of emerging RNA viruses in the testis**

Chapter 3: Expression of viral entry factors in the human testis: analysis of published single cell RNA-seq data

# Results

## I. Viral persistence of ZIKV in the testis

### Article 1:

Long-term Zika virus infection of non-sperm cells in semen

Mahé D, Bourgeau S, Frouard J, Joguet G, Pasquier C, Bujan L, Dejudcq-Rainsford N.  
Lancet Infect Dis. 2020 Dec;20(12):1371.



## Long-term Zika virus infection of non-sperm cells in semen

Zika virus is a mosquito-borne teratogenic virus that was unexpectedly found to persist in semen. Viral RNA has been detected up to 414 days after symptom onset<sup>1</sup> and infectious virus rescued up to 69 days after symptom onset.<sup>2</sup> During the last outbreak in 2015–16, sexual transmission of the Zika virus was reported in over 14 countries outside the epidemic zone.<sup>3</sup>

The cellular reservoirs of Zika virus and reasons for the sperm alterations that we reported in our prospective observational study<sup>4</sup> are unknown. Viral RNA is present in both the plasma and whole-cell fractions of semen,<sup>4</sup> and infectious virus is found in seminal plasma and spermatozoa,<sup>4</sup> with viral antigen found in spermatozoa in one patient 56 days after symptom onset.<sup>5</sup> We have also shown that Zika virus infects the human testis *ex vivo* and that productively infected sperm progenitors (ie, testicular germ cells) are shed in semen from acutely infected patients.<sup>6</sup> In this Correspondence, we report the characterisation of seminal non-sperm cells infected by Zika virus in three patients (patients 6, 13, and 15) from our published cohort,<sup>4</sup> for whom we collected longitudinal semen smears up to 160 days after symptom onset.

Through analysis of cell markers (DDX4, EPCAM, CD14, CD68, and MPO) and Zika virus antigens (envelope [E] and non-structural protein 1 [NS1]) by immunofluorescence microscopy (appendix), we found persistent shedding of Zika virus-infected testicular germ cells in the semen of all three patients. In patient 6, testicular germ cells were infected up to 160 days after symptom onset and represented the bulk of infected (E protein-positive) non-sperm cells in longitudinal samples at all timepoints. An estimated 21.6–52.1% of the

testicular germ cells released in semen were positive for E protein across the patients (appendix). In addition, in patient 6, detection of NS1 in testicular germ cells at 60 days after symptom onset showed ongoing Zika virus replication.

Up to 60.0% of exfoliated epithelial cells (as seen in patient 13 at 90 days after symptom onset), and up to 72.7% of monocytes or macrophages (as seen in patient 6 at 10–20 days after symptom onset) stained positive for E protein in the semen smears from the three patients. Infected epithelial cells and monocytes or macrophages were detected up to 90 days after symptom onset in patient 13 (appendix). We searched for infected Sertoli cells (positive for E protein and follicle-stimulating hormone receptor) in patients 6 and 13, but rare events at very early timepoints (7 days after symptom onset) were found exclusively in patient 13, and none were found at later timepoints (20, 60, and 90 days after symptom onset) in either patient. In leucocytospermic samples, a small proportion of neutrophils harboured E protein up to 60 days after symptom onset (appendix).

Altogether, these results show that human testicular germ cells are a persistent cell reservoir in which Zika virus actively replicates. Exfoliated infected epithelial cells and leucocytes that can originate from several sites within the genital tract (eg, the epididymis, vas deferens, seminal vesicles, prostate, or urethra) additionally contribute to prolonged viral shedding in semen. Persistent infection of non-sperm cells contributes to viral shedding and could explain the prolonged Zika virus excretion reported in semen from vasectomised men.<sup>3</sup> The data are important and highlight the need for further studies to decipher Zika virus localisation within the human male genital tract and its consequences for reproductive function and sexual transmission.

We declare no competing interests.

*Dominique Mahé, Salomé Bourgeau, Julie Frouard, Guillaume Joguet, Christophe Pasquier, Louis Bujan, \*Nathalie Dejuçq-Rainsford*  
nathalie.dejuçq-rainsford@inserm.fr

Institut National de la Santé et de la Recherche Médicale, École des Hautes Études en Santé Publique, Institut de Recherche en Santé, Environnement et Travail, Université de Rennes, F-35000 Rennes, France (DM, SB, JF, ND-R); Centre Caraïbéen de Médecine de la Reproduction and Centre d'Étude et de Conservation des Oeufs et du Spermé Humain (CECOS) CARAIBES, Centre Hospitalier Universitaire (CHU) de Pointe-à-Pitre, Pointe-à-Pitre, Guadeloupe, France (GJ); and Laboratoire de Virologie, Institut Fédératif de Biologie (CP), and Groupe de Recherche en Fertilité Humaine and CECOS Groupe d'Activité Médecine de la Reproduction, Hôpital Paule de Viguier (LB), CHU de Toulouse, Toulouse, France

- 1 Bujan L, Mansuy JM, Hamdi S, Pasquier C, Joguet G. 1 year after acute Zika virus infection in men. *Lancet Infect Dis* 2020; **20**: 25–26.
- 2 Arsuaga M, Bujalance SG, Díaz-Menéndez M, Vázquez A, Arribas JR. Probable sexual transmission of Zika virus from a vasectomised man. *Lancet Infect Dis* 2016; **16**: 1107.
- 3 Le Tortorec A, Matusali G, Mahé D, et al. From ancient to emerging infections: the odyssey of viruses in the male genital tract. *Physiol Rev* 2020; **100**: 1349–414.
- 4 Joguet G, Mansuy J-M, Matusali G, et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect Dis* 2017; **17**: 1200–08.
- 5 Mansuy JM, Suberbielle E, Chapuy-Regaud S, et al. Zika virus in semen and spermatozoa. *Lancet Infect Dis* 2016; **16**: 1106–07.
- 6 Matusali G, Houzet L, Satie A-P, et al. Zika virus infects human testicular tissue and germ cells. *J Clin Invest* 2018; **128**: 4697–710.

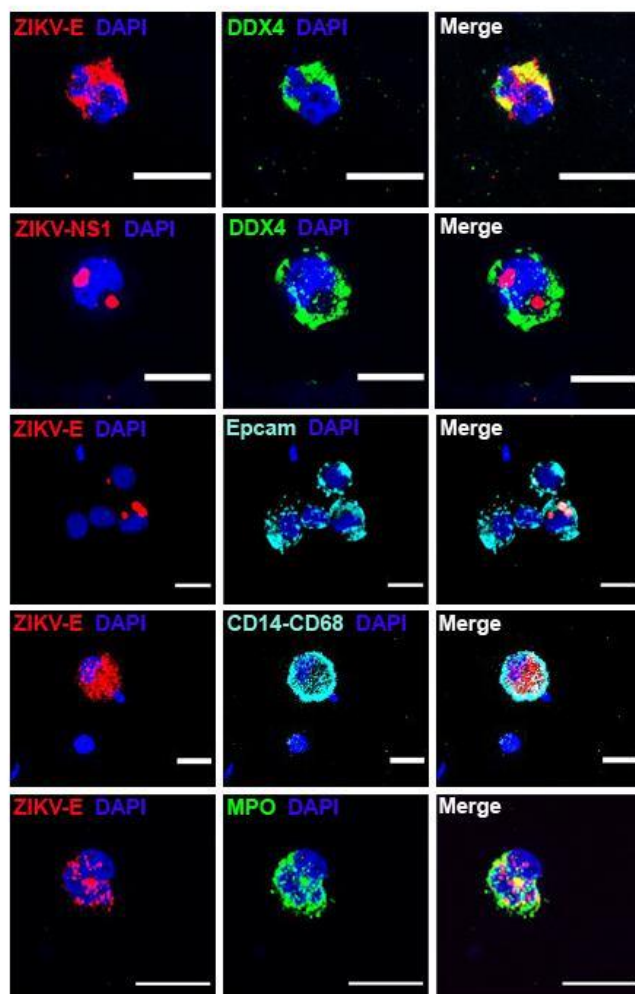
## Predictive performance of interferon- $\gamma$ release assays and tuberculin skin tests

Guozhong Zhou and colleagues<sup>1</sup> compared the performance of interferon- $\gamma$  release assays (IGRAs) and tuberculin skin tests for the prediction of incident tuberculosis and concluded that IGRAs had better predictive ability than tuberculin skin tests. We would like to express our concerns about the approaches used with regard to study design and data analysis and interpretation, which might explain

See Online for appendix

		d10-20	d60	d90	d160		
Patient 6	Zika vRNA (Log copies/mL)	Seminal plasma	5,5	6,1	5,5	3,4	
	Semen cells concentration (10 <sup>6</sup> /mL)	Total semen cells	5,3	6,5	5,3	3,3	
		Spermatozoa	1,8	1,04	1,98	9,3	
		NSC	1	0,2	0,5	0,5	
	% infected cells	Leucocytospermia	yes	no	no	no	
		Germ cells	29,9	<b>69,0</b>	52,1	<b>59,4</b>	#
		Epithelial cells	15,4	<b>13,8</b>	50,0	<b>12,5</b>	#
		Monocytes/macrophages	<b>72,7</b>	<b>4,3</b>	13,0	<b>3,8</b>	#
Neutrophils		1,9	<b>1,6</b>	0	<b>0</b>	#	
						<b>7,6</b>   <b>65,5</b>	
Patient 13	Zika vRNA (Log copies/mL)	Seminal plasma	8,9	4,9	3,3		
	Semen cells concentration (10 <sup>6</sup> /mL)	Total semen cells	8,4	3,6	3,4		
		Spermatozoa	26,6	11,5	7,9		
		NSC	2,4	1,8	0,1		
	% infected cells	Leucocytospermia	yes	yes	no		
		Germ cells	31,7	<b>46,9</b>	21,6	<b>37,9</b>	49,4
		Epithelial cells	<b>52,1</b>	<b>10,9</b>	<b>35,1</b>	<b>38,6</b>	<b>60,0</b>
		Monocytes/macrophages	#		10,8	<b>31,5</b>	15,4
Neutrophils		#		2,7	<b>7,4</b>	#	
						<b>66,1</b>   <b>20,3</b>	
Patient 15	Zika vRNA (Log copies/mL)	Seminal plasma	7,8	4,2	0		
	Semen cells concentration (10 <sup>6</sup> /mL)	Total semen cells	7,8	4,1	0		
		Spermatozoa	61	19	1,54		
		NSC	4,3	26,8	16		
	% infected cells	Leucocytospermia	yes	yes	yes		
		Germ cells	42,4	<b>43,5</b>	30,2	<b>32,3</b>	#
		Epithelial cells	<b>56,5</b>	<b>3,4</b>	25,5	<b>9,4</b>	#
		Monocytes/macrophages	#		0,8	<b>22,2</b>	#
Neutrophils		#		9,4	<b>5,6</b>	#	

NSC, Non sperm cells ; #, not available



**Appendix Table and Figure: Characterization and quantification of ZIKV-infected non-sperm cells in longitudinal semen samples from 3 ZIKV-infected patients for whom semen characteristics were published in a previous paper 4.**

The table shows viral loads, cell concentrations in semen and percentage of infected non-sperm cells within each cell type (black value) or related to the total number of infected cells (blue italic value) counted on the semen smears using NDPview software. Slides were immunolabeled as we described 6 , using the following combinations of antibodies: ZIKV E (envelope) / DDX4 (germ cells marker) / Epcam (epithelial cells marker) or ZIKV E /CD14 /CD68 (monocytes/macrophages marker) / MPO (neutrophils marker). For P6 d60, we performed additional staining with ZIKV NS1/DDX4. The percentage of infected cells within each cell type (black value) was obtained by quantifying cells double-labelled for ZIKV E and a specific cell marker versus cells single-labelled for this marker. The percentage of infected germ cells, epithelial cells, monocyte/macrophages and neutrophils related to the total number of infected cells/slide (blue italic value) was obtained by quantifying cells double-labelled with ZIKV and the cell marker of interest versus cells single-labelled with ZIKV. The picture shows examples of immunofluorescence detection on semen smears of ZIKV E (red) or NS1 (red, here for P6 60 days) together with specific cell markers (green or light blue). Nuclei are stained in blue with Dapi. Scale bars = 10µm.

## II. Viral replication of SARS-CoV-2 in the testis

### Article 2:

SARS-CoV-2 replicates in the human testis ex vivo  
and infects Leydig and Sertoli cells

Mahé Dominique\*, Bourgeau Salomé\*, da Silva Janaina, Schlederer Julie, Satie Anne-Pascale,  
Kuassivi Nadège, Mathieu Romain, Guillou Yves-Marie, Le Tortorec Anna, Pasquier Christophe,  
Plotton Ingrid, Bujan Louis, Dejuq-Rainsford Nathalie and the French COVID Cohort Study Group,  
*under review at The Journal of Clinical Investigations*

## **SARS-CoV-2 replicates in the human testis *ex vivo* and infects Leydig and Sertoli cells**

**Mahé Dominique\*<sup>1</sup>, Bourgeau Salomé\*<sup>1,2</sup>, da Silva Janaina<sup>1</sup>, Schleder Julie<sup>1</sup>, Satie Anne-Pascale<sup>1</sup>, Kuassivi Nadège<sup>1</sup>, Mathieu Romain<sup>3</sup>, Guillou Yves-Marie<sup>4</sup>, Le Tortorec Anna<sup>1</sup>, Pasquier Christophe<sup>5</sup>, Plotton Ingrid<sup>6</sup>, Bujan Louis<sup>7</sup>, Dejuccq-Rainsford Nathalie<sup>1</sup> and the French COVID Cohort Study Group**

<sup>1</sup> Institut National de la Santé et de la Recherche Médicale, Ecole des Hautes Etudes en Santé Publique, Institut de recherche en santé, environnement et travail, Université de Rennes, UMR\_S1085, Rennes, France

<sup>2</sup> University of CAS, Beijing 101408, China; CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai CAS, 320 Yueyang Road, Shanghai 200031, China

<sup>3</sup> Service d'Urologie, Centre Hospitalier Universitaire de Rennes, Rennes, France

<sup>4</sup> Service de Coordination des prélèvements, Centre Hospitalier Universitaire de Rennes, Rennes, France

<sup>5</sup> Laboratoire de virologie, Centre Hospitalier universitaire de Toulouse

<sup>6</sup> Institut National de la Santé et de la Recherche Médicale, Institut Cellules Souche et Cerveau (SBRI), UMR\_S1208, Bron, France

<sup>7</sup> Inserm 1203-Université Toulouse III et Montpellier DEFE (Développement Embryonnaire, Fertilité, Environnement) et CECOS Centre hospitalier Universitaire de Toulouse

### **Abstract:**

SARS-CoV-2, a beta-coronavirus first identified in China in December 2019, is a respiratory virus that quickly spread around the world, causing the current Covid-19 pandemic. It is now clear that SARS-CoV-2 infects several organs beyond the lungs. Alteration of semen parameters, testicular morphology and hormonal regulation have been described in Covid-19 patients, but the underlying mechanisms remain unknown. ACE2, the main entry receptor for SARS-CoV-2 is highly expressed in the testis, making this organ at risk of infection. Here we aimed to determine whether SARS-CoV-2 infects the testis and directly affects testicular cell functions. Using an *ex vivo* model, we demonstrate for the first time that SARS-CoV-2 replicates in the human testis and primarily infects Leydig cells and Sertoli cells, which both express ACE2 protein. The testicular tropism of SARS-CoV-2 was confirmed *in vivo* through the detection of infected Sertoli cells exfoliated in the semen of a Covid-19 patient. The infection of testis explants triggered an antiviral orientated innate immune response, the onset of which was followed by a decline in infectious virions release. While there was no major impact of the infection on the morphology or main hormonal functions of the testis during the 9 day of culture, we observed a decline of Leydig cell steroidogenic enzymes at the end of the culture period. Hormonal measurements in Covid-19 patients without co-morbidities revealed a transient hypogonadism suggestive of primary testicular failure. Altogether, these data reveal that SARS-CoV-2 replicates in the human testis and affects Leydig cells steroidogenic machinery.

## Introduction:

SARS-CoV-2, the causative agent of the ongoing Covid-19 pandemic, is a new beta-coronavirus first identified in China in December 2019. SARS-CoV-2 has rapidly spread worldwide and infected millions of people, generating a high death toll and long-term health damages. Although this emerging virus is primarily a respiratory virus, its pathogenicity extends beyond the lungs, with alterations in particular of the gastro-intestinal and uro-genital tracts (1, 2). It has progressively become clear that SARS-CoV-2 spreads to different organs through the infection of the vasculature (3). Studies in organoids and/or in deceased patients have demonstrated that SARS-CoV-2 infects a number of tissues other than lungs, including kidney (3, 4), capillary (3) and gastro-intestinal organs (5, 6). RNA-seq databases, Human Protein Atlas and several studies have collectively shown that the transcript and protein of ACE2, the main receptor used by SARS-CoV-2 to enter host cells, are highly expressed in the testis when compared to other organs, suggesting that the testes may be at risk of infection (7, 8).

In SARS-CoV-2-infected patients, alteration of semen parameters (e.g. decreased sperm count and mobility) ((9–14), others reviewed in (15)) and male hormones (9, 16, 17), others reviewed in (18)) have been reported in a number of studies, along with some cases of scrotal discomfort/orchitis (reviewed in (15)) and damaged testicular morphology described at autopsy of deceased patients (degenerated germ cells, reduced Leydig cell numbers, damaged Sertoli cells and inflammatory infiltrates) ((9, 19), others reviewed in (15)). Most studies except a few (11, 12, 20) failed to detect SARS-CoV-2 in semen, indicating that seminal excretion is rare (reviewed in (15)). Nonetheless, SARS-CoV-2 nucleic acids and proteins have been evidenced in the testes of a subset of deceased patients (39/71 in 7 studies) (2, 9, 19, 21), suggesting this organ is a target for SARS-CoV-2. However, most of these autopsy data are tainted with issues concerning the specificity of the techniques used, blood contaminations and/or altered tissue morphology. On the other hand, the absence of SARS-CoV-2 detection in the testes post-mortem can result from windows of tissue collection too far from acute infection and/or poor tissue and viral nucleic acids preservation (22). At present, whether SARS-CoV-2 infects the testis and directly alters testis functions and morphology, or whether these alterations result from indirect effects of the infection and systemic inflammation is unknown. Findings in animal models suggest that the testis might be a target for SARS-CoV-2. In transgenic mice expressing human ACE2 under the cytokeratin-18 promoter, SARS-CoV-2 virions were visualized in the testis (Sertoli cells), lung and brain using immunofluorescence and electron microscopy at day 6 post-intranasal infection (23). SARS-CoV-2 RNA was also transiently detected in the testes of Syrian hamster (up to day 4 post-intranasal inoculation in most animals, some up to day 28) and viral replication was evidenced over 72h in hamster testes infected *ex vivo* (24). No histopathological changes accompanied the testis infection in this study (24). In contrast, other authors reported a drastic drop of sperm count and testosterone levels immediately after SARS-CoV-2 infection of Syrian hamsters, together with massive long-term testis damages, albeit only a few testicular cells were infected by the virus (25, 26). Lately, SARS-CoV-2 has been detected in the testes of macaques using PET/CT imaging and immunofluorescence one to two weeks post-infection (27). However, these animal models cannot establish SARS-CoV-2 tropism for the human testis and there is still no formal evidence that SARS-CoV-2 replicates in this organ.

In the context of random detection of SARS-CoV-2 in organs from deceased patients and lack of animal models able to fully reproduce SARS-CoV-2 pathogenesis, *ex vivo* studies have been critical for uncovering the organ and cell tropism of SARS-CoV-2 in humans (1, 28). Here, we combined our *ex vivo* model of human testis, which relevance to infer testis tropism *in vivo* has been previously validated for a range of viruses (29–31) together with semen and serum samples from patients with Covid-19 in order to determine whether SARS-CoV-2 replicates in this organ, decipher its target cells and assess the effect of the infection on testis functions.

We reveal for the first time that SARS-CoV-2 replicates in the human testis and productively infects human testosterone-producing Leydig cells and germ cells-supporting Sertoli cells, which both express the SARS-CoV-2 main receptor ACE2. Using semen smear from a man infected with SARS-CoV-2, we confirmed the virus testicular tropism through the detection of infected Sertoli cells found exfoliated in semen during the acute stage of infection. The testis tissue immune response was poorly pro-inflammatory and consisted mainly of antiviral effectors up-regulation. Its onset coincided with the peak of infectious virions production, which decreased thereafter. There was no major impact of the infection on testis morphology. However, a decrease of Leydig cells' steroidogenic enzymes was noted at the end of the culture. The analysis of testosterone and LH blood levels in seven hospitalized patients with COVID-19 evidenced a transient primary hypogonadism in the first 3-10 days post-symptoms onset. Altogether, these results indicate that SARS-CoV-2 replicates in the human testis and point at an effect on Leydig cells' steroidogenic function.

## Results:

### **SARS-CoV-2 replicates in the human testis *ex vivo*.**

Testis explants from 7 uninfected donors (age range 29 to 69) were exposed to SARS-CoV-2 (BetaCoV/France/IDF0372/2020 isolated in France in 2020) as we previously described (29). SARS-CoV-2 replication was assessed by measuring viral RNA (vRNA) and infectious virions *de novo* release in testis explants culture supernatants over 3 day-periods during 9 days of culture (i.e. testis explants supernatants collected in full every 3 days). The vRNA levels in testis explants supernatants increased from a median of  $1,32 \cdot 10^7$  copies/ml (range  $6,27 \cdot 10^6$ - $3,74 \cdot 10^7$ ) measured at 3-day post-infection (dpi) to  $5,21 \cdot 10^8$  copies/ml (range  $8,89 \cdot 10^7$ - $1,12 \cdot 10^9$ ) at 6 dpi. At 9 dpi, vRNA release stabilized at a median of  $6,4 \cdot 10^8$  copies/ml (range  $2,63 \cdot 10^8$ - $1,82 \cdot 10^9$ ). The cumulated vRNA released over the whole 9-day culture period was at a median of  $1,55 \cdot 10^9$  copies/well (range  $5,82 \cdot 10^8$ - $2,38 \cdot 10^9$ ), thus above the level of viral inoculum ( $9,97 \cdot 10^7$  copies/well) (**Figure 1A**).

The production of infectious particles was measured by plaque forming assay in reporter VeroE6 cells. SARS-CoV-2 viral titer significantly increased from a median of  $6,84 \cdot 10^2$  PFU/ml (range  $8 \cdot 10^1$ - $1,97 \cdot 10^3$ ) in supernatants collected at 3 dpi to  $1,44 \cdot 10^4$  PFU/mL (range  $3,16 \cdot 10^3$ - $8,63 \cdot 10^4$ ) in supernatants collected at 6 dpi, whereas it decreased to  $8,6 \cdot 10^2$  PFU/ml (range  $4,2 \cdot 10^2$ - $6,4 \cdot 10^3$ ) in supernatants collected at 9 dpi (**Figure 1B**). The cumulated PFU released during the 9-day culture period ranged from  $4,63 \cdot 10^3$  to  $8,89 \cdot 10^4$ /well (viral inoculum  $3,5 \cdot 10^4$  PFU/well).

These data demonstrate that SARS-CoV-2 replicates in human testis explants to reach a peak of infectious virions production in between day 3 to 6 post-infection, while viral titer declines thereafter.

### **SARS-CoV-2 primarily replicates in Leydig cells in the interstitial tissue and infects Sertoli cells within the seminiferous tubules.**

To investigate the cellular localization of SARS-CoV-2 in the testis explants during the culture time course, RNAscope in situ hybridization (ISH) was undertaken on infected (**Figure 2A-C and E-G**) and mock-infected (**Figure 2D, H**) testis explants, using probes specific to the genomic RNA strand of SARS-CoV-2 (**Figure 2A-D**) or to the replicative viral strand (**Figure 2E-H**).

In line with the significant increase of SARS-CoV-2 RNA measured by RT-qPCR in testis explants' supernatants between 3 and 9 dpi, genomic vRNA labeling increased over time in testis explants (**Figure 2A-C**). Thus, while only a few genomic vRNA+ spots were detected in the interstitial tissue (IT) at 3dpi (**Figure 2A**), a much stronger staining appeared in the interstitium at 6 dpi, along with a spotty staining inside a few seminiferous tubules (ST) (**Figure 2B**). At 9 dpi, an intense staining for SARS-CoV-2 genomic RNA was present within several seminiferous tubules and their surroundings (**Figure 2C**), showing a progression of the infection inside the tubules over time. The detection of SARS-CoV-2 infection using antibodies against the structural nucleoprotein similarly revealed a progression of the staining inside the seminiferous tubules over time (**Suppl. Figure 1A-B**).

Consistent with the significant increase in infectious particles release in testis explants' supernatants in between 3 and 6 dpi, SARS-CoV-2 replicative RNA was detected *in situ* in infected testis explants from 6 dpi onwards (**Figure 2E-G**). At 6 dpi, foci composed of one or several productively infected cells were readily detected in the interstitial tissue and close to the seminiferous tubules lined by peritubular (**Figure 2E, F**). At 9 dpi, the number of interstitial foci decreased, while the number of seminiferous tubules harboring replicative vRNA increased (**Figure 2 G, I**).

To characterize the infected cell types, RNAscope ISH for genomic (G) and replicative (R) SARS-CoV-2 RNA were combined with fluorescent immunohistochemistry (IHC) against specific cell markers in testis explants. Immunofluorescence using antibodies against SARS-CoV-2 structural proteins or replicative double stranded RNA was undertaken as a complementary approach. In the interstitial compartment, numerous Cyp11A1+ Leydig cells were positive for both SARS-CoV-2 genomic and replicative RNA (**Figure 3A-B**), whereas SARS-CoV-2 RNA did not colocalize with CD68+/CD163+ resident macrophages (**Figure 3C**). Rare  $\alpha$ -SMA+ peritubular myoid cells surrounding the seminiferous tubules stained positive for SARS-CoV-2 genomic and replicative RNA (**Figure 3D-E**). In the seminiferous tubules, the large labeling for SARS-CoV-2 genomic RNA tended to obstruct cell marker detection by IHC and we therefore favored IF for co-localization with cell markers. Vimentin+ Sertoli cells harboring SARS-CoV-2 proteins and replicative vRNA were detected mainly at 9 dpi (**Figure 3F- G**). Only rare isolated DDX4+ germ cells were infected at 9 dpi, as observed with SARS-CoV-2 proteins and double stranded RNA labeling (**Figure 3H-I**). In line with the scarce detection of infected testicular germ cells, the exposure of germ cells freshly isolated from human testis to SARS-CoV-2 did not lead to infectious titer increase during the 4 days of *in vitro* culture. Only rare primary DDX4+ germ cells stained positive for double stranded RNA, indicating that germ cells are not a key target for SARS-CoV-2 (**Suppl. Figure 2**).

Altogether, these results indicate that SARS-CoV-2 primarily replicates in Leydig cells within the interstitial tissue, before spreading more widely into the seminiferous tubules, in which Sertoli cells represent the main targets.

#### **SARS-CoV-2 receptor ACE2 is highly expressed by both Leydig and Sertoli cells in testis explants.**

The expression in the testis of the host receptors and proteases previously described or suspected to mediate SARS-CoV-2 entry was investigated using RNA sequencing (RNA-seq) databases and RT-qPCR. RNA-seq data from 4 distinct studies (32–35) (**Figure 4A**) and RT-qPCR on testis explants (**Figure 4B**) concordantly showed the expression of ACE2 and alternative receptors BSG, AXL and NRP1 mRNAs in the testis, whereas TIM-1 showed very low expression levels and was mostly below the detection threshold in RT-qPCR. The proteases CTSL and CTSB transcripts were readily measured. Furin was detected in RNA-seq and was above RT-qPCR detection threshold in 3 out of 5 testis donors, while TMPRSS2 was below RT-qPCR detection threshold in 3 out of 5 donors and showed low expression in all RNA-seq studies (**Figure 4B**).

ACE2 protein localization was examined in the testis explants of 4 donors (age range 18-51) using IHC. ACE2 staining colocalized with Cyp11A1+ Leydig cells present within the interstitial tissue and bordering the seminiferous tubules (**Figure 5A**), as well as with vimentin+ Sertoli cells inside the seminiferous tubules (**Figure 5D**). In contrast, ACE2 was not detected in CD68/CD163+ macrophages (**Figure 5B**) nor in SMA+ peritubular myoid cells (**Figure 5C**). The vast majority of germ cells did not express ACE2 (**Figure 5E**), but a few DDX4+ germ cells stained positive, as occasionally detected using confocal microscopy (**Figure 5F**). The expression of ACE2 mRNA and ACE2 protein localization remained unchanged in infected *versus* uninfected testis explants during the culture period and was similar across patients, irrespective of their age (**Suppl. Figure 3A-B**). Consistent with RNA-seq and RT-qPCR data, TMPRSS2 protein expression in the testis was undetectable using IHC on testis tissue from all donors tested (**Suppl. Figure 3C**).

In summary, these data indicate that the main target cells of SARS-CoV-2 in the testis, i.e. Leydig cells and Sertoli cells, express high levels of ACE2. In contrast, ACE2 was undetectable in myoid peritubular cells, germ cells and macrophages.

#### **SARS-CoV-2 infects Sertoli cells found exfoliated in semen *in vivo***

A semen sample was collected from a man who presented mild symptoms (loss of smell, aches, nasal congestion without fever) five days ago and tested positive for SARS-CoV-2 (nasopharyngeal test) two days post-symptoms onset. The semen sample (seminal fluid and cell fraction) was positive for SARS-CoV-2. To search for infected testicular cells shed in semen, immunofluorescence was performed on semen smears using antibodies against SARS-CoV-2 nuclear protein and either Sertoli cell marker FSH receptor or germ cell marker DDX4. Confocal microscopy analysis evidenced intra-cytoplasmic staining of SARS-CoV-2 protein within a few Sertoli cells exfoliated in semen, whereas no infected germ cells nor spermatozoa could be detected (**Figure 6**).

The presence of infected Sertoli cells in the semen from this patient is in line with the detection of SARS-CoV-2 RNA and proteins in Sertoli cells *ex vivo* and demonstrates that SARS-CoV-2 infects the testis *in vivo*.

#### **The induction of antiviral effectors in SARS-CoV-2 infected testis explants precedes the decline in infectious virions release**

We next investigated the innate immune response of the human testis to SARS-CoV-2 infection *ex vivo* by measuring a panel of innate immune effectors and sensors in RT-qPCR. A range of antiviral effector genes (OAS1, MX1, RSAD2, IFIT1, ISG15), along with vRNA sensors MDA5 and RIG-I, were increased at 6 and 9 dpi in SARS-CoV-2 infected testis explants (**Figure 7A**), except for donor (T1) with low vRNA in the explants collected at 6 dpi (**Figure 7B**) and elevated innate response in the explants collected at 3 dpi (**Figure 7A**). IFN $\beta$  and pro-inflammatory cytokines IL1 $\beta$ , IL18, IL-6, TNF $\alpha$ , CCL5 and CXCL18 were not or only weakly increased at all time points. In contrast, CXCL10 and USP18, a negative regulator of type I IFN, were up-regulated from 6 dpi onwards. In concordance with our previous quantification of vRNA in the testis explants supernatants from the same donors (Figure 1), viral RNA levels within the explant tissues were elevated from 6 dpi onwards (**Figure 7B**).

Altogether, our data show that the testis innate response to SARS-CoV-2 infection is primarily antiviral, and that its onsets precedes the decline in infectious virus production measured in related supernatants (Figure 1B).

#### **SARS-CoV-2 infection decreases Leydig cells steroidogenic enzymes *ex vivo* and affects testosterone blood levels in men**

To assess the impact of SARS-CoV-2 in the testis, we first compared tissue morphology through H&E staining, apoptosis through cleaved-caspase 3 labeling in IHC, and cell viability through LDH release in supernatants, in infected versus mock-infected testis explants from 4 to 6 donors. We did not observe a major impact of the infection on testis architecture (**Figure 8A**), apoptosis (**Figure 8B**) and overall cell viability (**Figure 8D**) over time. We next measured the release of hormones produced by Leydig cells and Sertoli cells in infected versus non-infected testis explants, and quantified a range of cell specific markers in RT-qPCR at day 3, 6 and 9 of culture. Overall, testosterone levels were not significantly affected in infected *versus* uninfected testis supernatants from 5 to 6 donors during the culture time frame (**Figure 7A**) and androgen receptor and RHOX mRNA expression, which are regulated by testosterone signaling, were not significantly modified in infected explants (**Figure 9B-C**). However, testosterone levels showed a tendency to decrease in infected versus non-infected testis explants at day 9 post-infection ( $p=0,08$ , Mann Whitney), with a decrease observed in 4 out 6 donors (decrease of testosterone release of 41,85% to 75,61% compared to mock-infected explants from the same donors). The expression of genes responsible for testicular steroidogenesis revealed a concomitant significant decrease of the steroidogenic enzymes STAR and CYP11A1 mRNAs at 9 dpi, along with a global yet non-significant decrease of other Leydig cell markers in infected explants, except for INSL3 at 6 dpi (**Figure 9D and Suppl.**



**Figure 4A&B).** Germ cell markers (PLZF for spermatogonia, PGK2 for spermatocytes and PRM2 for spermatids) were not significantly modified by the infection at any time points (**Figure 9E and Suppl. Figure 4C, D**). The level of inhibin B, an hormone produced by Sertoli cell, in testis explants supernatants (**Figure 9F**) and that of transcripts encoding inhibin B and other Sertoli cell markers (FSH receptor and ZO-1) were not significantly affected by the infection during the culture time course (**Figure 9G and Suppl. Figure 4E, F**). Labeling for ZO-1, a marker of Sertoli cell tight junctions, did not show any significant modification of its distribution in the tubules from infected *versus* mock-infected explants (**Figure 9H**).

Overall, these data indicate that SARS-CoV-2 infection of the testis *ex vivo* specifically affects Leydig cells markers.

We next assessed the effect of SARS-CoV-2 on testis hormonal functions *in vivo*, by measuring testosterone and inhibin B and their respective pituitary hormonal regulators LH and FSH in serum samples from seven hospitalized COVID-19 patients, non-obese and without comorbidities. Testosterone levels in these patients were significantly lower than the reference range established in European and North American cohorts (36) at day 1-3 post hospitalization (dph, corresponding to 3-10 days post-symptoms onset, median 8 days), but significantly increased at 9 dph (ie 11-18 days post-symptoms onset) (**Figure 10A**). For both time points, LH levels were within published normal ranges (37, 38). However, LH levels were significantly higher at 1-3 dph than 9 dph. In line, the testosterone to LH ratio was significantly lower at 1-3 dph than 9 dph. Altogether these data suggest that the low testosterone levels at 3 dph resulted from primary testis failure, as evidenced by the relatively high LH levels at 3 versus 9 dph (**Figure 10, C**). In contrast, inhibin B and FSH levels remained within published normal ranges (37–39) and showed similar values at both time points (**Figure 10D, E**).

## Discussion:

Since the onset of the COVID-19 pandemic, and despite many speculations on the infection of the human testis by its causative agent, there has been no formal evidence of the tropism of SARS-CoV-2 for this organ. In this study we reveal for the first time that a beta strain of SARS-CoV-2 replicates in the human testis *ex vivo* and primarily infects Leydig cells in the interstitial tissue and Sertoli cells within the seminiferous tubules. The detection of infected Sertoli cells in the semen of a man infected by SARS-CoV-2 and hormonal measurement suggestive of primary testicular failure in patients suffering from Covid-19 further indicate that the testis is targeted by SARS-CoV-2.

Our results confirm that ACE2, the main cell entry receptor for SARS-CoV-2, is readily detected in the testis, consistent with previous studies (40, 41), and demonstrate ACE2 protein localization in Leydig and Sertoli cells, along with rare germ cells. In addition to ACE2 binding, SARS-CoV-2 requires the action of a protease onto the Spike protein, the chief one being TMPRSS2, to enter its host cells. In scRNA-seq studies, the detection of TMPRSS2 transcript has been restricted to spermatids and spermatogonia, with very little or absence of co-expression of TMPRSS2 and ACE2 (40, 42). Using IHC, we failed to detect TMPRSS2 in our human testis explants and only very levels of transcripts were measured by RT-qPCR. Altogether these data suggest that other proteases are responsible for SARS-CoV-2 infection of testicular cells. Furin has been proposed as an alternative protease for SARS-CoV-2 entry (43, 44), being able to cleave both the “priming site” between the S1 and S2 subunit of the Spike protein (45, 46), and the S2’ “triggering site” unveiling the fusion peptide responsible for viral entry. While our RT-qPCR on testis explants and published scRNA-seq data showed relatively low levels of Furin transcripts in the testis and in Leydig cells, respectively (41), there is indirect evidence of functional Furin in Leydig cells, as we previously demonstrated the efficient infection of Leydig cells by Mumps virus (47), which requires for its entry the action of Furin (48). Therefore, the infection of Leydig cells by SARS-CoV-2 observed *in situ* in testis explants from 3 dpi onwards might be supported by the cooperation of ACE2 and Furin at the plasma membrane. SARS-CoV-2 entry has also been found to occur in TMPRSS2 negative cells through the endocytosis of virions and processing of the viral spike protein by endolysosomal proteases such as CTSL (43). CTSL mRNA is readily detected in the testis, as showed by our RT-qPCR and by published RNA-seq data, and its protein has been localized in Sertoli cells and germ cells (49). The endolysosomal entry mechanism has been described as a “late pathway” of infection, being slower than the fusion at the plasma membrane triggered by TMPRSS2 (50). We hypothesize that a less efficient entry pathway might play a part in the delayed infection of Sertoli versus Leydig cells in testis explants. Rare peritubular myoid cells harbored SARS-CoV-2 RNA in our cultures despite their lack of ACE2 expression. Several alternative receptors have been proposed for SARS-CoV-2 including NRP1 (51), AXL (52) and BSG (53), which are expressed by peritubular cells along with CTSL and CTSB according to published scRNA-seq data (54). Alternatively, as spike-mediated cell-to-cell transmission was recently found to represent a critical feature in SARS-CoV-2 infection (55), SARS-CoV-2 entry could occur in a receptor-independent way, as demonstrated for other coronaviruses (46). In this scenario, SARS-CoV-2 virions budding from Leydig cells in close proximity to the seminiferous tubules could infect peritubular myoid cells without the need of ACE2.

The relevance for Covid-19 infected men of our *ex vivo* findings of testis infection by a beta strain of SARS-CoV-2 is supported by the presence of SARS-CoV-2 nucleocapsid protein in Sertoli cells exfoliated 5 days post-symptoms onset in the semen from a man with mild symptoms, who tested positive in December 2020. We previously reported that testicular germ cells, which are sheltered from acquired immunity in the testis, are persistently infected by ZIKV in the semen from men who recovered from the infection (30). Here we did not detect any SARS-CoV-2 positive testicular germ cells in the semen of the patient tested, which is in line with the rarity of germ cell infection in the testis explants. While most authors have failed to detect SARS-CoV-2 in the fluid fraction of semen (composed at 90% of seminal vesicles and prostatic secretions), some studies reported the presence of SARS-CoV-2 RNA in whole semen (i.e. seminal fluid and cell fraction) from a subset of individuals during the acute stage

of infection (11, 12, 20, 56) and up to 81 days post symptoms in one convalescent man recovering from a severe Covid-19 (20). While two independent studies failed to detect SARS-CoV-2 in prostatic fluid (15), further investigations are needed to explore the infection of the male genital tract by SARS-CoV-2 and determine whether other semen-producing organs beside the testes are infected. Nevertheless, according to current reports, the seminal shedding of SARS-CoV-2 appears infrequent and no infectious viral particles have been rescued from semen (20), suggesting that sexual transmission is unlikely.

Interestingly, we observed a significant decrease in the release of infectious SARS-CoV-2 virions by testis explants between 6 and 9 dpi (median 4,14log PFU/ml decrease equivalent to 94% decrease), which contrasts with our previous finding of sustained production of infectious ZIKV by testis explants and by isolated germ cells (29). The analysis of variants other than the beta strain of SARS-CoV-2 used in our study would be of interest to compare potential modification of cell tropism in the testis but was not possible within the time frame of this study due to limited access to testis samples. Of note a study using different strains of SARS-CoV-2 in Syrian hamsters (Wuhan strain, delta and omicron) showed similar alterations in the testis (11). The infectious titer decrease in testis explants over time might be due to several non-exclusive mechanisms, including cell death or metabolic dysfunction by the main producing cell population, efficient control of viral replication by the cellular antiviral response, and failure to produce mature virions by the main cell types infected at these later time points, i.e. Sertoli cells. Since no increased cell mortality was observed in infected explants over time, as assessed by LDH release and *in situ* detection of cleaved-caspase 3, the cellular antiviral response could be at play. SARS-CoV-2 was previously found to be remarkably sensitive to the cell antiviral responses even once infection is established (57, 58) and antiviral responses mediated by type I IFN are crucial for the successful control of SARS-CoV-2 infection in patients (59). It is noteworthy that the decrease in infectious virions occurred just after the induction of antiviral effectors in the testis explants. Among the ISGs induced in the testis, OAS1 and Mx1 are strong inhibitors of SARS-CoV-2 replication in patients (60). A negative impact of SARS-CoV-2 infection on the metabolism of productively infected cells, as suggested by the trend for decreased expression of Leydig cell markers at 9 dpi in RT-qPCR, might additionally participate to the infectious titer decrease. Another potential mechanism underlying the limited infectivity of testis supernatants at day 9 could be an impaired release of infectious viral particles by Sertoli cells, which represent the main cell type harboring replicative viral RNA *in situ* at this time point. In line with this hypothesis, Campos et al. showed that a commercial human Sertoli cell line only produced low titers of SARS-CoV-2 (24). Moreover, several elements from the literature suggest that SARS-CoV-2 may only transiently infect the testis *in vivo*. In a model of Syrian hamsters infected *in vivo*, SARS-CoV-2 was detected in testes up to 4 dpi by RT-qPCR in all animals but resolved thereafter in most animals (61), whereas lungs were infected for longer duration and to higher levels. In patients, the inconsistent detection of viral material in testes at autopsy (2, 9, 19, 21) and of vRNA shedding in semen ((11, 12, 20) ; reviewed in (15)) also suggest that SARS-CoV-2 does not replicate for extended duration in the male genital tract. Our observation of decreased virions production during the testis culture time span supports this notion, although cases of viral persistence may occur, as reported in one patient up to 81 days post-symptoms (20).

In infected testis explants, the transcripts encoding MDA5 (considered the most important for sensing CoV RNA), ISG-15 (which activates MDA5 in the context of SARS-CoV2 infection), (reviewed in (60, 62) and RIG-I (which role during SARS-CoV-2 infection is controversial) increased from 6 dpi, indicating activation of pathogen sensing pathways by viral replication. LGP2, a potentiator of MDA5-mediated IFN response (62), was inconsistently increased in the explants. In contrast, TLR3, another dsRNA sensor, and TLR2, which sensing of SARS-CoV-2 envelope protein was required for inflammatory cytokines release in patients (63), were not up-regulated. The absence of a consistent and strong transcriptional induction of leading pro-inflammatory cytokines such as IL-1b, IL-6 and TNF $\alpha$  indicated a minimal pro-inflammatory response in testis explants infected by SARS-CoV-2. As for ZIKV, CXCL-10 was the only pro-inflammatory chemokine notably up-regulated by SARS-CoV-2 infection of the testis *ex vivo* (29). There was little to no IFN $\beta$  mRNA up-regulation in infected testis explants, which is similar

to findings in other *ex vivo* models infected by SARS-CoV-2 (60). This could be due to a disruption of the IFN response by SARS-CoV-2, which proteins were found to reduce or delay IFN expression through a range of mechanisms (62, 64). Of note, we observed a similar lack of IFN $\beta$  induction in testis explants infected with Zika (29). This lack of IFN induction could thus also represent a specific feature necessary to the homeostasis of the human testis, since an over-expression of IFN $\beta$  in this organ triggers germ cell apoptosis and sterility (65). Nevertheless, despite poor IFN $\beta$  induction, ISGs such as OAS1 and Mx1 were up-regulated in testis explants, as in lung organoids (66) and critically ill patients (67). In animal models, an IFN-independent induction of ISGs has been reported upon SARS-CoV-2 infection ((60) for review), while in human cells, IFN signaling was impaired by SARS-CoV-2, which notably involved host retro-control enhancement (64). Both USP-18 and ISG15, which act together as negative regulators of IFN signaling (68), were increased in infected testis explants, whereas ACE2 mRNA expression, which is directly stimulated by IFN (69), was unchanged, which support an IFN-independent induction of antiviral effectors in the testis. Interestingly, the detection of ISGs upregulation in SARS-CoV-2 infected testis explants by RT-qPCR occurred from 6 dpi onwards in most donors and preceded the decline in infectious viral particles release, although we could not establish correlations between infection and ISGs mRNA levels.

Despite active replication in our *ex vivo* model, the overall testis tissue morphology and viability were unmodified by the SARS-CoV-2 beta strain infection during the 9-day culture time frame. Even in highly infected areas, the *in-situ* detection of cleaved-caspase 3 positive cells did not reveal increased apoptosis. The study of the replication capacities and effects on testis functions of other SARS-CoV-2 variants would be of interest to assess potential differences. In deceased patients who suffered from severe COVID-19, severe morphological damages including germ cell degeneration, Sertoli cell vacuolization and detachment from tubular basement membrane indicative of testis blood barrier disruption have been reported, along with immune cell infiltrates (9, 19, 70). However, SARS-CoV-2 nucleic acids or proteins have only been occasionally detected in the testis of these patients (2, 9, 19, 21) suggesting either an indirect effect of the infection or a long-term consequence of previous replication in the testis. Similar testicular damages and infiltrates of immune cells in the interstitium and seminiferous tubules, were recently described in the early stage of SARS-CoV-2 infection in Syrian hamsters despite a low frequency of infected cells and irrespective of the SARS-CoV-2 strains used (25), as well as in one SARS-CoV-2 infected macaque (27). In contrast, in another study in Syrian hamster, in which SARS-CoV-2 was transiently detected in the testis, no histopathological changes were noted (24). It is established that inflammation of the testis (orchitis) can disrupt the blood-testicular barrier and break the immune-privilege, leading to autoimmunity against spermatogenic cells, as exemplified following infection by Mumps virus, a testis-tropic virus (71). Orchitis, which has been occasionally reported in patients infected with SARS-CoV-2, can also occur in the presence of elevated systemic inflammation following infection with non-testis tropic virus such as Influenza virus (71). In moderate to severely ill patients with Covid-19, the association between elevated markers of systemic inflammation and low levels of testosterone levels concomitantly to low levels of LH further point at an indirect effect of the infection onto the testis (17, 19, 72). These elements together with our *ex vivo* findings suggest that secondary effects of the infection, such as prolonged high levels of systemic inflammation and medications, represent the most likely culprits for the drastic alterations of testicular morphology and function observed in patients with severe Covid-19. Nevertheless, a direct effect of SARS-CoV-2 replication onto the testis should not be ruled out. A significant decrease in Leydig cells' steroidogenic enzymes was observed towards the end of the culture in infected testis explants, suggesting a direct effect of the SARS-CoV-2 strain onto this infected cell type. Moreover, our analysis of blood testosterone and LH levels in seven hospitalized patients suffering from Covid-19 without co-morbidities showed low testosterone levels and low testosterone to LH ratio in the first few days post-symptoms onset, suggesting a testicular origin. This alteration was transient, as testosterone levels and testosterone to LH ratio resumed to normal ranges within a week. Other authors have reported elevated LH concentrations associated with low to normal testosterone levels (the latter

defined as compensated hypogonadism) in a subset of Covid-19 patients, indicating primary testicular failure (15, 17). In contrast, Sertoli cell markers including inhibin B release were unaffected in infected testis explants, and our measurements of inhibin B and FSH in patients during the early stage of infection showed values within normal ranges that remained stable at the two time points tested, unlike testosterone levels. In large cohorts of men with moderate to severe Covid-19, FSH levels globally remained within normal values during the acute stage of infection (16) and inhibin B was unchanged in most patients (73). Overall these data suggest a transient effect of SARS-CoV-2 on Leydig cells. Given the anti-inflammatory properties of testosterone, an early decrease in testosterone might contribute to elevated inflammatory level. How SARS-CoV-2 impacts Leydig cells is unknown. A possibility might be an effect mediated by ACE2 internalization and downregulation at the surface of infected Leydig cells upon SARS-CoV-2 binding, as described in other models (74). ACE2 plays a key role in the equilibrium of the renin-angiotensin system (RAS), a system of finely tuned agonists and antagonists that regulate blood pressure. In addition to ACE2, several RAS components such as Mas, a G protein coupled receptor, have been described in the testis and notably in Leydig cells (75, 76). While the role of RAS in the physiology of the testis is unclear, the deletion of Mas in mice affected the expression of steroidogenic enzymes such as STAR (77), which was also decreased in our model. In addition to the modulation of testosterone production, a suspected physiological function of ACE2 in Leydig cells is to balance the interstitial fluid volume by regulating the local vascular system (78). This function could obviously not be assessed in our *ex vivo* model but its modification might impact testis morphology *in vivo*. Of note, the expansion of the interstitial space observed in the testis of SARS-CoV-2 infected Syrian hamsters, an indicator of an elevated volume of testis interstitial fluid, suggested increased testis capillaries permeability (24, 79). This might favor the infiltration of leukocytes in the interstitial space and in turn testis inflammation. The downregulation of ACE2 expression in Leydig cells has also been correlated with spermatogenesis impairment in men (78). In line, alterations of sperm parameters (e.g. sperm conc, sperm number, motility) have been reported in patients with Covid-19 even with mild symptoms (13), although these alterations were more pronounced in severe cases (10, 15). Sperm alterations were maintained at distance from acute infection in one longitudinal study on 5 patients (14). However, beside a potential direct effect of virus infection of the testis, acute illness with fever, one of the most common symptoms of COVID-19 may be involved in those alterations, as elevated body temperature is known to affect spermatogenesis (71). The medications used for the treatment of COVID-19 can additionally have adverse effect on semen parameters (18).

In conclusion, our study demonstrates for the first time that a beta strain of SARS-CoV-2 replicates in the human testis and that the virus may directly affect testicular functions. These data pave the ways for further studies to evaluate the impact of testis infection by contemporary strains of SARS-CoV-2 and its consequences on fertility. The long-term follow-up of semen parameters and hormonal levels in infected men with mild symptoms or asymptomatic (i.e. without high inflammation and fever or medication) are warranted.

## Materials and methods:

### Virus

The SARS-CoV-2 beta strain used was isolated during the 2020 outbreak in France and passaged three times in Vero cells (BetaCoV/France/IDF0372/2020, European Virus Archive). VeroE6 cells were maintained as already described (29). To produce viral stocks, VeroE6 cells were infected at a MOI of  $10^{-4}$  in serum free medium with  $1\mu\text{g/ml}$  TPCK-trypsin (Sigma-Aldrich) for 2 hours and cultured for 3 days in complete medium at a final serum concentration of 5%. Supernatants were then harvested, centrifuged, filtered ( $0,45\ \mu\text{m}$ ), aliquoted and frozen at  $-80^{\circ}\text{C}$ .

### Organotypic culture of human testis explants and infection

Human testis samples, obtained from deceased ( $n=6$ ) or prostate cancer patients ( $n=1$ ) at Rennes University Hospital, were dissected into  $3\text{mm}^3$  explants and cultured as we previously described (29). For infection, explants (4 sections/well) were transferred in  $500\ \mu\text{l}$  of serum free culture medium in the presence or absence of  $10^5$  TCID<sub>50</sub> SARS-CoV-2 ( $7\times 10^4$  PFU corresponding to  $2,9\times 10^8$  vRNA copies). After an overnight incubation, explants were washed 3 times in PBS, transferred onto polyethylene terephthalate insert ( $3\ \mu\text{m}$  high-density pores) in 12-well plates (2 explants/well) containing 1 ml of culture medium. Six hours later, the medium was changed again to further wash away potential residual virus input (time 0 for sample collection). For each experimental condition, 3 wells were tested and explants were cultured and processed as we previously described (29).

### Isolation and infection of testicular germ cells

Primary testicular cells (TGC) were prepared as we previously described (29). Upon isolation and overnight culture, non-adherent germ cells were collected and incubated in the presence or absence of SARS-CoV-2 at an MOI of 1 (corresponding to  $1.4 \times 10^6$  TCID<sub>50</sub>/million cells) for 2 hours at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . Cells were trypsinized for 5 min at  $37^{\circ}\text{C}$  before inactivation and then cultured onto 6-well plates at a density of 1 million cells per ml in supplemented StemPro-34 (Invitrogen).

### Patients samples

A semen sample was collected 5 days post-symptoms onset from a man with mild symptoms infected with SARS-CoV-2 in December 2020. Semen smears were prepared as we previously described (29) and the semen sample frozen (GERMETHEQUE national Biobank BB-0033-00081) until viral analyze. The Ct values for SARS-CoV-2 in seminal plasma and seminal cells were 20.5 and 21 respectively.

For hormonal assays, serum samples were obtained from a subset of seven patients without co-morbidities (age range 35-51 years old) enrolled in the C20-05 French Covid Cohort, a cohort of COVID-19 hospitalized patients (NCT04262921, (80)) between March 2020 and January 2021 in hospitals throughout France upon SARS-CoV-2 positive RT-qPCR in nasopharyngeal swabs. Recorded information included time since symptom onset (3-12 days with a median of 8 days), absence of co-morbidities, BMI (under 30) as well as clinical and biological markers. All patients were discharged alive from hospital except for one, who died due to pulmonary fibrosis following a discontinuation of treatment a month after admission.

### RT-qPCR

Total RNA was extracted using QIAmp vRNA (for supernatants) and RNeasy isolation kit (for tissues and cells), and treated with DNase (all from QIAGEN). RNA extracted from culture supernatants was subjected to RT-qPCR using GoTaq Probe 1-Step RT-qPCR System (Promega) and SARS-CoV-2 primers and probes as follows: HKU-ORF1b-nsp14 forward TGGGGTTTTACAGGTAACCT, reverse AACACGCTTAACAAAGCACTC and probe TAGTTGTGATGCAATCATGACTAG (81). A full-length viral genome RNA (EVA, isolate SARS-CoV-2/human/ITA/INMI1/2020) was used as standard and serial dilutions of a known number of copies of vRNA was systematically run. Primers for the relative quantification of

steroidogenesis enzymes mRNA, testicular cell markers mRNA, and innate immune response effector genes mRNA are listed in the **Supplementary Table 1** and were either used previously (29) or designed using the Primer-BLAST tool. Total RNA extracted from tissues and cells was reverse transcribed using the iScript cDNA Synthesis Kit and then subjected to RT-qPCR using the iTaq SYBR green mix (all from Bio-rad) as previously described (29).

#### **Quantification of infectious viral titer**

Infectious viruses were quantified by plaque assay as described (82). Briefly,  $2.5 \times 10^5$  VeroE6 cells were seeded in 12-well plates and allowed to grow to confluence for 24h. Medium was then removed, and cells were incubated with 125 $\mu$ l of serial dilutions of culture supernatants for 1 h at 37°C. Cells were overlaid with 0.8ml of a 1:1 mixture of DMEM 2x with 4%SVF and 2%Methylcellulose in ddH<sub>2</sub>O and incubated at 37°C for 4 days. The overlay media were washed away and cells were fixed in 4% paraformaldehyde for 1h before staining with a 1% (w/v) crystal violet solution in 20% ethanol. The number of plaques was then counted and virus titer was defined as the number of plaque-forming units per ml.

#### **RNAscope ISH, IHC and histology assessment**

Testis explants were fixed in 4% formaldehyde and embedded in paraffin. RNAscope in situ hybridization (ISH) and dual fluorescence RNAscope ISH-IHC were performed as previously described (29). For RNAscope, antisense probes (nucleotide 21,563-25,384, catalog 848561) and sense probes (nucleotide 21,631-23,303, catalog 845701) targeting the viral spike encoding sequence were obtained from Advanced Cell Diagnostics. For dual fluorescence RNAscope ISH-IHC or single IHC, antibodies and specific conditions are specified in the **Supplementary Table 2**. Sections of mock-infected testis explants or sections stained with isotype antibody control were systematically used as negative control. Colorimetric stained sections were examined and photographed under a light microscope (Olympus BX51) or captured with a scanner NanoZoomer 2.0 RS (Hamamatsu, Tokyo, Japan) at 40 $\times$  magnification. Fluorescent images were acquired with the Zeiss Axio Imager system connected to Zen software or with the SP8 confocal system (Leica) connected to LAS software and analyzed using Fiji software.

#### **Bioinformatic analysis of RNA-sequencing data**

Adult human testis bulk RNA-sequencing (32–35) datasets were obtained from the ReproGenomic Viewer website (54) and were plotted using average FPKM values of all individual samples for each dataset.

#### **Tissue viability assay**

Global tissue viability was assessed by measuring the lactate dehydrogenase (LDH) release in culture medium using the enzymatic fluorometric assay CytoTox-ONE Homogeneous Integrity Assay (Promega) according to the manufacturer's instructions.

#### **Hormones quantification**

Total testosterone (T) blood concentration was measured using an in-house liquid chromatography-mass spectrometry after liquid extraction using Agilent's 1290 Infinity II LC System and MSMS 6495. Mean intra-assay coefficients of variation were 3.7 and 3.3% for T concentrations of 0.080 and 0.274 nmol/l respectively; inter-assay coefficients of variation were 4.71 and 5.98% for T concentrations of 2.254 and 8.934 nmol/L, respectively. Inhibin B blood concentration was measured using Beckman Coulter's Gen II ELISA Kit (DSL-10684100) according to the manufacturers' instructions. The limit of quantification was 5ng/L. Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) blood concentration were assayed by Abbott's ARCHITECT kits and according to manufacturer's instructions. The coefficients of variation at 20mIU/L were respectively 3.6% and 6.9% for LH and 1.9% and 7.6%, for FSH.

#### **Immunocytofluorescence**

Semen smears from donors and testicular germ cells put onto polylysine-coated glass coverslips, were fixed in 4% paraformaldehyde for 20 min at room temperature. Immunocytofluorescence was performed as previously described (29). Viral material was detected with antibodies against SARS-CoV-2 nucleoprotein (1µg/ml, GeneTex, 3851) or viral double strand dsRNA (1µg/ml, anti-dsRNA J2, Scicons). Infected cell characterization was performed using rabbit anti-DDX4 (5µg/ml, Abcam, ab13840) and anti-FSHR (10µg/ml, Origene, TA313897). Isotype control antibodies or non-infected cells were used as negative controls. Images were acquired with the SP8 confocal system (Leica) connected to LAS software and analyzed using Fiji software.

### **Statistics**

When comparing independent sets of samples, we used unpaired Mann-Whitney tests (two sets to compare) or Kruskal-Wallis tests followed by Dunn tests (more than two sets to compare). When repeated measurements along time data were paired, Wilcoxon signed-rank test (for two repetitions), or Friedman test followed by Dunn test (more than two repetitions) were implemented. Data expressed as fold change (ratio between infected and their respective control) were compared to 1 using One Sample Wilcoxon test. Patient hormonal levels, when outside of the normal range, were compared to the minimal reference values using One Sample Wilcoxon test. Statistical significance threshold was set at 0.05. All statistical analyzes were performed using GraphPad Prism 6 software and are specified in the figure legends.

### **Study approval**

Normal testes were obtained at autopsy of organ donors, or for one patient, after orchiectomy, and processed within 2 hours of surgery. The procedure was approved by Ethics Committee Ouest V, Rennes, France (authorization DC-2016-2783) and the French National Agency for Bio- medical Research (authorization PF S09-015). Semen sample produced by masturbation was obtained from a SARS-CoV-2 infected donor living in France, at day 5 after onset of symptoms upon written informed consent (clinical study approved by the institutional ethics review board: CPP Sud Méditerranée III, 2020.06.04 bis\_ 20.05.29.61705). Blood samples for hormone assays were obtained from the French COVID cohort (authorization 2020-A00256-33) after consent of each participant or its surrogate.

### **Author contributions:**

NDR designed the study, raised funding, contributed to critical analysis of the data and wrote the manuscript. DM and SB performed most experiments, analyzed data and wrote the manuscript. JdS performed experiments and analyzed data. RM contributed to testis samples. JS, NK and ALT contributed to experiments and tools. CP performed virological analyses. IP performed hormonal assays. LB contributed to study design and semen samples collection and analyses.

### **Acknowledgments:**

This project was funded by MSD and Inserm-REACTing. SB received stipends from Rennes University and the CAS-TWAS president's international fellowship initiative. JdS was supported by MSD funding.

We are most grateful to all the team of the "Service de coordination des prélèvements" at Rennes University hospital and to the organ donors and their families. We thank Nathalie Costet for advice on statistical analyses.

The authors thank the GERMETHEQUE national Biobank and particularly Nathalie Moinard PhD, Noéline Merle and Myriam Daudin MD for patient and sample management. Jean Michel Mansuy PhD is also acknowledged for patient semen viral analyzes.

We thank the Scientific Advisory Board and the members of the C20-05 French Covid Cohort study group for providing raw data and clinical and biological data, as well as the participants and patients who participated to this study. The French COVID cohort investigators group



includes Marie BARTOLI, Alpha DIALLO (ANRS, Paris, France), Soizic LE MESTRE, Christelle PAUL, Ventzislava PETROV-SANCHEZ (ANRS-MIE, Paris, France), Catherine CHIROUZE (CHRU Jean Minjot, Besançon, France), Claire ANDREJAK (CHU Amiens, France), Denis MALVY (CHU Bordeaux, France), François GOEHRINGER, Patrick ROSSIGNOL (CHU Nancy, France), Manuel ETIENNE (CHU Rouen, France), Tristan GIGANTE, Morgane GILG, Bénédicte ROSSIGNOL (F-CRIN INI-CRCT, Nancy, France), Marine BELUZE (F-CRIN Partners Platform, Paris, France), Delphine BACHELET, Krishna BHAVSAR, Lila BOUADMA, Minerva CERVANTES-GONZALEZ, Anissa CHAIR, Charlotte CHARPENTIER, Léo CHENARD, Camille COUFFIGNAL, Marie-Pierre DEBRAY, Diane DESCAMPS, Xavier DUVAL, Philippine ELOY, Marina ESPOSITO-FARESE, Aline-Marie FLORENCE, Jade GHOSN, Isabelle HOFFMANN, Ouifiya KAFIF, Antoine KHALIL, Nadhem LAFHEJ, Cédric LAOUÉNAN, Samira LARIBI, Minh LE, Quentin LE HINGRAT, Sophie LETROU, France MENTRÉ, Gilles PEYTAVIN, Valentine PIQUARD, Carine ROY, Marion SCHNEIDER, Richa SU, Coralie TARDIVON, Jean-François TIMSIT, Sarah TUBIANA, Benoit VISSEAU (Hôpital Bichat, Paris, France), Dominique DEPLANQUE (Hôpital Calmette, Lille, France), Jean-Sébastien HULOT (Hôpital Européen Georges Pompidou, Paris, France), Olivier PICONE (Hôpital Louis Mourier, Colombes, France), François ANGOULVANT (Hôpital Necker, Paris, France), Amal ABROUS, Sandrine COUFFIN-CADIERGUES, Fernanda DIAS DA SILVA, Hélène ESPEROU, Ikram HOUAS, Salma JAAFOURA, Aurélie PAPADOPOULOS (Inserm sponsor, Paris, France), Alexandre GAYMARD, Bruno LINA, Manuel ROSA-CALATRAVA (Inserm UMR 1111, Lyon, France), Céline DORIVAL (Inserm UMR 1136, Paris, France), Jérémie GUEDJ, Guillaume LINGAS, Nadège NEANT (Inserm UMR 1137, Paris, France), Laurent ABEL (Inserm UMR 1163, Paris, France), Sylvie BEHILILL, Vincent ENOUF, Hugo MOUQUET, Sylvie VAN DER WERF (Pasteur Institute, Paris, France), Yves LEVY, Aurélie WIEDEMANN (Vaccine Research Institute (VRI), Inserm UMR 955, Créteil, France). The French Covid Cohort is sponsored by the Institut national de la santé et de la recherche médicale (INSERM) and funded through the REACTing (REsearch & ACTion emergING infectious diseases) consortium, the Ministry of Health and Social Affairs, the Ministry of Higher Education and Research dedicated COVID19 fund and PHRC n°20-0424. Funding sources are not involved in the study design, data acquisition, data analysis, data interpretation or manuscript writing.

Experiments were conducted in part on L3, MRic, and H2P2 platforms at Biosit federative structure (Université de Rennes, CNRS, Inserm, Biosit [Biologie, Santé, Innovation Technologique de Rennes] – UMS 3480, US\_S 018).

Address correspondence to : Nathalie Dejuccq-Rainsford, IRSET-Inserm U1085, 9 avenue du Pr Léon Bernard, F-35000 Rennes, France. Phone : 33.2.2323.5069 ; Email : [nathalie.dejuccq-rainsford@univ-rennes1.fr](mailto:nathalie.dejuccq-rainsford@univ-rennes1.fr)

## References

1. Gausson A et al. Evidence of SARS-CoV-2 Infection in Cells, Tissues, and Organs and the Risk of Transmission Through Transplantation. *Transplantation* 2021;105(7):1405–1422.
2. Yao XH et al. A cohort autopsy study defines COVID-19 systemic pathogenesis. *Cell Res.* 2021;31(8):836–846.
3. Monteil V et al. Inhibition of SARS-CoV-2 Infections in Engineered Human Tissues Using Clinical-Grade Soluble Human ACE2. *Cell* 2020;181(4):905-913.e7.
4. Puelles VG et al. Multiorgan and Renal Tropism of SARS-CoV-2. *N. Engl. J. Med.* 2020;383(6):590–592.
5. Zhou J et al. Infection of bat and human intestinal organoids by SARS-CoV-2. *Nat. Med.* 2020;26(7):1077–1083.
6. Lamers MM et al. SARS-CoV-2 productively infects human gut enterocytes. *Science* 2020;369(6499):50–54.
7. Stanley KE, Thomas E, Leaver M, Wells D. Coronavirus disease-19 and fertility: viral host entry protein expression in male and female reproductive tissues. *Fertil. Steril.* 2020;114(1):33–43.
8. Wang Z, Xu X. scRNA-seq Profiling of Human Testes Reveals the Presence of the ACE2 Receptor, A Target for SARS-CoV-2 Infection in Spermatogonia, Leydig and Sertoli Cells. *Cells* 2020;9(4). doi:10.3390/CELLS9040920
9. Enikeev D et al. Prospective two-arm study of testicular function in patients with COVID-19. *Andrology* [published online ahead of print: February 6, 2022]; doi:10.1111/ANDR.13159
10. Holtmann N et al. Assessment of SARS-CoV-2 in human semen—a cohort study. *Fertil. Steril.* 2020;114(2):233–238.
11. Li D, Jin M, Bao P, Zhao W, Zhang S. Clinical Characteristics and Results of Semen Tests Among Men With Coronavirus Disease 2019. *JAMA Netw. open* 2020;3(5):e208292.
12. Gacci M et al. Semen impairment and occurrence of SARS-CoV-2 virus in semen after recovery from COVID-19. *Hum. Reprod.* 2021;36(6):1520–1529.
13. Pazir Y et al. Impaired semen parameters in patients with confirmed SARS-CoV-2 infection: A prospective cohort study. *Andrologia* 2021;53(9). doi:10.1111/AND.14157
14. Best JC et al. Evaluation of SARS-CoV-2 in Human Semen and Effect on Total Sperm Number: A Prospective Observational Study. *World J. Mens. Health* 2021;39(3):489–495.
15. He Y et al. Effect of COVID-19 on Male Reproductive System - A Systematic Review. *Front. Endocrinol. (Lausanne).* 2021;12. doi:10.3389/FENDO.2021.677701
16. Ma L et al. Evaluation of sex-related hormones and semen characteristics in reproductive-aged male COVID-19 patients. *J. Med. Virol.* 2021;93(1):456–462.
17. Schroeder M et al. High estradiol and low testosterone levels are associated with critical illness in male but not in female COVID-19 patients: a retrospective cohort study. *Emerg. Microbes Infect.* 2021;10(1):1807–1818.
18. Guo J, Sheng K, Wu S, Chen H, Xu W. An Update on the Relationship of SARS-CoV-2 and Male Reproduction. *Front. Endocrinol. (Lausanne).* 2021;12. doi:10.3389/FENDO.2021.788321
19. Ma X et al. Pathological and molecular examinations of postmortem testis biopsies reveal SARS-CoV-2 infection in the testis and spermatogenesis damage in COVID-19 patients. *Cell. Mol. Immunol.* 2021;18(2):487–489.
20. Purpura LJ et al. SARS-CoV-2 RNA Shedding in Semen and Oligozoospermia of Patient with Severe Coronavirus Disease 11 Weeks after Infection. *Emerg. Infect. Dis.* 2022;28(1):196.
21. Poma AM et al. COVID-19 autopsy cases: detection of virus in endocrine tissues. *J. Endocrinol. Invest.* 2022;45(1):209–214.
22. Song C et al. Absence of 2019 novel coronavirus in semen and testes of COVID-19 patients†. *Biol. Reprod.* 2020;103(1):4–6.
23. Ullah I et al. Live imaging of SARS-CoV-2 infection in mice reveals that neutralizing antibodies require Fc function for optimal efficacy. *Immunity* 2021;54(9):2143-2158.e15.
24. Campos RK et al. SARS-CoV-2 Infects Hamster Testes. *Microorganisms* 2021;9(6).

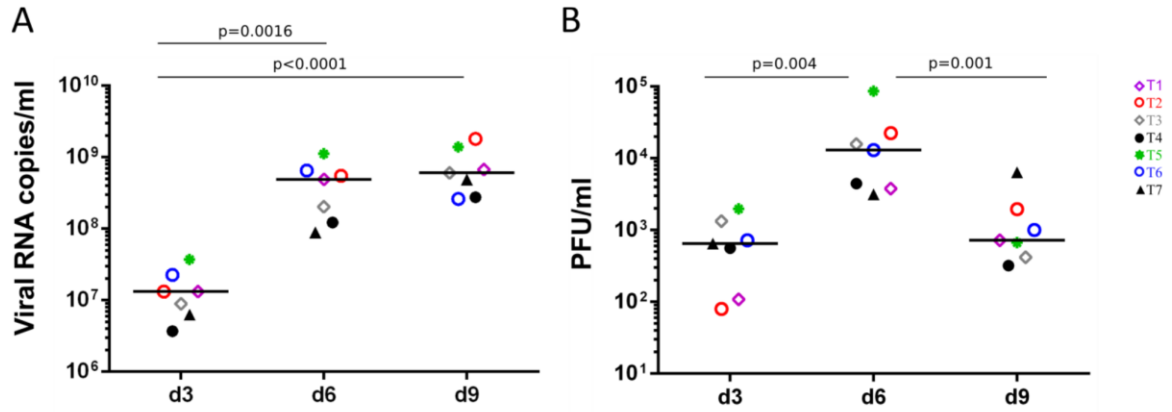
doi:10.3390/MICROORGANISMS9061318

25. Li C et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections by intranasal or testicular inoculation induces testicular damage preventable by vaccination in golden Syrian hamsters. *Clin. Infect. Dis.* [published online ahead of print: February 18, 2022]; doi:10.1093/CID/CIAC142
26. Dejuqc-Rainsford N. Is SARS-CoV-2-induced testicular damage in hamsters relevant?. *Nat. Rev. Urol.* 2022;1.
27. Madden P et al. An immunoPET probe to SARS-CoV-2 reveals early infection of the male genital tract in rhesus macaques. *Res. Sq.* [published online ahead of print: April 8, 2022]; doi:10.21203/RS.3.RS-1479315/V1
28. Clevers H. COVID-19: organoids go viral. *Nat. Rev. Mol. Cell Biol.* 2020;21(7):355–356.
29. Matusali G et al. Zika virus infects human testicular tissue and germ cells. *J. Clin. Invest.* 2018;128(10):4697–4710.
30. Mahé D et al. Long-term Zika virus infection of non-sperm cells in semen. *Lancet Infect. Dis.* 2020;20(12):1371.
31. Roulet V et al. Susceptibility of human testis to human immunodeficiency virus-1 infection in situ and in vitro. *Am. J. Pathol.* 2006;169(6):2094–103.
32. Jégou B, Sankararaman S, Rolland AD, Reich D, Chalmel F. Meiotic Genes Are Enriched in Regions of Reduced Archaic Ancestry. *Mol. Biol. Evol.* 2017;34(8):1974–1980.
33. Djureinovic D et al. The human testis-specific proteome defined by transcriptomics and antibody-based profiling. *Mol. Hum. Reprod.* 2014;20(6):476–488.
34. Soumillon M et al. Cellular Source and Mechanisms of High Transcriptome Complexity in the Mammalian Testis. *Cell Rep.* 2013;3(6):2179–2190.
35. Brawand D et al. The evolution of gene expression levels in mammalian organs. *Nature* 2011;478(7369):343–348.
36. Travison TG et al. Harmonized Reference Ranges for Circulating Testosterone Levels in Men of Four Cohort Studies in the United States and Europe. *J. Clin. Endocrinol. Metab.* 2017;102(4):1161–1173.
37. Benderradji H et al. Defining reference ranges for serum anti-Müllerian hormone (AMH) on a large cohort of normozoospermic adult men. *J. Clin. Endocrinol. Metab.* [published online ahead of print: April 9, 2022]; doi:10.1210/CLINEM/DGAC218
38. Plotton I et al. Réévaluation des valeurs de références des gonadotrophines plasmatique chez l'homme avec fonction testiculaire normale sur automate ARCHITECT. *Ann. Endocrinol. (Paris)*. 2013;74(4):318–319.
39. Plotton I, Garby L, Morel Y, Lejeune H. Decrease of anti-Mullerian hormone in genetic spermatogenic failure. *Andrologia* 2012;44(5):349–354.
40. Wang Z, Xu X. scRNA-seq Profiling of Human Testes Reveals the Presence of the ACE2 Receptor, A Target for SARS-CoV-2 Infection in Spermatogonia, Leydig and Sertoli Cells. *Cells* 2020;9(4). doi:10.3390/CELLS9040920
41. Zhou L et al. SARS-CoV-2 Targets by the pscRNA Profiling of ACE2, TMPRSS2 and Furin Proteases. *iScience* 2020;23(11). doi:10.1016/J.ISCI.2020.101744
42. Pan F et al. No evidence of severe acute respiratory syndrome-coronavirus 2 in semen of males recovering from coronavirus disease 2019. *Fertil. Steril.* 2020;113(6):1135–1139.
43. Takeda M. Proteolytic activation of SARS-CoV-2 spike protein. *Microbiol. Immunol.* 2022;66(1):15–23.
44. Papa G et al. Furin cleavage of SARS-CoV-2 Spike promotes but is not essential for infection and cell-cell fusion. *PLoS Pathog.* 2021;17(1). doi:10.1371/JOURNAL.PPAT.1009246
45. Coutard B et al. The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res.* 2020;176. doi:10.1016/J.ANTIVIRAL.2020.104742
46. Jaimes JA, André NM, Chappie JS, Millet JK, Whittaker GR. Phylogenetic Analysis and Structural Modeling of SARS-CoV-2 Spike Protein Reveals an Evolutionary Distinct and Proteolytically Sensitive Activation Loop. *J. Mol. Biol.* 2020;432(10):3309–3325.
47. Le Tortorec A et al. Antiviral responses of human Leydig cells to mumps virus infection

- or poly I:C stimulation. *Hum. Reprod.* 2008;23(9):2095–2103.
48. Ueo A et al. Lysosome-Associated Membrane Proteins Support the Furin-Mediated Processing of the Mumps Virus Fusion Protein. *J. Virol.* 2020;94(12). doi:10.1128/JVI.00050-20
  49. Gye MC, Kim ST. Expression of cathepsin L in human testis under diverse infertility conditions. *Arch. Androl.* 2004;50(3):187–191.
  50. Koch J et al. TMPRSS2 expression dictates the entry route used by SARS-CoV-2 to infect host cells. *EMBO J.* 2021;40(16). doi:10.15252/EMBJ.2021107821
  51. Daly JL et al. Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science (80-. ).* 2020;370(6518):861–865.
  52. Wang S et al. AXL is a candidate receptor for SARS-CoV-2 that promotes infection of pulmonary and bronchial epithelial cells. *Cell Res.* 2021;31(2):126–140.
  53. Wang K et al. CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. *Signal Transduct. Target. Ther.* 2020;5(1). doi:10.1038/S41392-020-00426-X
  54. Darde TA et al. The ReproGenomics Viewer: a multi-omics and cross-species resource compatible with single-cell studies for the reproductive science community. *Bioinformatics* 2019;35(17):3133–3139.
  55. Zeng C et al. SARS-CoV-2 spreads through cell-to-cell transmission.. *Proc. Natl. Acad. Sci. U. S. A.* 2022;119(1). doi:10.1073/PNAS.2111400119/-DCSUPPLEMENTAL
  56. Delaroché L et al. Evaluation of SARS-CoV-2 in semen, seminal plasma, and spermatozoa pellet of COVID-19 patients in the acute stage of infection. *PLoS One* 2021;16(12):e0260187.
  57. Lokugamage KG et al. Type I Interferon Susceptibility Distinguishes SARS-CoV-2 from SARS-CoV. *J. Virol.* 2020;94(23). doi:10.1128/JVI.01410-20
  58. Hatton CF et al. Delayed induction of type I and III interferons mediates nasal epithelial cell permissiveness to SARS-CoV-2. *Nat. Commun.* 2021;12(1). doi:10.1038/S41467-021-27318-0
  59. Park A, Iwasaki A. Type I and Type III Interferons - Induction, Signaling, Evasion, and Application to Combat COVID-19. *Cell Host Microbe* 2020;27(6):870–878.
  60. Lowery SA, Sariol A, Perlman S. Innate immune and inflammatory responses to SARS-CoV-2: Implications for COVID-19. *Cell Host Microbe* 2021;29(7):1052–1062.
  61. Campos RK et al. SARS-CoV-2 Infects Hamster Testes. *Microorganisms* 2021;9(6). doi:10.3390/MICROORGANISMS9061318
  62. Jouvenet N, Goujon C, Banerjee A. Clash of the titans: interferons and SARS-CoV-2. *Trends Immunol.* 2021;42(12):1069–1072.
  63. Zheng M et al. TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. *Nat. Immunol.* 2021;22(7):829–838.
  64. Ribero MS, Jouvenet N, Dreux M, Nisole S. Interplay between SARS-CoV-2 and the type I interferon response. *PLoS Pathog.* 2020;16(7). doi:10.1371/JOURNAL.PPAT.1008737
  65. Satie AP et al. Excess Type I Interferon Signaling in the Mouse Seminiferous Tubules Leads to Germ Cell Loss and Sterility. *J. Biol. Chem.* 2011;286(26):23280.
  66. Youk J et al. Three-Dimensional Human Alveolar Stem Cell Culture Models Reveal Infection Response to SARS-CoV-2. *Cell Stem Cell* 2020;27(6):905-919.e10.
  67. Galani IE et al. Untuned antiviral immunity in COVID-19 revealed by temporal type I/III interferon patterns and flu comparison. *Nat. Immunol.* 2021;22(1):32–40.
  68. Perng YC, Lenschow DJ. ISG15 in antiviral immunity and beyond. *Nat. Rev. Microbiol.* 2018;16(7):423.
  69. Ziegler CGK et al. SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues. *Cell* 2020;181(5):1016.
  70. Yang M et al. Pathological Findings in the Testes of COVID-19 Patients: Clinical Implications. *Eur. Urol. Focus* 2020;6(5):1124–1129.
  71. Le Tortorec A et al. From ancient to emerging infections: The odyssey of viruses in the male genital tract. *Physiol. Rev.* 2020;100(3):1349–1414.
  72. Dhindsa S et al. Association of Circulating Sex Hormones With Inflammation and

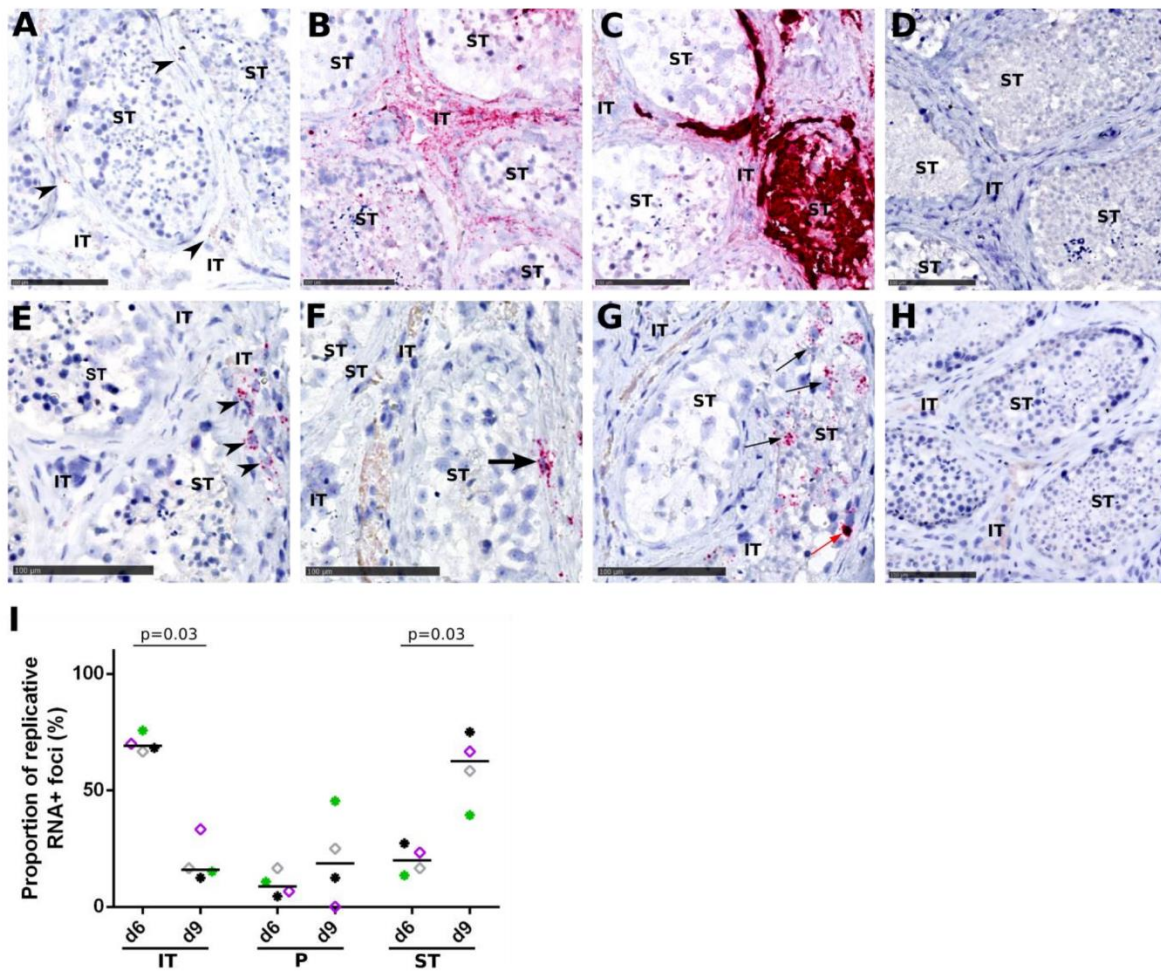
- Disease Severity in Patients With COVID-19. *JAMA Netw. open* 2021;4(5). doi:10.1001/JAMANETWORKOPEN.2021.11398
73. Moreno-Perez O et al. Male pituitary-gonadal axis dysfunction in post-acute COVID-19 syndrome-Prevalence and associated factors: A Mediterranean case series. *Clin. Endocrinol. (Oxf)*. 2022;96(3):353–362.
74. Gurwitz D. Angiotensin receptor blockers as tentative SARS-CoV-2 therapeutics. *Drug Dev. Res.* 2020;81(5):537–540.
75. Leal MC et al. The role of angiotensin-(1-7) receptor Mas in spermatogenesis in mice and rats. *J. Anat.* 2009;214(5):736–743.
76. Reis AB et al. Angiotensin (1-7) and its receptor Mas are expressed in the human testis: Implications for male infertility. *J. Mol. Histol.* 2010;41(1):75–80.
77. Xu P, Santos RAS, Bader M, Alenina N. Alterations in gene expression in the testis of angiotensin-(1–7)-receptor Mas-deficient mice. *Regul. Pept.* 2007;138(2–3):51–55.
78. Pan PP, Zhan QT, Le F, Zheng YM, Jin F. Angiotensin-converting enzymes play a dominant role in fertility. *Int. J. Mol. Sci.* 2013;14(10):21071–21086.
79. Dejuq-Rainsford N. Is SARS-CoV-2-induced testicular damage in hamsters relevant?. *Nat. Rev. Urol.* [published online ahead of print: March 31, 2022]; doi:10.1038/S41585-022-00589-Y
80. Yazdanpanah Y. Impact on disease mortality of clinical, biological, and virological characteristics at hospital admission and overtime in COVID-19 patients. *J. Med. Virol.* 2021;93(4):2149–2159.
81. Pizzorno A et al. Characterization and Treatment of SARS-CoV-2 in Nasal and Bronchial Human Airway Epithelia. *Cell reports. Med.* 2020;1(4). doi:10.1016/J.XCRM.2020.100059
82. Case JB, Bailey AL, Kim AS, Chen RE, Diamond MS. Growth, detection, quantification, and inactivation of SARS-CoV-2. *Virology* 2020;548:39–48.
83. Wang C et al. Investigation, treatment and monitoring of late-onset hypogonadism in males: ISA, ISSAM, EAU, EAA and ASA recommendations. *Eur. J. Endocrinol.* 2008;159(5):507–514.

## Figures and legends



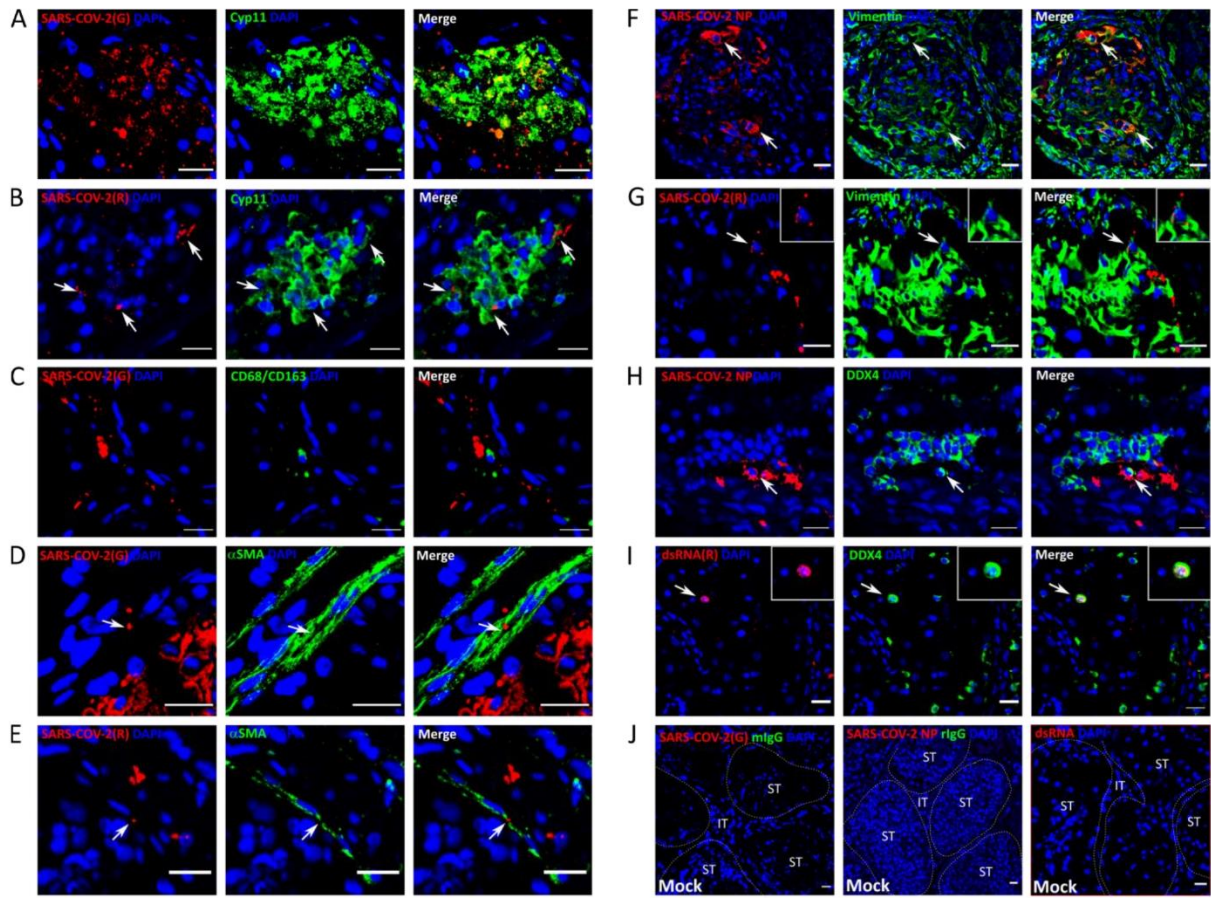
**Figure 1. SARS-CoV-2 replicates in human testis explants.**

Human testis explants from 6 post-mortem (T1-6) and one orchidectomy (T7) donors were infected overnight at a MOI of 1 (corresponding to  $7 \cdot 10^4$  PFU and  $3 \cdot 10^8$  vRNA copies) with a low-passage SARS-CoV-2 strain isolated in 2020 in France (IDF0372\_2020\_C2). Explants were thoroughly washed and cultured on inserts with 1mL medium/well for 10 days. Media was fully removed and changed every 3 days. Each of the time points (day 4, 7, 10) therefore represents *de novo* viral release over a 3-day-culture period. **(A)** SARS-CoV-2 RNA levels were quantified by RT-qPCR against ORF1b-nsp14 mRNA in culture supernatants. vRNA was consistently below detection threshold in mock-infected explants' supernatants. **(B)** Viral titers were measured by infectivity assay on VeroE6 cells. Each symbol represents the median value of all infected wells for a defined donor (same symbol throughout the figures). Horizontal bars represent the median values for all donors. Mock-infected explants were all negative. Statistical analyses were performed using Friedman-Dunn non-parametric comparison.



**Figure 2. SARS-CoV-2 infects interstitial tissue and seminiferous tubules in human testis explants.**

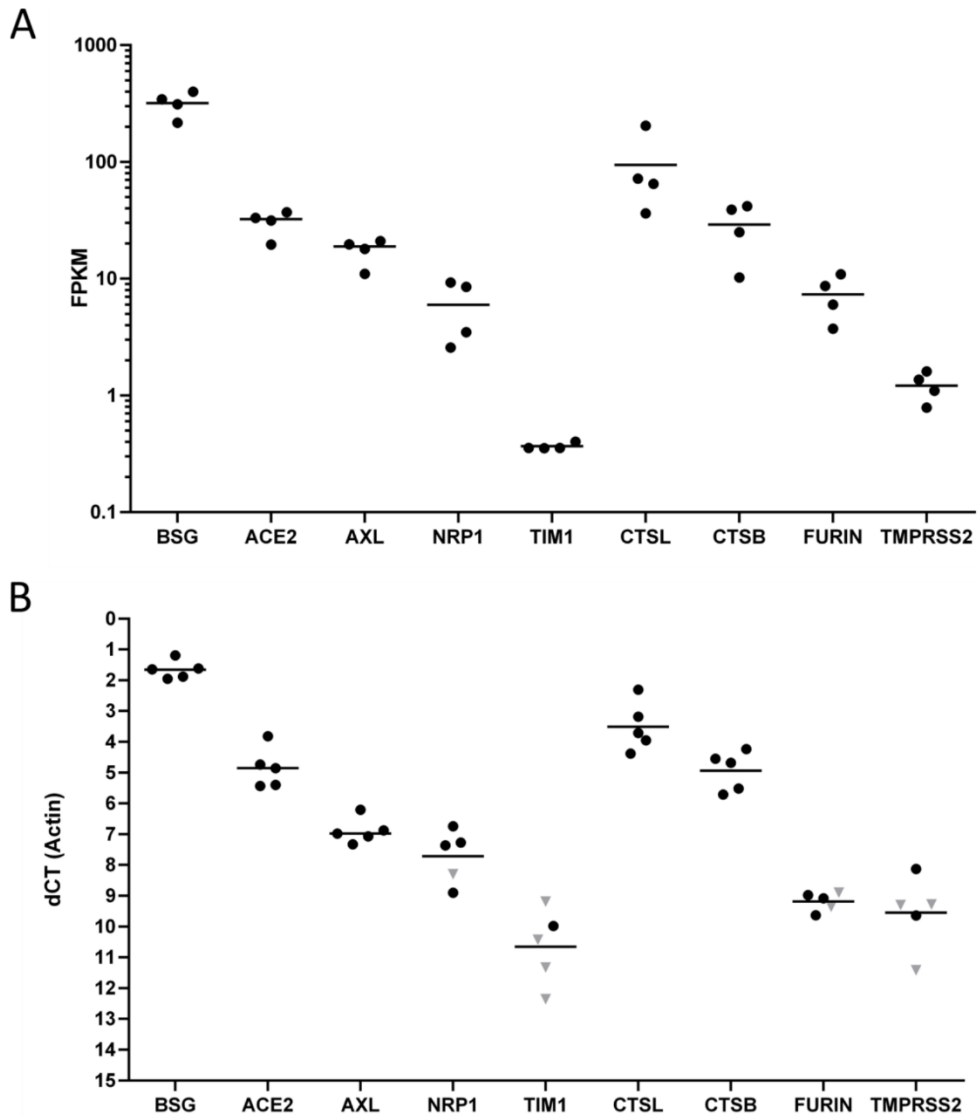
The first row shows representative images of RNAScope ISH using a probe targeting SARS-CoV-2 genomic RNA in infected (A, B, C) versus mock-infected (D) testis explants at day 3 (A), 6 (B) and 9 (C) post-infection. The second row shows representative images of RNAScope ISH using a probe targeting SARS-CoV-2 replicative RNA at day 6 (E, F) and 9 (G) post-infection and in control mock-infected testis explants (H). SARS-CoV-2 staining increased in the interstitial tissue (IT) between day 3 and 6 pi (e.g. A, E) and became prominent in seminiferous tubules (ST) at day 9 (e.g. C, G). Black arrow heads point at infected cells in the interstitial tissue (A, E). Thick black arrow point at infected cells in the extracellular matrix surrounding the seminiferous tubules (F). Thin black arrows point at infected cells in the seminiferous tubules (G). The thin red arrow points at an infected germ cell (G). Scale bars: 100  $\mu$ m. The number of infected cell foci in the interstitial tissue (IT), peritubular connective tissue surrounding seminiferous tubules (P) and number of infected seminiferous tubules (ST) were quantified in at least 3 whole sections from 4 donors labeled with SARS-CoV-2 replicative RNA on day 6 and 9 p.i. (I). \*P < 0.05 (Mann-Whitney non-parametric comparison).



**Figure 3. Identification of SARS-CoV-2 infected cells in human testis explants.**

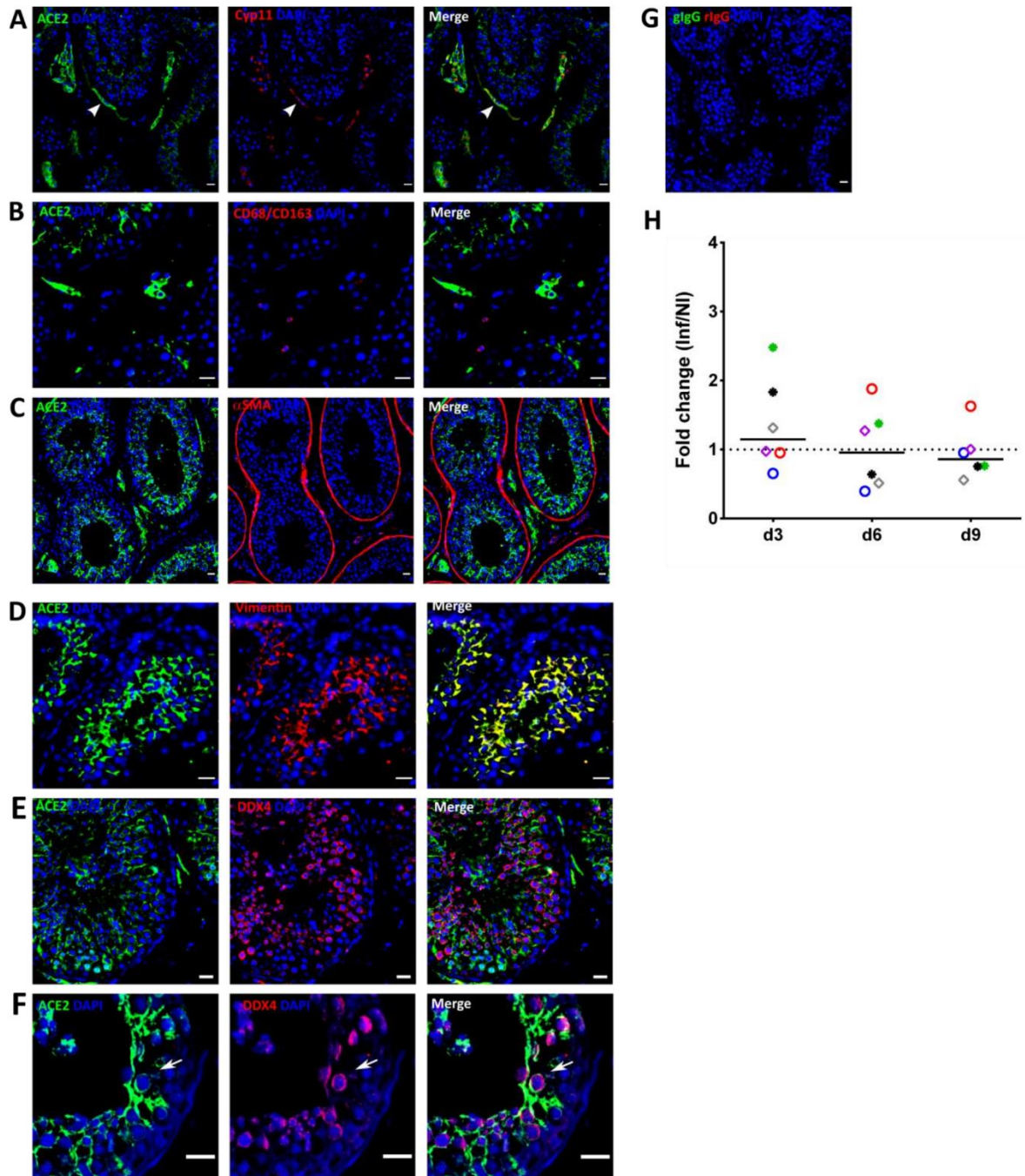
RNA scope ISH using probes targeting SARS-CoV-2 genomic (G) RNA (A, B, D) or SARS-CoV-2 replicative (R) RNA (C, E, G) was coupled with immunofluorescence for cell markers to label Cyp11A1+ Leydig cells (A, B), CD68/CD163+ macrophages (C),  $\alpha$ SMA+ peritubular cells (D, E), Vimentin+ Sertoli cells (F, G) and DDX4+ germ cells (H, I). IHC against SARS-CoV-2 nucleoprotein (NP) (F, H) or double-stranded RNA (dsRNA) (I) was undertaken to further characterize infected cells in the seminiferous tubules. No staining for SARS-CoV-2 by ISH or IHC and for cell markers isotypes were ever observed in mock-infected testis (J). Nuclei are stained in blue. Scale bars: 20 $\mu$ m.





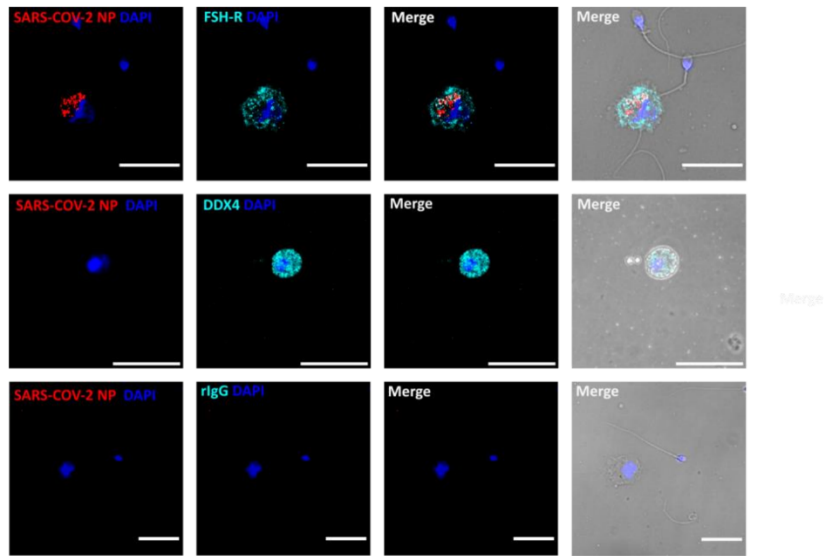
**Figure 4. Expression of putative entry factors for SARS-CoV-2 infection in the human testis.**

**(A)** Expression levels of known entry factors for SARS-CoV-2 in the testis. Shown are the mRNA expression levels from publicly available bulk RNA sequencing data, represented as Fragment Per Kilobase Million (FPKM) values. Each dot represents the mean FPKM value obtained for testis samples from different donors ( $n=1$  to  $8$ ) in each of the four studies (32–35) (testis from 13 donors in total). Horizontal bars represent median values. **(B)** Relative expression levels of entry factors for SARS-CoV-2 in the testis. mRNA expression was quantified by RT-qPCR and is presented as the ratio of cycle threshold (Ct) values for each entry factors versus actin in testis explants. Each dot represents a non-infected testis explant from a distinct donor. Horizontal bars represent median values. Grey triangles represent samples with a raw Ct value below 29 for the entry factor.



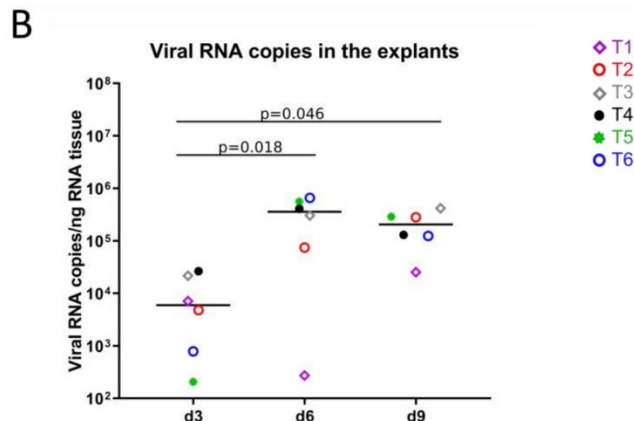
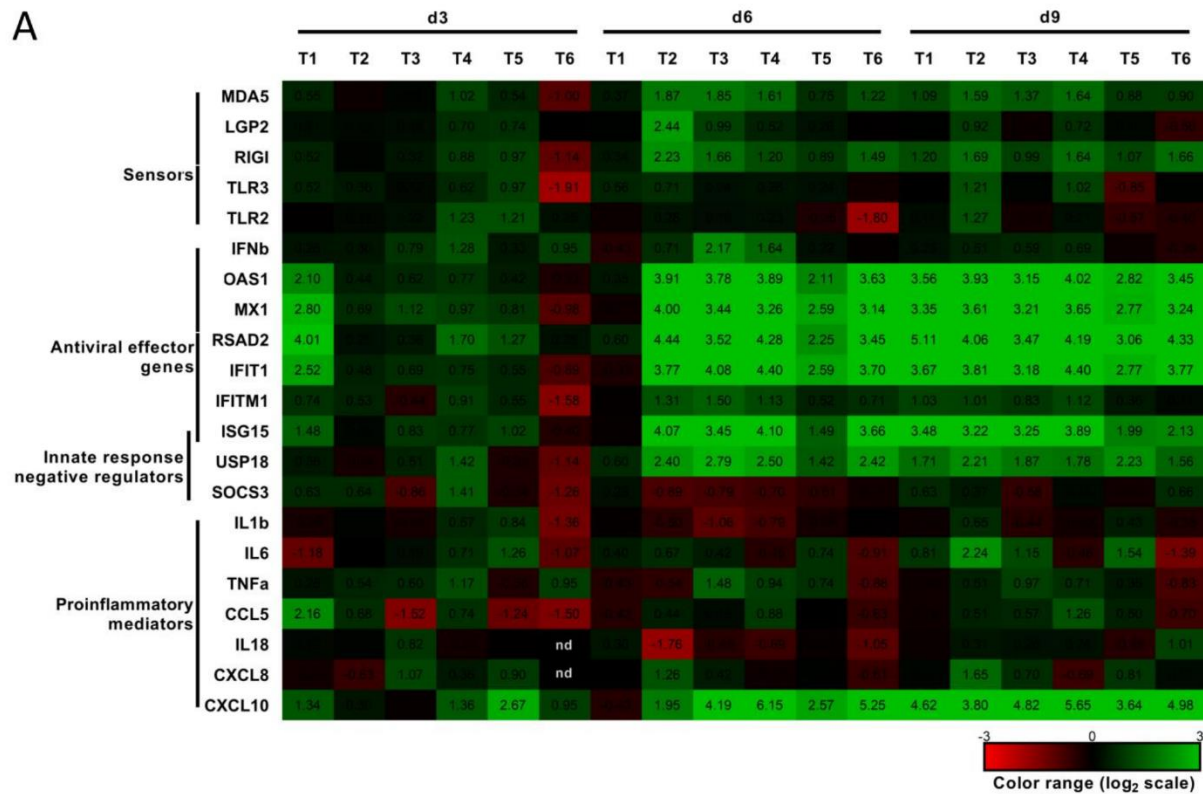
**Figure 5. ACE2 is mostly expressed by Leydig and Sertoli cells in the human testis.**

(A-G) Representative images of immunofluorescent labelling of ACE2 and cell markers in non-infected testis explants (n=4 independent donors). No ACE2 colocalization was observed with CD68/CD163+ macrophages (B). ACE2 was expressed by Cyp11A1+ Leydig cells localized in the interstitial tissue and bordering the seminiferous tubules (white arrow head) (B). SMA+ peritubular cells were negative for ACE2 (C). In the seminiferous tubules, ACE2 was expressed by Vimentin+ Sertoli cells (D), whereas DDX4+ germ cells were overall negative (E), except from the rare ACE2/DDX4 co-labelling detected in isolated germ cells within a few seminiferous tubules (white arrow) (F). Nuclei are stained in blue. Goat and rabbit IgG isotopes are shown as representative negative controls (G). Scale bars: 20µm (H) Fold change in ACE2 mRNA levels were measured by RT-qPCR in testis explants infected by SARS-CoV-2 (Inf) versus mock infected (NI) testis along the 9-day culture period. Each symbol represents a different donor. Bars represent median values. No significant difference was found using the One Sample Wilcoxon test.



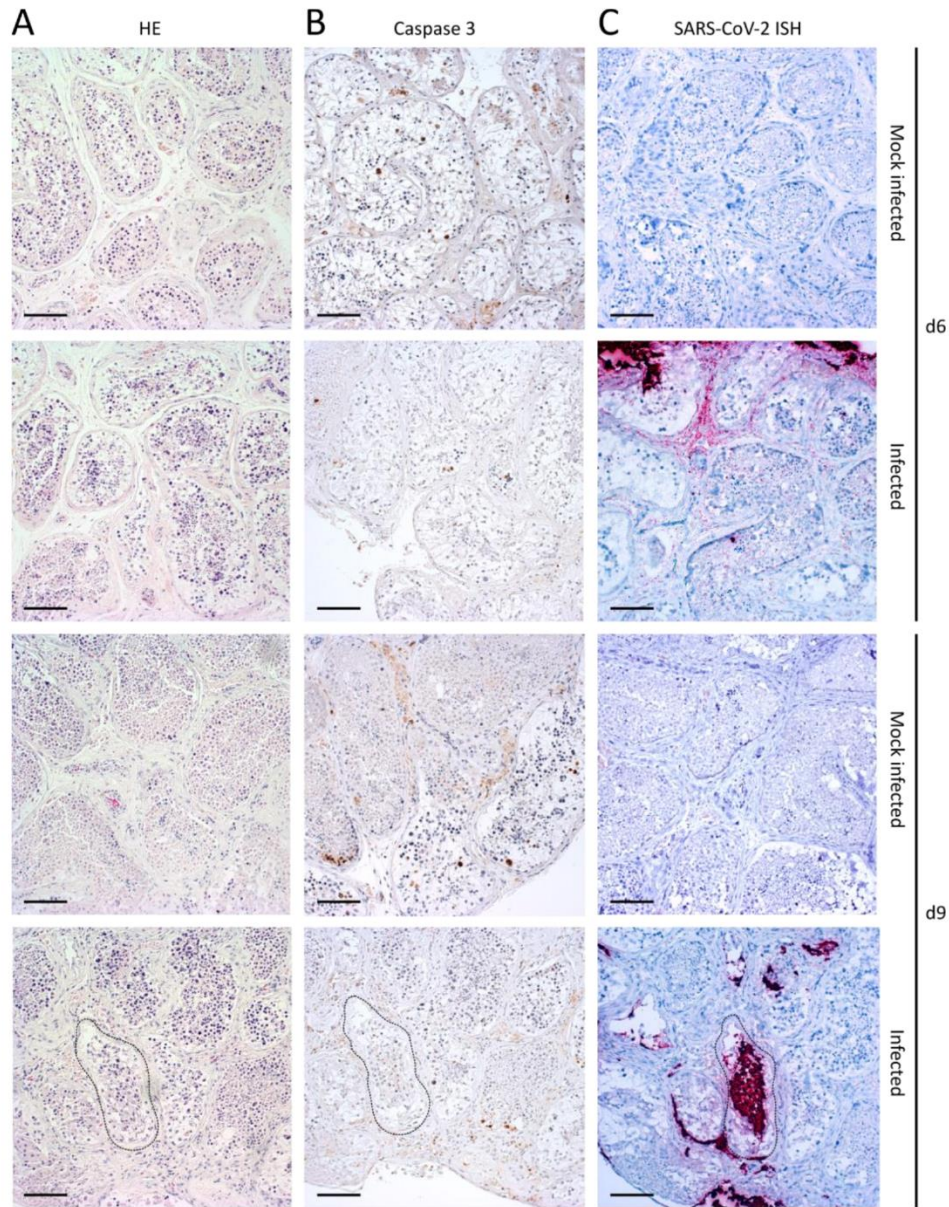
**Figure 6. Infected Sertoli cell detection in semen from a patient acutely infected by SARS-CoV-2 with mild symptoms.**

Immunofluorescence against the SARS-CoV-2 NP protein and cell markers for Sertoli cells (FSH-R) or germ cells (DDX4) were used to label semen cell smears from a SARS-CoV-2 infected patient at day 5 post-symptoms onset (first two rows). The bottom panel shows semen smear from a non-infected individual stained with anti-SARS-CoV-2 antibody and rabbit IgG isotype as a negative control. Nuclei are stained in blue. In the merge panels, brightfield images are included. Scale bars: 20 $\mu$ m



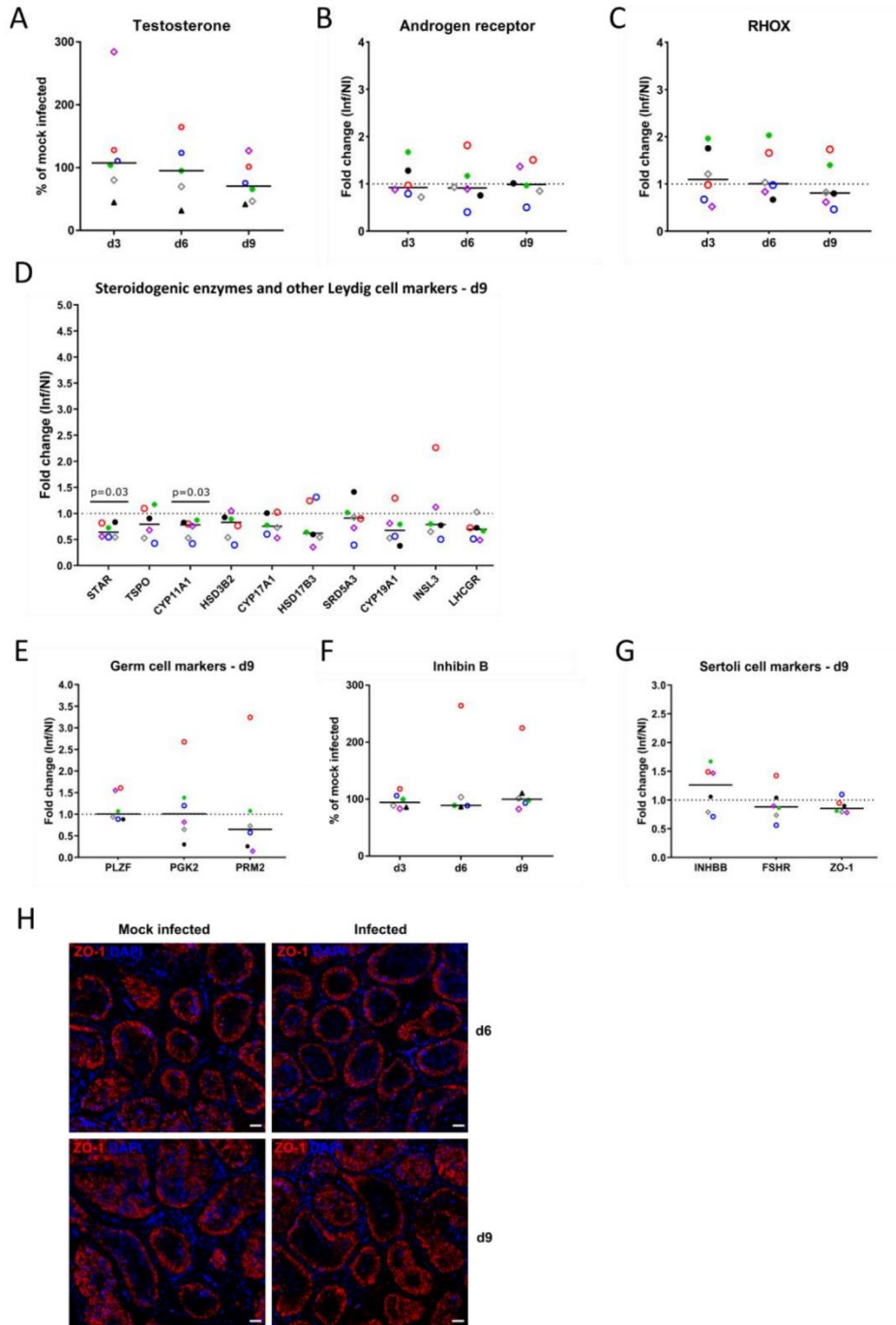
**Figure 7. SARS-CoV-2 infection induces antiviral effector genes expression in human testis explants.**

(A) Innate immune gene expression determined by RT-qPCR in testis explants from 6 donors infected with SARS-CoV-2 for 3, 6 and 9 dpi (d3, d6, d9). Heatmap shows log<sub>2</sub>-transformed expression ratios between SARS-infected and time-matched mock-infected controls. Green indicates upregulation and red downregulation of mRNA compared with controls. (B) Viral loads in the testicular explants tissues used in (A), detected by RT-qPCR against ORF1b-nsp14 mRNA after total RNA extraction. Mock-infected explants were all negative. Each symbol represents a different donor and horizontal bars represent median values. Statistical analysis was performed using nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test.



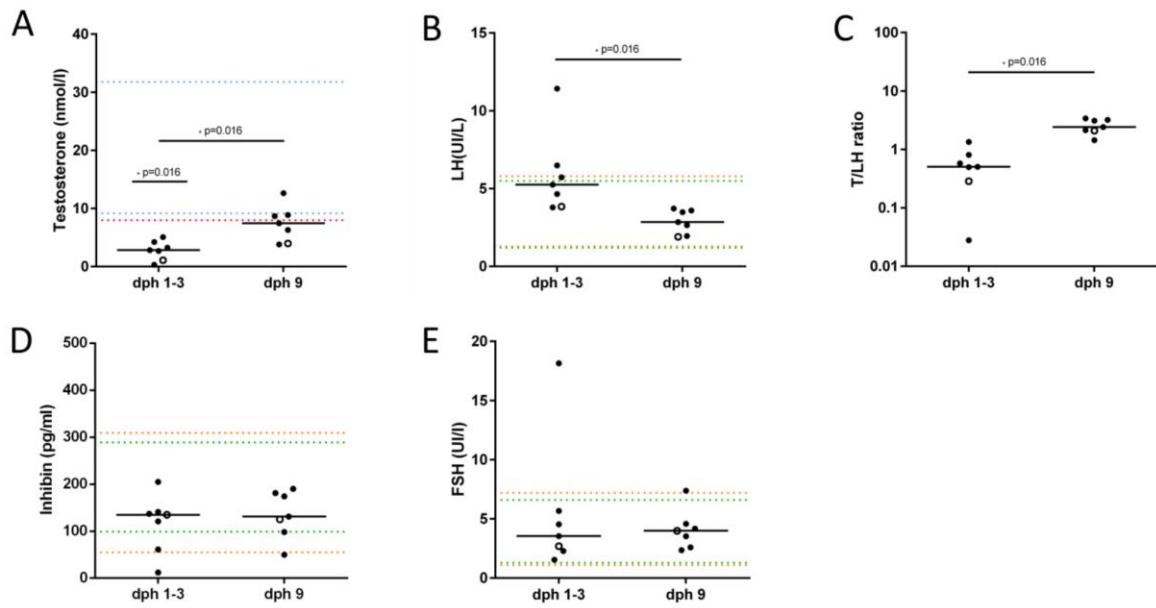
**Figure 8. SARS-CoV-2 infection does not alter human testis explant morphology or cell viability *ex vivo*.**

Representative images of Hematoxylin-Eosin histological staining (A), cleaved Caspase-3 apoptosis labelling (B) and RNAScope ISH against SARS-CoV-2 genomic RNA (C) in infected and mock-infected testis explants at 6 and 9 dpi (5  $\mu$ m serial sections were used for the latter). (D) LDH release in testis supernatant from 4 donors expressed as percent of mock-infected explants on the corresponding day of culture. Each symbol represents a different donor and horizontal bars represent median values. No significant difference was found using Mann-Whitney non-parametric test. Scale bars: 100  $\mu$ m.



**Figure 9. SARS-CoV-2 infection alters Leydig cells steroidogenic enzymes expression ex vivo.**

(A) Testosterone release by testis explants from 6 donors, expressed as percent of mock-infected explants on the corresponding day of culture. No significant difference was found using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. (B, C) Fold change in androgen receptor (B) and RHOX (C) mRNA levels measured by RT-qPCR in testis explants infected by SARS-CoV-2 versus mock infected testis up to 9 dpi. No significant difference was found using the One Sample Wilcoxon Test. (D) Expression profile of steroidogenic enzymes and other Leydig cell markers (INSL3, LHCGR) determined by RT-qPCR at 9 dpi. Values are expressed as fold changes of SARS-CoV-2 infected testis (Inf) versus mock-infected testis (NI). Statistical analyses were performed using One Sample Wilcoxon test. (E) Expression profile of specific markers for spermatogonia (PLZF), spermatocytes (PGK2) and spermatids (PRM2) at 9 dpi. No significant difference was found using the One Sample Wilcoxon Test. (F) Inhibin B release by testis explants, expressed as percent of mock-infected explants on the corresponding day of culture. No significant difference was found using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. (G) Expression profile of Sertoli cell markers. No significant difference was found using the One Sample Wilcoxon Test. (H) IHC of Sertoli cell tight junction-associated protein ZO-1 in infected versus non-infected explants at 6 and 9 dpi. Nuclei are stained in blue. Scale bars: 50µm. (I) Fold change in basal ZO-1 distribution percentage, as counted after ZO-1 IHC in testis explants infected by SARS-CoV-2 versus mock infected testis along the 10-day culture period. No significant difference was found using One Sample Wilcoxon test. Each symbol represents a different donor, horizontal bars represent median values.



**Figure 10. Testosterone levels are transiently below reference range in patients acutely infected by SARS-CoV-2.**

Levels of sex hormones measured in the serum from seven SARS-CoV-2 infected patients without co-morbidities (age range: 18-51) at day 1-3 and 9 post-hospitalization. Testosterone (A), Luteinizing Hormone (LH) (B), T/LH ratio (C), Inhibin B (D), and Follicle Stimulating Hormone (FSH) (E) levels measured by mass spectrometry or immunoassays are represented. All patients were discharged alive except one (empty circle). Blue dotted lines are harmonized reference ranges for circulating testosterone levels in European and North American cohorts (36). Red dotted line is the consensus threshold level of testosterone for hypogonadism reported by Wang et al., 2008 (83). Green dotted lines are reference ranges for hormone levels in a French cohort of normozoospermic adult men (37). Orange dotted lines are Lyon University hospital inhouse reference ranges for adult men with normal spermatogenesis (38, 39). Statistical analysis was performed using One Sample Wilcoxon Test for testosterone levels with the reference values comparison and Wilcoxon matched-pairs signed-rank test to compare hormone levels between times.

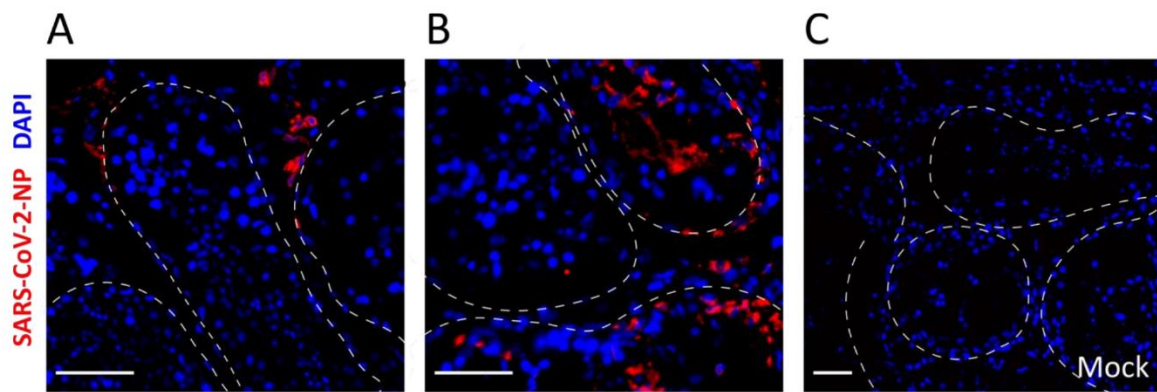
**Supplementary Table 1.** Primers used for RT-qPCR.

Gene	Forward primer	Reverse primer
ACE2	GTGGTGGGAGATGAAGCGAGAG	AGAGATGCGGGGTCACAGTATG
ACTB	TACAGCTTCACCACCACGG	TGCTCGAAGTCCAGGGCGA
AR	TACGGGGACATGCGTTTGGAG	ACAGATCAGGCAGGTCTTCTGG
AXL	TGGTCAGCCAGCTCAGAATCAC	AGGTCTGATGTCCCAGAAACACC
BSG	CTTGAATGACAGCGCCACAGAG	TCGTCCGAGTCCACCTTGAAC
CCL5	TGCTGCTTTGCCCTACATTGCC	TTTCGGGTGACAAAGACGACTG
CTSB	TGACTGGGGTGACAATGGCTTC	TTCCAGCCACCCTTCTGATTC
CTSL	ATCACAGTTTAGAGGCACAGTGG	CCTCCATCCTTCTTCATTCATGCC
CXCL10	GTGGCATTCAAGGAGTACCTCTC	CGTGGACAAAATTGGCTTGCGAG
CYP11A1	AGACCTGGAAGGACCATGTG	TCCTCGAAGGACATCTTGCT
CYP17A1	GTGGAGACCACCACCTCTGT	GCTGAAACCCACATTCTGGT
CYP19A1	CCAGGTCCTGGCTACTGCAT	GATCCCCATCCACAGGAATCT
FSHR	TGCCATTGAACTGAGGTTTG	GGTCCCCAAATCCTGAAAT
FURIN	ATGGGACGCTGACCAAGTTCAC	AAGCCTTCCTCGCACACCACAC
HKU-ORF1b-nsp14	TGGGGTTTTACAGGTAACCT	AACACGCTTAACAAAGCACTC
HSD17B3	TCCTTGGCCTCTCTACTCCA	AGACAGCATATGGGGTCAGC
HSD3B2	GCCTGTTGGTGAAGAGAAG	GCAGGCTCTTTTCAGGAATG
IFIT1	CTTGAGCCTCCTTGGGTTCTGTC	GTTCTCAAAGTCAGCAGCCAGTC
IFITM1	CCGTGAAGTCTAGGGACAGGAAG	CACAGAGCCGAATACCAGTAACAG
IFNb	CAGAAGCTCCTGTGGCAATTG	TCCTCAGGGATGTCAAAGTTCA
IL1b	CACGATGCACCTGTACGATCAC	ACATGGAGAACACCCTTGTTC
IL6	ACACAGACAGCCACTCACCTC	TGTTTTCTGCCAGTGCCTCTTTG
INHBB	AGCCTCCAGGATACCAGCAA	TCTCCGACTGACAGGCATTTG
INSL3	GCGACCGTGAGTTGCTACAG	GGTCCCAGCGTGAGATTACTG
ISG15	CGAACCTCTGAGCATCCTGGTG	CCTCGAAGGTCAGCCAGAACAG
LGP2	CCCACCATGTCAATGTGAACCC	CCTTGAAGACTTTGTTGATGACCAC
LHCGR	GCCGGTCTCACTCGACTATC	TCATTAAGTCCTCTGAAAGCTTGA
MDA5	AAAGCACTGCAAAAGAAGTGTGC	TGCACCATCATTGTTCCCAAG
MX1	CGTTAGCCGTGGTGATTTAGCAG	ATCCTTCAATCCCAGCAGCTC
NRP1	ACCTTGGTGGGATTGCTGTGG	GTATCCTGGCGTGCTCCCTGTTTC
OAS1	CATGTGCTGCCTGCCTTTCATG	TCGATGAGCTTGACATAGATTTGGG
PGK2	ACCAGAGGATCAAGGCTTCC	TCAGGCCGACCTAGATGACT
PLZF	CTCATTCAAGCGGGTGCCAAAG	AGTATGGGTCTGCCTGTGTGTC
PRM2	GCAGAAGGCGCAAAAGAC	TCTGCATGTTCTCTTCTGCTGGT
RHOX	CACGAAGTTCACGCTGTTGC	TTCCGCAAGTTCCTTCTTGTG
RIGI	GACACAGAGAGTCTGGCAAAGAG	CTTTGTCTGGCATCTGGAACACC
RSAD2	ACATGACGGAACAGATCAAAGCAC	AGCATCTTCTCCACAATTCTCACC
SOCS3	TAAGGGGTAAAGGGCGCAAAGG	CCTGGTTGGCTTCTTGTGCTTG
STAR	GGCTGGCATGGCCACAGAC1	TTGGGCAGCCACCCCTTGA
TIM1	AGAAACCCACCCTACGACTG	CGGTGTCATTCCCCTCTGTTGTG
TLR2	GACCGCAATGGTATCTGCAAGG	CTGTTGTTGGACAGGTCAAGGC
TLR3	ACTGAACCATGCACTCTGTTTGC	AAGGCACCTATCCGTTCTTTCTG
TMPRSS2	TATCGACAAATGAGGGCAGACG	TCATGCACGGGGAAGCAAAAC
TNFa	CTGTAGCCCATGTTGTAGCAAACC	TCTCTCAGCTCCACGCCATTG
TSPO	GGCTTCACAGAGAAGGCTG	ACTGACCAGCAGGAGATCCA
USP18	ACTCCTTGATTTGCGTTGAC	TTTCCCACGGGTCTTCTT
ZO-1	TTATTTGGGCTGTGGCGTGAG	CCTCCATTGCTGTGCTCTTGG



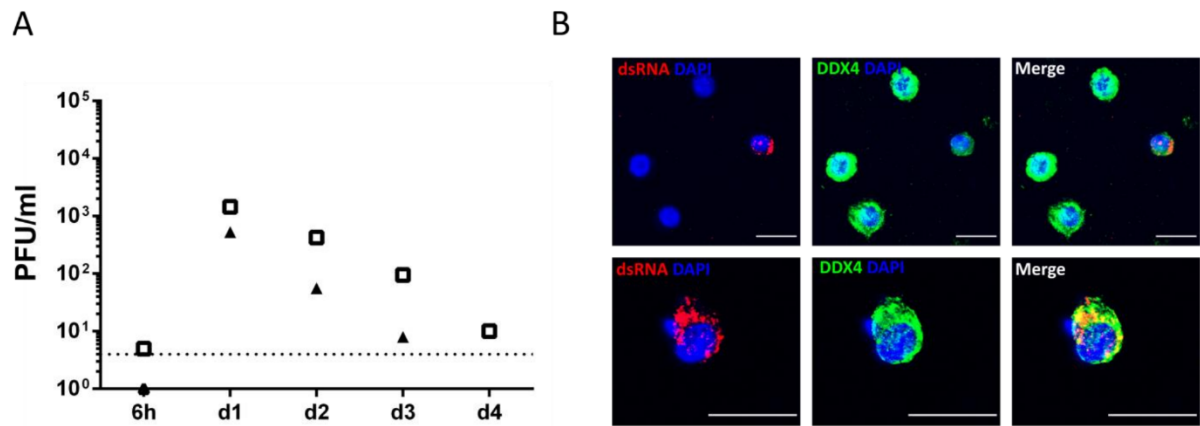
**Supplementary Table 2.** Antibodies used for immunostainings.

Antigen	Isotype, clone	Company, order	Concentration	Demasking buffer	Blocking buffer
αSMA	Mouse IgG2a, #1A4	Dako, #M0814	1.7 µg/mL	Citrate pH6	PBS 10% BSA
ACE2	Goat IgG, polyclonal	R&D, #AF933	5 µg/mL	Citrate pH6	PBS 10% BSA
Cleaved Caspase 3	Rabbit IgG, polyclonal	Cell Signaling, #9661	0.35 µg/mL	EDTA pH8	TBS 10% BSA
CD163	Mouse IgG1, #10D6	Leica, #NCL-L-CD163	0.5 µg/mL	Citrate pH6	PBS 10% BSA
CD68	Mouse IgG1, #KP1	Dako, #M0814	1.85 µg/mL	Citrate pH6	PBS 10% BSA
CYP11A1	Rabbit IgG, polyclonal	Sigma #HPA016436	0.5 µg/mL	Citrate pH6	PBS 10% BSA
DDX4 (for IHC)	Rabbit IgG, polyclonal	Abcam, #ab13840	2 µg/mL	Citrate pH6	PBS 2% BSA
DDX4 (for RNAscope)	Rabbit IgG, polyclonal	Sigma, #HPA037764	1 µg/mL	Citrate pH6	TBS 10% Chicken serum
dsRNA	Mouse IgG2ak, #J2	Scicons, #RNT-SCI-10010200	1 µg/mL	Citrate pH6	TBS 10% Chicken serum
SARS-NP	Mouse IgG2b, #3851	Genetex, #GTX36802	1 µg/mL	Citrate pH6	PBS 10% BSA
TMPRSS2	Rabbit IgG, #EPR3862	Abcam, #ab109131	0.5 µg/mL	EDTA pH9	TBS 10% Chicken serum
Vimentin (for IHC)	Rabbit IgG, #EPR3776	Abcam, #ab92547	0.78 µg/mL	Citrate pH6	∅
Vimentin (for RNAscope)	Rabbit IgG, polyclonal	Sigma, #HPA001762	1 µg/mL	Citrate pH6	TBS 5% BSA
ZO1	Mouse IgG1, #1A12	Invitrogen #33-9100	1 µg/mL	EDTA pH8	∅



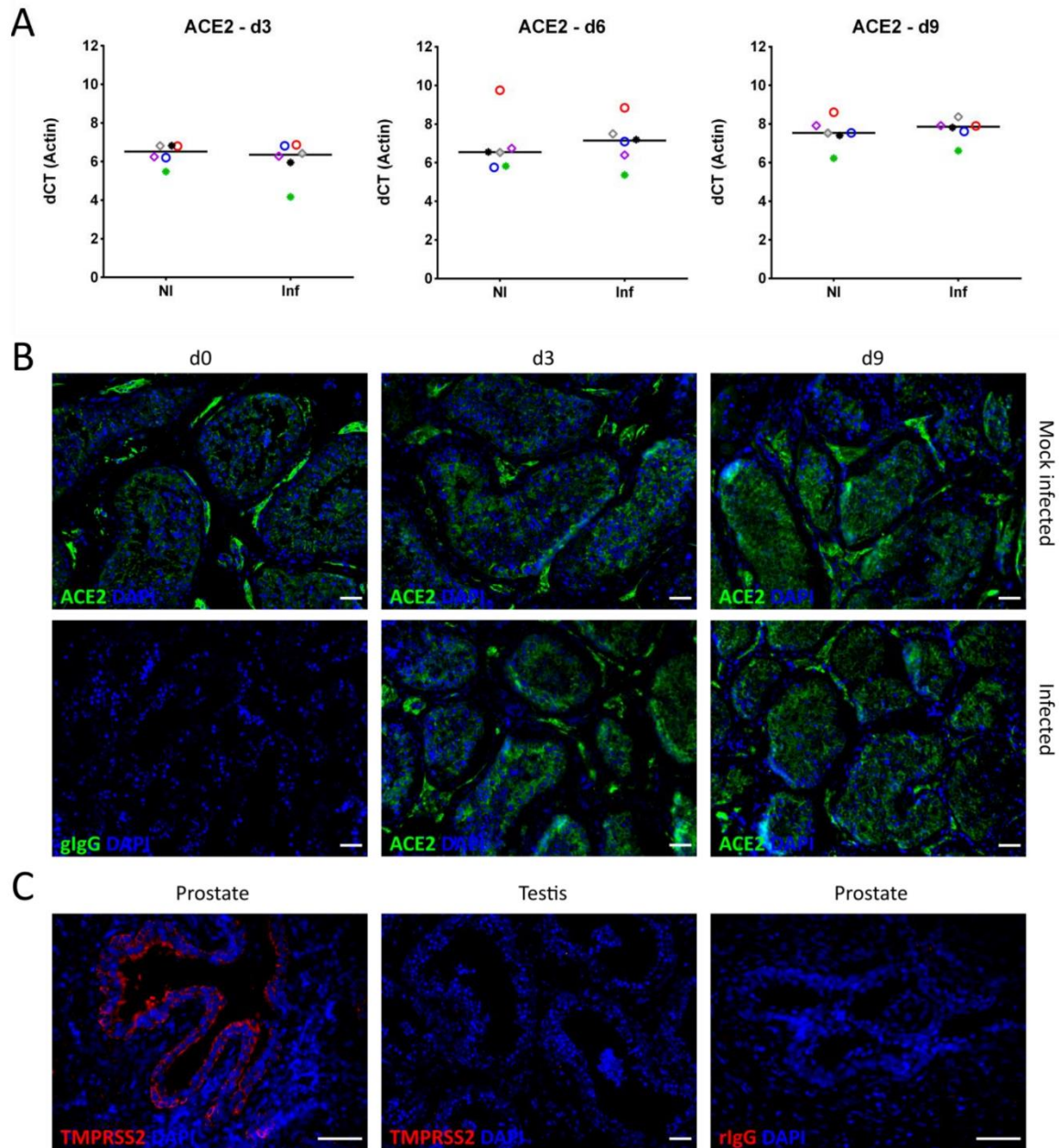
**Supplemental Fig.1. SARS-CoV-2 infection and progression through Nucleoprotein (NP) viral protein detection.**

IHC using a specific antibody against the SARS-CoV-2 nucleoprotein (NP) was performed on testis explants at day 6 (A) and day 9 (B) post-infection, showing a progression of the staining almost exclusively localized within the interstitium at day 6 to within the seminiferous tubules at day 9. No staining was ever observed in mock-infected testis explants (C). Nuclei are stained in blue. Scale bars: 50  $\mu$ m.



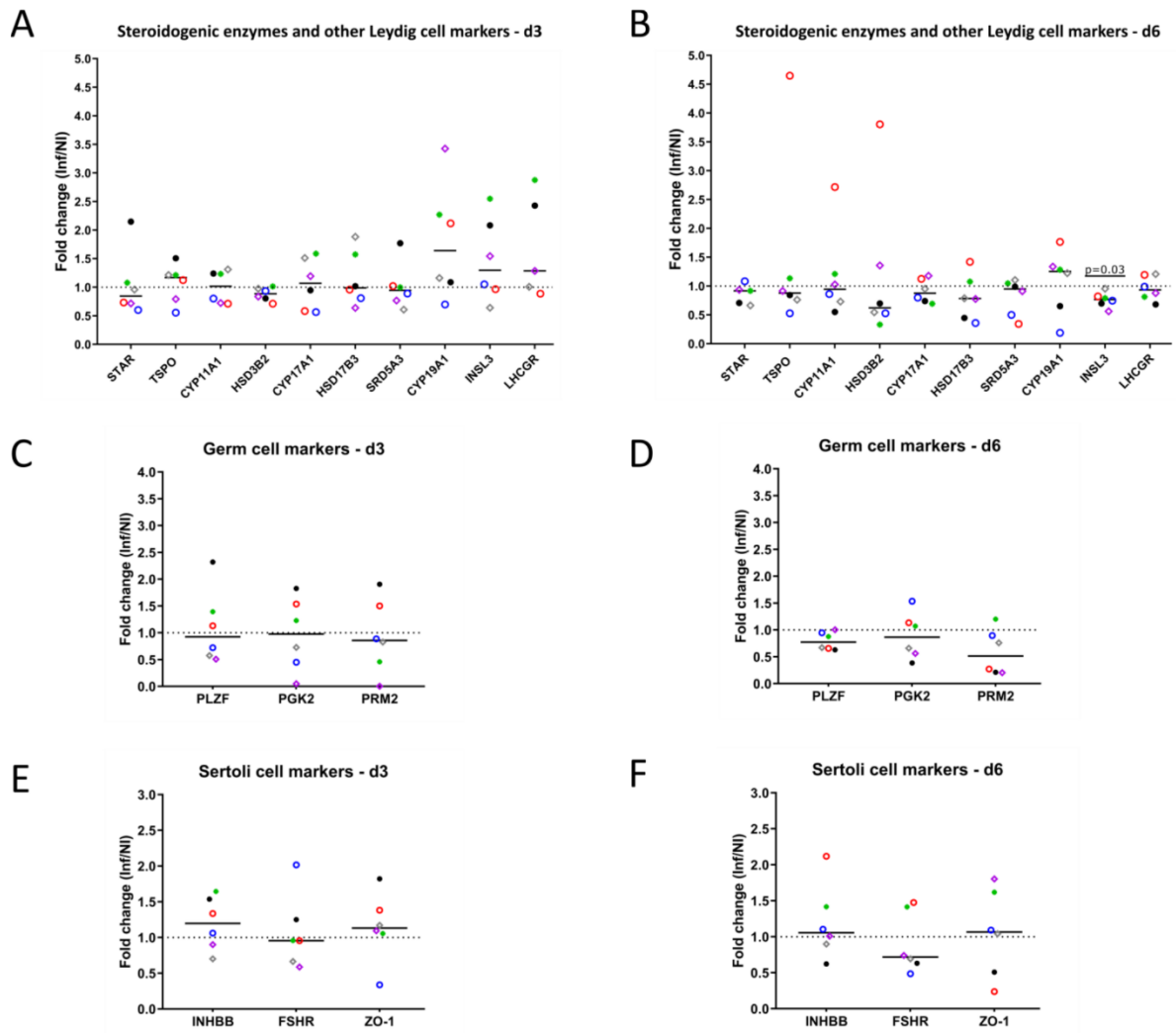
**Supplemental Fig.2. SARS-CoV-2 infection of isolated testicular germ cells.**

Primary testicular germ cells (TGC) isolated from seminiferous tubules were infected 2 hours with SARS-CoV-2 (MOI of 1, corresponding to  $1.4 \times 10^6$  TCID<sub>50</sub> per million cells), and cultured for 4 days as we previously described (29). **(A)** Viral titers were measured by infectivity assay on VeroE6 cells. The 2 donors tested are represented by a distinct symbol. The dotted line indicates the detection limit. **(B)** SARS-CoV-2 replicative RNA was localized in germ cells by IHC using an antibody targeting double-stranded RNA (dsRNA), an intermediary of SARS-CoV-2 replication coupled with an antibody against the germ cell marker DDX4. Nuclei are stained in blue. Scale bars: 20µm.



**Supplemental Fig.3. ACE2 expression during culture of infected and mock infected testis explants and absence of detection of TMPRSS2 protein in the testis.**

**(A)** Relative expression levels of ACE2 mRNA in the mock-infected (NI) and infected (Inf) testis explants at day 3 (d3), day 6 (d6) and day 9 (d9) of culture. mRNA expression was quantified by RT-qPCR and is presented as the ratio of cycle threshold (Ct) values for ACE2 versus actin in testis explants. Each dot represents a different donor and horizontal bars represent median values. No significant difference was found using Mann Whitney test. **(B)** Representative images of immunofluorescent labelling of ACE2 in mock-infected and infected testis explants at day 0 (d0), day 3 (d3) and day 9 (d9) of culture. No changes in intensity or localization of the staining was observed over the culture and infection course. No staining for ACE2 isotypes (glgG) was ever observed in infected or mock-infected testis. **(C)** Representative images of immunofluorescent labelling of TMPRSS2 in non-infected prostate and testis. TMPRSS2 staining was found in epithelial cells of the prostate, whereas no staining was observed for TMPRSS2 in testis explants. Staining specificity in the prostate was controlled using isotype (rlgG). Nuclei are stained in blue. Scale bars: 50 $\mu$ m.



**Supplemental Fig.4. Testicular cell markers expression in testis explants infected by SARS-CoV-2.**

(A, B) Expression profile of transcripts encoding steroidogenic enzymes and other Leydig cell markers; (C, D) markers of spermatogonia (PLZF), spermatocytes (PGK2) and spermatids (PRM2); (E, F) and markers of Sertoli cells, determined by RT-qPCR at 3 (A, C, E) and 6 (B, D, F) dpi. Values are expressed as fold changes of SARS-CoV-2 infected testis (Inf) versus mock-infected testis (Ni). Each symbol represents a different donor and horizontal bars represent median values. Statistical analyses were performed using One Sample Wilcoxon test.

# III. Viral entry factors of emerging RNA viruses in the testis

## Chapter 3:

Expression of viral entry factors in the human testis:  
analysis of published single cell RNA-seq data

Bourgeau Salomé\*, Chan Sock Peng Edith\*, Mahé Dominique, Rolland Antoine, Barreteau Andreas,  
Chalmel Frédéric\*, Dejuçq-Rainsford Nathalie\*, *in preparation*.

## **Expression of viral entry factors in the human testis: analysis of published single cell RNA-seq data**

Bourgeau Salomé<sup>1,2\*</sup>, Chan Sock Peng Edith<sup>1\*</sup>, Mahé Dominique<sup>1</sup>, Rolland Antoine<sup>1</sup>, Barreteau Andreas<sup>1</sup>, Chalmel Frédéric<sup>1\*</sup>, Dejucq-Rainsford Nathalie<sup>1\*</sup>.

<sup>1</sup> Institut National de la Santé et de la Recherche Médicale, Ecole des Hautes Etudes en Santé Publique, Institut de recherche en santé, environnement et travail, Université de Rennes, UMR\_S1085, Rennes, France

<sup>2</sup> University of CAS, Beijing 101408, China; CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai CAS, 320 Yueyang Road, Shanghai 200031, China

### **Introduction**

The Male Genital Tract (MGT) is the target of several viral infections <sup>1</sup>. Over thirty contemporary and emerging viruses have been reported in semen but for most, the shedding source is undetermined. Recently, two emergent viruses responsible for acute infections (Zika and Ebola) were unexpectedly sexually transmitted by men who recovered from the infection. Prolonged excretion in semen of viral RNA and infectious virus was found to occur for months to years after viral clearance in the blood <sup>2-5</sup>. The sexual transmission of Ebola virus by survivors up to 5 years post-recovery has been associated with the resurgence of epidemics foci <sup>1,6</sup>, highlighting the dramatic consequences of viral persistence in semen, and the crucial need to anticipate it. The extended shedding of ZIKV in semen led to the transmission of the virus by infected travelers in 14 countries outside the geographic zone of the insect vector <sup>1</sup>. The presence of ZIKV in semen has also been suspected to enhance in utero transmission, as evidenced in a mouse model <sup>7</sup>. As illustrated by the HIV pandemic, semen is an important vector for viral dissemination. The prolonged shedding in semen of acute emerging viruses therefore represents a major concern for future epidemics.

As detailed in the result section of this manuscript, we uncovered ZIKV-infected testicular germ cells up to 160 days post symptoms onset in the semen of men who recovered from Zika virus infection <sup>8</sup>; Testicular germ cells represented the majority of infected cells in semen and harbored ZIKV for the longest duration (as compared with seminal macrophages and epithelial cells, which were only infected up to 90 days post-symptoms). This demonstrates that the testis is an important reservoir for ZIKV shedding in semen <sup>8</sup>. The testis is an immune-privilege organ, which together with a dampened innate immune response against certain viruses such as ZIKV (as evidenced by our team), could represent an ideal shelter for viruses, and in turn sustain prolonged seminal excretion. Moreover, viral infections of the testis can impact spermatogenesis and fertility and affect the production of male hormones, as showed for Mumps virus <sup>1,9</sup>. Lately, we showed that SARS-CoV-2 can infect the human testis and disrupt the expression of steroidogenic enzymes. All these elements point at the importance of predicting the potential tropism of emerging viruses for the testis and determining the mechanisms involved in this infection, in order to anticipate and prevent or cure these infections. RNA viruses are the most prevalent among emerging pathogens and there is growing evidence in both humans and animals that several RNA viruses are shed in semen and can infect testicular cells <sup>1</sup>. Therefore, we sought here to evaluate the susceptibility of the human testis

to infection by a selection of emerging RNA viruses. The first step of viral infection is the viral entry, which consists in the recognition and binding of a virus to its cell receptor(s), and in some cases, involves the cleavage of the virus fusion protein by specific cellular proteases. Viral tropism, and in turn cell susceptibility, are thus firstly driven by the existence and availability of such host factors (HF). In order to predict the capability of the human testis to support the entry steps of emergent RNA viruses, we conducted a review of the literature on the proven EF used by viruses from a range of viral families, which have been reported in the male genital tract and semen of men <sup>1</sup>. We then bioinformatically evaluated the expression of these EF in the human testis, using an already published single cell RNA sequencing (scRNA-seq) dataset <sup>10</sup>.

## Material and Methods

We focused our study on viruses that had been previously reported in human semen or testis <sup>1</sup> and included other members of their genus involved in human diseases.

For each virus, the infected human organs and cells reported in the literature were searched (*Annexe 1*). When organ tropism was not clearly stated, it was inferred from the origin of the infected cell types reported, as displayed on Figure 1. For each virus, only attachment and entry factors for which usage has been demonstrated by *gain or loss of function experiments* (e.g. antibody blocking or other competing agents, genetic manipulations) have been included. Attachment receptors, entry receptors and fusion proteases are presented distinctively. For the sake of clarity, when collected data regarding tropism and host factor usage were redundant for several viruses of the same genus, only one representative virus was shown. This way, Junin virus (JUNV) is representative of the New World Mammarenaviruses (Machupo virus MACV, Sabia virus SABV, Guanarito virus GTOV); and Seoul virus (SEOV) is clustered with Puumala virus (PUUV). Enteroviruses were represented under their species names, except for specific viruses for which more detailed information were available. These are displayed alone.

The false color heatmap was generated using the *pheatmap* package implemented in R (Version 4.1.1) (Figure 1). The scRNA-seq dataset (expression data, t-SNE dimensionality reduction and associated metadata) published by Wang et al., 2018 <sup>10</sup> was downloaded via the ReproGenomics Viewer <sup>11,12</sup> and integrated into the *Seurat* package (v. 4.0.5) <sup>13</sup>. Only the samples from fertile donors were used. Briefly cell annotation was based on the metadata provided by the authors with minor changes: the different stages/types of spermatogonia, spermatocytes and spermatids were grouped together under the term “Spermatogonia”, “Spermatocytes” and “Spermatids” respectively. The dot plot figure was produced by using the FlexDotPlot package <sup>14</sup> and the cowplot package.

## Results

Figure 1 shows the viral host factors (i.e. attachment and entry receptors, proteases) used by the different viruses of interest and presents the virus genus, organ tropism and reported presence in human semen or testis. It permits the visualization, at once, of the various host factors used by the members of the genus of each emergent virus detected in semen or in testis. It also indicates their



tropism for cutaneous (e.g. keratinocytes, dermal fibroblast), musculoskeletal (e.g. skeletal myocytes, smooth muscle cells), hepatic (e.g. hepatocytes, Kupffer cells), endothelial (e.g. endothelial cells, pericytes), neural (e.g. neurons, astrocytes), renal (e.g. tubular cells, podocytes), gastrointestinal (e.g. enterocytes, Paneth cells) and respiratory (e.g. alveolar cells, ionocytes) tissues.

These data evidence the usage of both common and distinct entry factors within a specific genus. For instance, within the Flavivirus, ZIKV, DENV, YFV and WNV use AXL for infection, whereas this receptor has not been demonstrated as an entry factor for JEV. All Flaviviruses use HAVCR1 (TIM-1) and CD209 (DC-SIGN) to some extent for viral entry, except YFV. Similarly, the Togaviruses CHIKV, SINV and WEEV use BSG, whereas by BSG knock out in HEK293T cells had no impact on VEEV, ONNV, MAYV and RRV infections<sup>15</sup>. In Betacoronaviruses, SARS-CoV-1 does not harbor a motif for Furin cleavage, unlike SARS-CoV-2 and MERS-CoV<sup>16</sup>. However, all viruses of the genus use TMPRSS2. CTSL and CTSB promote Ebola viruses (EBOV) entry into target cells, whereas Marburg virus (MARV) is not cleaved by these proteases<sup>17</sup>.

Figure 2 shows the expression within the various cell populations of the human testis of the transcripts encoding the host factors displayed in Figure 1. This figure has two main purposes: (i) in case of viruses already known to infect the human testis, such as ZIKV, it entitles to easily visualize candidate entry factors within the different testicular cell populations; and (ii) for emerging viruses not yet identified in the testis, it helps to predict the potential for infection of the testicular cell populations.

Concerning (i), WNV was reported to infect human Sertoli cells *in vivo*<sup>1</sup>. WNV is able to use AXL, HAVCR1, TIMD4, MERTK, TYRO3, CD209, CLEC4M, the Integrin  $\alpha V\beta 3$  (represented by its two subunits ITGA5 and ITGB3), RPSA and CLDN1 as entry receptors (Figure 1). As showed in Figure 2, Sertoli cells express several of these receptors. AXL is particularly expressed in Sertoli cells and testicular macrophages. MERKT, TYRO3, CLEC4M are over expressed in Sertoli cells compared to other cell types. RPSA, and to a lesser extent TIMD4, are broadly expressed by all cell types. Surprisingly, CLDN1 mRNA was not readily detected in Sertoli cells, although this protein is involved in the tight junctions between Sertoli cells that form the blood-testis-barrier. On the contrary, HAVCR1 is not expressed in testicular cells apart from a few spermatids. Interestingly, the expression of the two subunits of the  $\alpha V\beta 3$  Integrin in each cell type is not consistent. These data suggest that WNV might infect Sertoli cells through binding to AXL, MERKT, TYRO3, CLEC4M, RPSA or TIMD4 principally, a hypothesis that will need to be verified by *in vitro* experiments. The expression of AXL, RPSA and TIMD4 in macrophages also suggests these cells may support the entry of WNV.

We demonstrated that ZIKV mostly infect CD68/CD163+ macrophages and DDX4+ germ cells in the human testis *ex vivo*<sup>18</sup>. ZIKV uses AXL, HAVCR1, TYRO3, NCAM1, EGFR, the Integrin  $\alpha V\beta 5$ , and HSPA4 for viral entry into target cells. Testicular macrophages express AXL to high levels, as well as the two subunits of  $\alpha V\beta 5$  when compared to other cell types, which may support their infection. The main receptors for ZIKV entry (e.g. AXL, TYRO3, HAVCR1) are absent from Testicular Germ Cells (TGC). However, HSPA4 and NCAM1, which are transcribed in Spermatogonia Stem Cells (SSC) and

spermatids, constitute potential candidates. As mentioned above, many receptors are shared among viruses of the same genus, especially within the Flaviviruses and some receptors are suspected to be panflaviviral (e.g. TAM, TIM and C-type lectins receptors)<sup>19</sup>. Thus TIMD4 (used by DENV and WNV), HSP90AA1/AB1 (used by DENV and JEV respectively) and RPSA, which are expressed by TGC, might contribute to their infection by ZIKV and require further investigation.

Additionally, we showed that SARS-CoV-2 infects the human testis *ex vivo*, targeting primarily Leydig and Sertoli cells, and to a lesser extent Peritubular Myoid Cells (PMC) and germ cells. Besides its main receptor ACE2, SARS-CoV-2 was shown to use AXL, HAVCR1, BSG, VIM, TFRC and NRP1 to infect cells. To prime infection, SARS-CoV-2 needs to be cleaved by cellular proteases, and uses with different efficiencies TMPRSS2, TMPRSS4 / 11A / 13 / 11D / 11E / 11F, Furin, CTSL and CTSB. Figure 2 indicates that ACE2 is only transcribed in Sertoli cells, which is inconsistent with our immunohistochemistry (IHC) results depicting ACE2 in Leydig, Sertoli and a few germ cells. As we did not observe ACE2 in PMC, other receptor(s) are likely involved in their infection. PMC express AXL, BSG, VIM, TRFC, NRP1, which thus constitute candidate receptors to explore. Consistent with our IHC results, TMPRSS2 appears little transcribed in the human testis, and only in a few SSC and spermatid. In line, we did not evidence protein expression of TMPRSS2 in the testis by IHC. CTSL, CTSB, and Furin are broadly expressed in testicular cells, a finding we confirmed in RT-qPCR on testis explants, and may trigger the priming of the SARS-CoV-2 spike protein.

Regarding the prediction of the potential infection of the testis by emerging viruses (ii), DENV and CHIKV represent interesting examples. Case reports have recorded the presence of these viruses in human semen and the subsequent sexual transmission of DENV<sup>1,20</sup>. To our knowledge, the infection of the testis by these viruses has never been reported. DENV principal receptors are TAM (AXL, MERTK, TYRO3), C-type lectins (CD209, CLEC4M, CLEC5A) and MRC1. Testicular macrophages express CD209, CLEC5A and MRC1; Leydig cells express AXL and TYRO3; PMC express AXL and CD209, Sertoli cell express AXL, MERTK, TYRO3, CD209 CLEC4M and MRC1. Therefore, somatic cells appear well equipped to support DENV infection. Additionally, DENV alternative receptors RPSA, PHB, and HSPA5 / A4 / 90AA1 are broadly expressed in the testis, including in TGC. CHIKV uses MXRA8, BSG, PHB, RPSA, TSPAN9, EPS15 and FUZ as receptors. While MXRA8 and TSPAN9 are mainly expressed in Leydig cells and PMC, FUZ is transcribed in spermatids; BSG, PHB, RPSA and EPS15 are broadly transcribed in the testicular cells. Therefore, based on the transcription of these receptors, the human testis represents a potential target for both DENV and CHIKV. Since putative target cell populations include cells localized within the intratubular compartments, the testis could represent a source of viral shedding in semen.

A focus on the steroidogenic Leydig cells shows that they specifically transcribe RNASEK and EFN3, the receptors of RVFV and of the Henipaviruses (HeV, NiV) respectively. Sertoli cells, which support and protect germ cells, transcribe CLEC4M, also a receptor for RVFV and TOSV. The vulnerable SSC and spermatogonia, situated out of the blood-testis-barrier, transcribe TSPAN9 and MET, which act as receptors for SFV and LASV, respectively.

Regarding viruses related (same genus) to those that have been detected in the male genital tract, the CD55 receptor of the Sin Nombre Virus (SNV); the BSG receptor of the Ross River Virus (RRV) and Western Equine Encephalitis Virus (WEEV); the LAMP1 and TRFC receptors of the New World Mammarenaviruses (JUNV) are all broadly expressed in the different testicular cell types. This suggests that the testis might be susceptible to these viruses, which have never been reported in the human MGT or semen. This observation raises the question of the potential infection of the human testis by these understudied viruses. Interestingly, the same observation can be made regarding the cell receptors used by Enteroviruses. Indeed, several receptors of Rhinoviruses HRV (LDLR, VLDLR, LRP1) and Echoviruses (FCGRT) are expressed in the somatic cells of the testis. Receptors of PoV (PVR); of EV-A71 (WARS1, PHB); of lineage A Cocksackie viruses, CV-A (SCARB2); and of lineage B Cocksackie viruses, CV-B (B2M, DNM2, ARF6) are expressed by all cell types in the testis.

Overall, this first analysis seeks to give an overview of the host factors used for viral entry by emerging single-strand RNA viruses, and their transcription in human testicular cells. The data presented here will need to be strengthened by the analysis of additional scRNA-seq data and by the validation of the expression of the receptors of interest in RT-qPCR, IHC or flow cytometry experiments. Complementary analyses of the equipment in viral restrictions factors of testicular cells will further contribute to apprehend the susceptibility of the human testis to a range of viruses, and in turn help predicting the infection of this organ. The human testis being a potential source of viral persistence and seminal shedding, this knowledge is important for anticipating the risk (or lack) of persistence and sexual transmission for these viral genera.

## Figures Legends

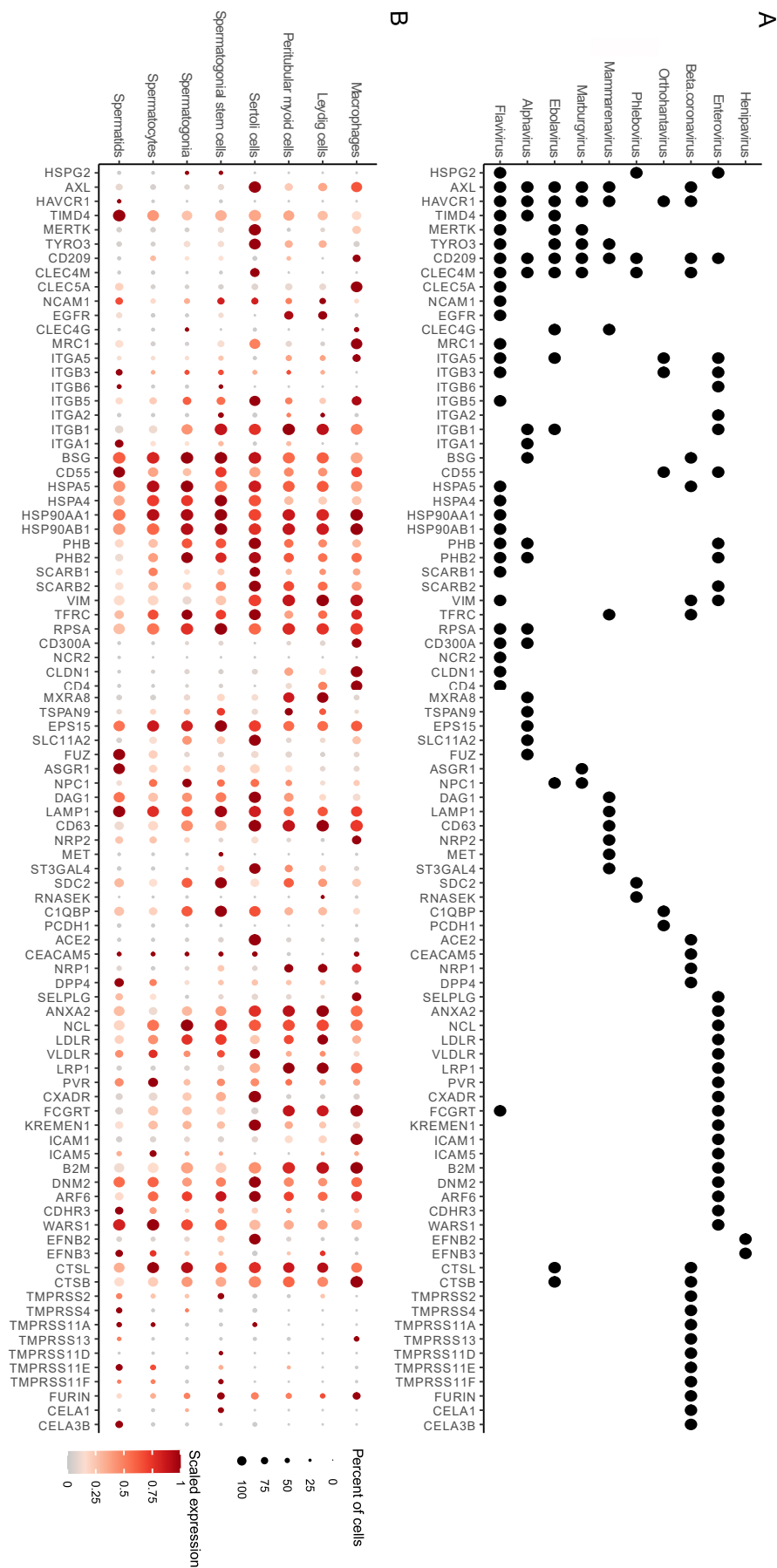
### Figure 1. Host factors used by emerging single strand RNA viruses for viral entry.

Viruses identifications (ID) are displayed on the X axis of the heatmap. The entry factors are displayed on the Y axis. The entry factors are designated by their NCBI official gene symbols. For each virus ID, the type of entry factor (EF) was color-coded: attachment receptors (yellow); entry receptors (red); and, proteases (blue). Column labels represent the association of each virus with its known tropism(s) (black) and its viral genera color-coded as indicated in the legend.

### Figure 2. Expression in the human testis of host factors used by emerging single strand RNA viruses for viral entry.

Panels A and B: Host factors (HF) are displayed on the X axis. Upper dot plot (A) represents the virus genera usage of the different HF. A dot is displayed if at least one virus of the genus uses the concerned EF. Bottom dot plot (B) represents the relative expression of EF in major testicular cell types. Size of dots corresponds to the percentage of cells expressing a given gene. The averaged expression values of each gene were scaled between [0;1] using a 0-max scaling (0=0; the maximum value = 1).





**Figure 2: Expression in the human testis of host factors used by emerging single strand RNA viruses for viral entry.**

**Annexes**

<b>Order</b>	<i>Amarillovirales</i>				
<b>Family</b>	<i>Flaviviridae</i>				
<b>Genus</b>	<i>Flavivirus</i>				
<b>Virus</b>	<b>ZIKV</b>	<b>DENV</b>	<b>YFV</b>	<b>WNV</b>	<b>JEV</b>

<b>Tropism (Virus-specific)</b>	21–26	27,28	29	30	31,32
<b>Tropism (Global)</b>	33–35				
<b>Receptors (Virus-specific)</b>	36–45	22,46–50		51–54	55
<b>Receptors (Global)</b>	19,34,49,56,57				

<b>Order</b>	<i>Martellivirales</i>							
<b>Family</b>	<i>Togaviridae</i>							
<b>Genus</b>	<i>Alphavirus</i>							
<b>Virus</b>	<b>CHIKV</b>	<b>RRV</b>	<b>SFV</b>	<b>SINV</b>	<b>MAYV</b>	<b>ONNV</b>	<b>VEEV</b>	<b>WEEV</b>

<b>Tropism (Virus-specific)</b>	58,59	60		61,62				
<b>Tropism (Global)</b>	15,63–67							
<b>Receptors (Virus-specific)</b>	15,46,50,68–70							
<b>Receptors (Global)</b>	65,67(p8),71							

<b>Order</b>	<i>Mononegavirales</i>	
<b>Family</b>	<i>Filoviridae</i>	
<b>Genus</b>	<i>Ebolavirus</i>	<i>Marbugvirus</i>
<b>Virus</b>	<b>EBOV</b>	<b>MARV</b>

<b>Tropism (Virus-specific)</b>	72,73	74,75
<b>Tropism (Global)</b>	76	
<b>Receptors (Virus-specific)</b>	17,77–84	85,86
<b>Receptors (Global)</b>	75,87,88(p1),89,90	

<b>Order</b>	<i>Arenaviridae</i>				<i>Bunyvirales</i>			<i>Hantaviridae</i>			
<b>Family</b>	<i>Mammarenavirus</i>				<i>Phenuiviridae</i>			<i>Orthohantavirus</i>			
<b>Genus</b>	<i>Mammarenavirus</i>				<i>Phlebovirus</i>			<i>Orthohantavirus</i>			
<b>Virus</b>	<b>LASV</b>	<b>LUJV</b>	<b>LCMV</b>	<b>JUNV</b>	<b>RVFV</b>	<b>TOSV</b>	<b>PTV</b>	<b>ANDV</b>	<b>SNV</b>	<b>HTNV</b>	<b>PUUV</b>

<b>Tropism (Virus-specific)</b>	91,92	93		94	95,96	97		98		99	
<b>Tropism (Global)</b>	100–105										
<b>Receptors (Virus-specific)</b>	106–109		110	111					99,112		
<b>Receptors (Global)</b>	101,113–123										

<b>Order</b>	<i>Nidovirales</i>		
<b>Family</b>	<i>Coronaviridae</i>		
<b>Genus</b>	<i>Beta-coronavirus</i>		
<b>Virus</b>	<b>SARS-CoV-1</b>	<b>SARS-CoV-2</b>	<b>MERS-CoV</b>

<b>Tropism (Virus-specific)</b>	124	125,126	127,128
<b>Tropism (Global)</b>	129-131		
<b>Receptors (Virus-specific)</b>	132-139	140-146,147(p14),148-151	
<b>Receptors (Global)</b>	152-159		

<b>Order</b>	<i>Picornavirales</i>										
<b>Family</b>	<i>Picornaviridae</i>										
<b>Genus</b>	<i>Enterovirus</i>										
<b>Virus</b>	<b>EV-A71</b>	<b>CV-A</b>	<b>Echovirus</b>	<b>CV-B</b>	<b>PoV</b>	<b>CV-C</b>	<b>EV-D68</b>	<b>EV-D70</b>	<b>HRV-A</b>	<b>HRV-B</b>	<b>HRV-C</b>

<b>Tropism (Virus-specific)</b>	160		161,162	163,164	165		166	167	168,169		
<b>Tropism (Global)</b>	170-175										
<b>Receptors (Virus-specific)</b>			176	177-179			180		168		
<b>Receptors (Global)</b>	171,174,181-183										



<b>Order</b>	<i>Mononegavirales</i>	
<b>Family</b>	<i>Paramyxoviridae</i>	
<b>Genus</b>	<i>Henipavirus</i>	
<b>Virus</b>	<b>HeV</b>	<b>NiV</b>

<b>Tropism (Global)</b>	184,185	186,187
<b>Receptors (Virus-specific)</b>		188
<b>Receptors (Global)</b>	189,190	

## References

1. Le Tortorec A, Matusali G, Mahé D, et al. From Ancient to Emerging Infections: The Odyssey of Viruses in the Male Genital Tract. *Physiological reviews*. 2020;100(3):1349-1414. doi:10.1152/PHYSREV.00021.2019
2. Blitvich BJ, Magalhaes T, Viridiana Laredo-Tiscareño S, Foy BD. Sexual Transmission of Arboviruses: A Systematic Review. *Viruses*. 2020;12(9). doi:10.3390/V12090933
3. Bujan L, Mansuy JM, Hamdi S, Pasquier C, Joguet G. 1 year after acute Zika virus infection in men. *The Lancet Infectious diseases*. 2020;20(1):25-26. doi:10.1016/S1473-3099(19)30678-4
4. Schindell BG, Webb AL, Kindrachuk J. Persistence and Sexual Transmission of Filoviruses. *Viruses*. 2018;10(12). doi:10.3390/V10120683
5. Teixeira T, Oliveira Y, Bernardes F, et al. Viral infections and implications for male reproductive health. *Asian journal of andrology*. 2021;23(4):335-347. doi:10.4103/AJA.AJA\_82\_20
6. Keita AK, Koundouno FR, Faye M, et al. Resurgence of Ebola virus in 2021 in Guinea suggests a new paradigm for outbreaks. *Nature*. 2021;597(7877):539-543. doi:10.1038/s41586-021-03901-9
7. Duggal NK, McDonald EM, Ritter JM, Brault AC. Sexual transmission of Zika virus enhances in utero transmission in a mouse model. *Scientific reports*. 2018;8(1). doi:10.1038/S41598-018-22840-6
8. Mahé D, Bourgeau S, Frouard J, et al. Long-term Zika virus infection of non-sperm cells in semen. *The Lancet Infectious Diseases*. 2020;20(12):1371. doi:10.1016/S1473-3099(20)30834-3
9. Le Goffic R, Mouchel T, Ruffault A, Patard JJ, Jégou B, Samson M. Mumps virus decreases testosterone production and gamma interferon-induced protein 10 secretion by human Leydig cells. *J Virol*. 2003;77(5):3297-3300. doi:10.1128/jvi.77.5.3297-3300.2003
10. Wang M, Liu X, Chang G, et al. Single-Cell RNA Sequencing Analysis Reveals Sequential Cell Fate Transition during Human Spermatogenesis. *Cell Stem Cell*. 2018;23(4):599-614.e4. doi:10.1016/j.stem.2018.08.007
11. Darde TA, Lecluze E, Lardenois A, et al. The ReproGenomics Viewer: a multi-omics and cross-species resource compatible with single-cell studies for the reproductive science community. *Bioinformatics*. 2019;35(17):3133-3139. doi:10.1093/bioinformatics/btz047
12. Darde TA, Sallou O, Becker E, et al. The ReproGenomics Viewer: an integrative cross-species toolbox for the reproductive science community. *Nucleic Acids Res*. 2015;43(W1):W109-116. doi:10.1093/nar/gkv345

13. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. *Cell*. 2021;184(13):3573-3587.e29. doi:10.1016/j.cell.2021.04.048
14. Leonard S, Lardenois A, Tarte K, Rolland AD, Chalmel F. FlexDotPlot: a universal and modular dot plot visualization tool for complex multifaceted data. *Bioinformatics Advances*. 2022;2(1):vbac019. doi:10.1093/bioadv/vbac019
15. De Caluwé L, Coppens S, Vereecken K, et al. The CD147 Protein Complex Is Involved in Entry of Chikungunya Virus and Related Alphaviruses in Human Cells. *Frontiers in Microbiology*. 2021;12:352. doi:10.3389/FMICB.2021.615165/BIBTEX
16. Johnson BA, Xie X, Kalveram B, et al. Furin Cleavage Site Is Key to SARS-CoV-2 Pathogenesis. *bioRxiv*. Published online August 26, 2020:2020.08.26.268854. doi:10.1101/2020.08.26.268854
17. Gnirß K, Kühl A, Karsten C, et al. Cathepsins B and L activate Ebola but not Marburg virus glycoproteins for efficient entry into cell lines and macrophages independent of TMPRSS2 expression. *Virology*. 2012;424(1):3-10. doi:10.1016/J.VIROL.2011.11.031
18. Matusali G, Houzet L, Satie AP, et al. Zika virus infects human testicular tissue and germ cells. *The Journal of clinical investigation*. 2018;128(10):4697-4710. doi:10.1172/JCI121735
19. Perera-Lecoin M, Meertens L, Carnec X, Amara A. Flavivirus entry receptors: an update. *Viruses*. 2013;6(1):69-88. doi:10.3390/V6010069
20. Martins EB, Silva MFB, Tassinari WS, et al. Detection of Chikungunya virus in bodily fluids: The INOVACHIK cohort study. *PLoS neglected tropical diseases*. 2022;16(3):e0010242. doi:10.1371/JOURNAL.PNTD.0010242
21. Agrelli A, de Moura RR, Crovella S, Brandão LAC. ZIKA virus entry mechanisms in human cells. *Infection, Genetics and Evolution*. 2019;69:22-29. doi:10.1016/J.MEEGID.2019.01.018
22. Cruz-Oliveira C, Freire JM, Conceição TM, Higa LM, Castanho MARB, Da Poian AT. Receptors and routes of dengue virus entry into the host cells. *FEMS microbiology reviews*. 2015;39(2):155-170. doi:10.1093/FEMSRE/FUU004
23. Gavino-Leopoldino D, Figueiredo CM, da Silva MOL, et al. Skeletal Muscle Is an Early Site of Zika Virus Replication and Injury, Which Impairs Myogenesis. *Journal of virology*. 2021;95(22). doi:10.1128/JVI.00904-21
24. Hamel R, Dejarnac O, Wichit S, et al. Biology of Zika Virus Infection in Human Skin Cells. *Journal of virology*. 2015;89(17):8880-8896. doi:10.1128/JVI.00354-15
25. Miner JJ, Diamond MS. Zika virus pathogenesis and tissue tropism. *Cell host & microbe*. 2017;21(2):134. doi:10.1016/J.CHOM.2017.01.004
26. Retallack H, Di Lullo E, Arias C, et al. Zika virus cell tropism in the developing human brain and inhibition by azithromycin. *Proceedings of the National Academy of Sciences of the United*

- States of America*. 2016;113(50):14408-14413. doi:10.1073/PNAS.1618029113/-/DCSUPPLEMENTAL
27. Begum F, Das S, Mukherjee D, Mal S, Ray U. Insight into the Tropism of Dengue Virus in Humans. *Viruses*. 2019;11(12). doi:10.3390/V11121136
  28. Reyes-del Valle J, Salas-Benito J, Soto-Acosta R, del Angel RM. Dengue Virus Cellular Receptors and Tropism. *Current Tropical Medicine Reports*. 2014;1(1):36-43. doi:10.1007/S40475-013-0002-7/TABLES/1
  29. Monath TP, Barrett ADT. Pathogenesis and pathophysiology of yellow fever. *Advances in virus research*. 2003;60:343-395. doi:10.1016/S0065-3527(03)60009-6
  30. Lim SM, Koraka P, Osterhaus ADME, Martina BEE. West Nile Virus: Immunity and Pathogenesis. *Viruses*. 2011;3(6):811. doi:10.3390/V3060811
  31. Johnson RT, Burke DS, Elwell M, et al. Japanese encephalitis: immunocytochemical studies of viral antigen and inflammatory cells in fatal cases. *Annals of neurology*. 1985;18(5):567-573. doi:10.1002/ANA.410180510
  32. Lannes N, Neuhaus V, Scolari B, et al. Interactions of human microglia cells with Japanese encephalitis virus. *Virology Journal*. 2017;14(1):1-12. doi:10.1186/S12985-016-0675-3/FIGURES/6
  33. Gould EA, Solomon T. Pathogenic flaviviruses. *Lancet (London, England)*. 2008;371(9611):500-509. doi:10.1016/S0140-6736(08)60238-X
  34. Laureti M, Narayanan D, Rodriguez-Andres J, Fazakerley JK, Kedzierski L. Flavivirus Receptors: Diversity, Identity, and Cell Entry. *Frontiers in Immunology*. 2018;9:2180. doi:10.3389/FIMMU.2018.02180
  35. Pierson TC, Diamond MS. The continued threat of emerging flaviviruses. *Nature microbiology*. 2020;5(6):796-812. doi:10.1038/S41564-020-0714-0
  36. Giraldo MI, Xia H, Aguilera-Aguirre L, et al. Envelope protein ubiquitination drives entry and pathogenesis of Zika virus. *Nature 2020 585:7825*. 2020;585(7825):414-419. doi:10.1038/s41586-020-2457-8
  37. Kim SY, Zhao J, Liu X, et al. Interaction of Zika Virus Envelope Protein with Glycosaminoglycans. *Biochemistry*. 2017;56(8):1151-1162. doi:10.1021/ACS.BIOCHEM.6B01056
  38. Liu S, Delalio LJ, Isakson BE, Wang TT. AXL-Mediated Productive Infection of Human Endothelial Cells by Zika Virus. *Circulation research*. 2016;119(11):1183-1189. doi:10.1161/CIRCRESAHA.116.309866
  39. Meertens L, Labeau A, Dejarnac O, et al. Axl Mediates ZIKA Virus Entry in Human Glial Cells and Modulates Innate Immune Responses. *Cell reports*. 2017;18(2):324-333. doi:10.1016/J.CELREP.2016.12.045

40. Nowakowski TJ, Pollen AA, Di Lullo E, Sandoval-Espinosa C, Bershteyn M, Kriegstein AR. Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in Neural Stem Cells. *Cell stem cell*. 2016;18(5):591-596. doi:10.1016/J.STEM.2016.03.012
41. Richard AS, Shim BS, Kwon YC, et al. AXL-dependent infection of human fetal endothelial cells distinguishes Zika virus from other pathogenic flaviviruses. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114(8):2024-2029. doi:10.1073/PNAS.1620558114
42. Sabino C, Bender D, Herrlein ML, Hildt E. The Epidermal Growth Factor Receptor Is a Relevant Host Factor in the Early Stages of The Zika Virus Life Cycle In Vitro. *Journal of Virology*. 2021;95(20). doi:10.1128/JVI.01195-21
43. Sirohi D, Kuhn RJ. Zika Virus Structure, Maturation, and Receptors. *The Journal of Infectious Diseases*. 2017;216(suppl\_10):S935-S944. doi:10.1093/INFDIS/JIX515
44. Srivastava M, Zhang Y, Chen J, et al. Chemical proteomics tracks virus entry and uncovers NCAM1 as Zika virus receptor. *Nature communications*. 2020;11(1). doi:10.1038/S41467-020-17638-Y
45. Wang S, Zhang Q, Tiwari SK, et al. Integrin  $\alpha\beta 5$  Internalizes Zika Virus during Neural Stem Cells Infection and Provides a Promising Target for Antiviral Therapy. *Cell reports*. 2020;30(4):969. doi:10.1016/J.CELREP.2019.11.020
46. Carnec X, Meertens L, Dejarnac O, et al. The Phosphatidylserine and Phosphatidylethanolamine Receptor CD300a Binds Dengue Virus and Enhances Infection. *Journal of Virology*. 2016;90(1):92. doi:10.1128/JVI.01849-15
47. Chen Y, Maguire T, Hileman RE, et al. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nature medicine*. 1997;3(8):866-871. doi:10.1038/NM0897-866
48. Liu P, Ridilla M, Patel P, et al. Beyond attachment: Roles of DC-SIGN in dengue virus infection. *Traffic (Copenhagen, Denmark)*. 2017;18(4):218-231. doi:10.1111/TRA.12469
49. Meertens L, Carnec X, Lecoin MP, et al. The TIM and TAM Families of Phosphatidylserine Receptors Mediate Dengue Virus Entry. *Cell host & microbe*. 2012;12(4):544. doi:10.1016/J.CHOM.2012.08.009
50. Sharma A, Vasanthapuram R, M Venkataswamy M, Desai A. Prohibitin 1/2 mediates Dengue-3 entry into human neuroblastoma (SH-SY5Y) and microglia (CHME-3) cells. *Journal of Biomedical Science*. 2020;27(1):1-17. doi:10.1186/S12929-020-00639-W/FIGURES/11
51. Chu JH, Ng ML. Interaction of West Nile virus with alpha v beta 3 integrin mediates virus entry into cells. *The Journal of biological chemistry*. 2004;279(52):54533-54541. doi:10.1074/JBC.M410208200

52. Davis CW, Nguyen HY, Hanna SL, Sánchez MD, Doms RW, Pierson TC. West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. *Journal of virology*. 2006;80(3):1290-1301. doi:10.1128/JVI.80.3.1290-1301.2006
53. Hershkovitz O, Rosental B, Rosenberg LA, et al. NKp44 Receptor Mediates Interaction of the Envelope Glycoproteins from the West Nile and Dengue Viruses with NK Cells. *The Journal of Immunology*. 2009;183(4):2610-2621. doi:10.4049/JIMMUNOL.0802806
54. Martina BEE, Koraka P, van den Doel P, Rimmelzwaan GF, Haagmans BL, Osterhaus ADME. DC-SIGN enhances infection of cells with glycosylated West Nile virus in vitro and virus replication in human dendritic cells induces production of IFN-alpha and TNF-alpha. *Virus research*. 2008;135(1):64-71. doi:10.1016/J.VIRUSRES.2008.02.008
55. Yun SI, Lee YM. Early Events in Japanese Encephalitis Virus Infection: Viral Entry. *Pathogens*. 2018;7(3). doi:10.3390/PATHOGENS7030068
56. Hu T, Wu Z, Wu S, Chen S, Cheng A. The key amino acids of E protein involved in early flavivirus infection: viral entry. *Virology Journal* 2021 18:1. 2021;18(1):1-12. doi:10.1186/S12985-021-01611-2
57. Tabata T, Petitt M, Puerta-Guardo H, et al. Zika Virus Targets Different Primary Human Placental Cells, Suggesting Two Routes for Vertical Transmission. *Cell host & microbe*. 2016;20(2):155-166. doi:10.1016/J.CHOM.2016.07.002
58. Fox JM, Diamond MS. Immune-mediated protection and pathogenesis of chikungunya virus. *Journal of immunology (Baltimore, Md : 1950)*. 2016;197(11):4210. doi:10.4049/JIMMUNOL.1601426
59. Matusali G, Colavita F, Bordi L, et al. Tropism of the Chikungunya Virus. *Viruses*. 2019;11(2). doi:10.3390/V11020175
60. Krejbich-Trotot P, Belarbi E, Ralambondrainy M, et al. The growth of arthralgic Ross River virus is restricted in human monocytic cells. *Virus Research*. 2016;225:64. doi:10.1016/J.VIRUSRES.2016.09.007
61. Adouchief S, Smura T, Sane J, Vapalahti O, Kurkela S. Sindbis virus as a human pathogen—epidemiology, clinical picture and pathogenesis. *Reviews in Medical Virology*. 2016;26(4):221-241. doi:10.1002/RMV.1876
62. Xu J, Nash RJ, Frey TK. Cellular responses to Sindbis virus infection of neural progenitors derived from human embryonic stem cells. *BMC Res Notes*. 2014;7:757. doi:10.1186/1756-0500-7-757
63. Cappuccio L, Maise C. Infection of Mammals and Mosquitoes by Alphaviruses: Involvement of Cell Death. *Cells* 2020, Vol 9, Page 2612. 2020;9(12):2612. doi:10.3390/CELLS9122612
64. Gardner CL, Burke CW, Tesfay MZ, Glass PJ, Klimstra WB, Ryman KD. Eastern and Venezuelan Equine Encephalitis Viruses Differ in Their Ability To Infect Dendritic Cells and Macrophages:

- Impact of Altered Cell Tropism on Pathogenesis. *Journal of Virology*. 2008;82(21):10634. doi:10.1128/JVI.01323-08
65. Holmes AC, Basore K, Fremont DH, Diamond MS. A molecular understanding of alphavirus entry. *PLoS Pathogens*. 2020;16(10). doi:10.1371/JOURNAL.PPAT.1008876
  66. Suhrbier A, Jaffar-Bandjee MC, Gasque P. Arthritogenic alphaviruses—an overview. *Nature Reviews Rheumatology* 2012 8:7. 2012;8(7):420-429. doi:10.1038/nrrheum.2012.64
  67. Zhang R, Kim AS, Fox JM, et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. *Nature* 2018 557:7706. 2018;557(7706):570-574. doi:10.1038/s41586-018-0121-3
  68. Bernard E, Salignat M, Gay B, et al. Endocytosis of Chikungunya Virus into Mammalian Cells: Role of Clathrin and Early Endosomal Compartments. *PLoS ONE*. 2010;5(7). doi:10.1371/JOURNAL.PONE.0011479
  69. van Duijl-Richter MKS, Hoornweg TE, Rodenhuis-Zybert IA, Smit JM. Early Events in Chikungunya Virus Infection—From Virus CellBinding to Membrane Fusion. *Viruses* 2015, Vol 7, Pages 3647-3674. 2015;7(7):3647-3674. doi:10.3390/V7072792
  70. Schnierle BS. Cellular Attachment and Entry Factors for Chikungunya Virus. *Viruses*. 2019;11(11). doi:10.3390/V11111078
  71. Song H, Zhao Z, Chai Y, et al. Molecular Basis of Arthritogenic Alphavirus Receptor MXRA8 Binding to Chikungunya Virus Envelope Protein. *Cell*. 2019;177(7):1714-1724.e12. doi:10.1016/J.CELL.2019.04.008
  72. Martines RB, Ng DL, Greer PW, Rollin PE, Zaki SR. Tissue and cellular tropism, pathology and pathogenesis of Ebola and Marburg viruses. *The Journal of Pathology*. 2015;235(2):153-174. doi:10.1002/PATH.4456
  73. Takada A. Filovirus Tropism: Cellular Molecules for Viral Entry. *Frontiers in Microbiology*. 2012;3(FEB). doi:10.3389/FMICB.2012.00034
  74. Hofmann-Winkler H, Kaup F, Pöhlmann S. Host Cell Factors in Filovirus Entry: Novel Players, New Insights. *Viruses*. 2012;4(12):3336. doi:10.3390/V4123336
  75. Shimojima M, Takada A, Ebihara H, et al. Tyro3 family-mediated cell entry of Ebola and Marburg viruses. *Journal of virology*. 2006;80(20):10109-10116. doi:10.1128/JVI.01157-06
  76. Burk R, Bollinger L, Johnson JC, et al. Neglected filoviruses. *FEMS Microbiology Reviews*. 2016;40(4):494. doi:10.1093/FEMSRE/FUW010
  77. Alvarez CP, Lasala F, Carrillo J, Muñoz O, Corbí AL, Delgado R. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. *Journal of virology*. 2002;76(13):6841-6844. doi:10.1128/JVI.76.13.6841-6844.2002
  78. Brindley MA, Hunt CL, Kondratowicz AS, et al. Tyrosine kinase receptor Axl enhances entry of Zaire ebolavirus without direct interactions with the viral glycoprotein. *Virology*. 2011;415(2):83-94. doi:10.1016/J.VIROL.2011.04.002

79. Gramberg T, Hofmann H, Möller P, et al. LSECtin interacts with filovirus glycoproteins and the spike protein of SARS coronavirus. *Virology*. 2005;340(2):224-236. doi:10.1016/J.VIROL.2005.06.026
80. Marzi A, Möller P, Hanna SL, et al. Analysis of the interaction of Ebola virus glycoprotein with DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) and its homologue DC-SIGNR. *The Journal of infectious diseases*. 2007;196 Suppl 2(Suppl 2). doi:10.1086/520607
81. Rogers KJ, Brunton B, Mallinger L, et al. IL-4/IL-13 polarization of macrophages enhances Ebola virus glycoprotein-dependent infection. *PLOS Neglected Tropical Diseases*. 2019;13(12):e0007819. doi:10.1371/JOURNAL.PNTD.0007819
82. Schornberg KL, Shoemaker CJ, Dube D, et al.  $\alpha 5\beta 1$ -Integrin controls ebolavirus entry by regulating endosomal cathepsins. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(19):8003. doi:10.1073/PNAS.0807578106
83. Simmons G, Reeves JD, Grogan CC, et al. DC-SIGN and DC-SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells. *Virology*. 2003;305(1):115-123. doi:10.1006/VIRO.2002.1730
84. Takada A, Watanabe S, Ito H, Okazaki K, Kida H, Kawaoka Y. Downregulation of beta1 integrins by Ebola virus glycoprotein: implication for virus entry. *Virology*. 2000;278(1):20-26. doi:10.1006/VIRO.2000.0601
85. Becker S, Spiess M, Klenk HD. The asialoglycoprotein receptor is a potential liver-specific receptor for Marburg virus. *Journal of General Virology*. 1995;76(2):393-399. doi:10.1099/0022-1317-76-2-393/CITE/REFWORKS
86. Marzi A, Gramberg T, Simmons G, et al. DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. *Journal of virology*. 2004;78(21):12090-12095. doi:10.1128/JVI.78.21.12090-12095.2004
87. Brunton B, Rogers K, Phillips EK, et al. TIM-1 serves as a receptor for Ebola virus in vivo, enhancing viremia and pathogenesis. *PLoS Neglected Tropical Diseases*. 2019;13(6). doi:10.1371/JOURNAL.PNTD.0006983
88. Carette JE, Raaben M, Wong AC, et al. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature*. 2011;477(7364):340-343. doi:10.1038/NATURE10348
89. Kondratowicz AS, Lennemann NJ, Sinn PL, et al. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(20):8426-8431. doi:10.1073/PNAS.1019030108
90. Takada A, Fujioka K, Tsuiji M, et al. Human Macrophage C-Type Lectin Specific for Galactose and N -Acetylgalactosamine Promotes Filovirus Entry. *Journal of Virology*. 2004;78(6):2943-2947. doi:10.1128/JVI.78.6.2943-2947.2004/ASSET/7F9B686F-1E47-472A-8296-1328C6E267E8/ASSETS/GRAPHIC/ZJV0060413630004.JPEG



91. Baize S, Kaplon J, Faure C, Pannetier D, Georges-Courbot MC, Deubel V. Lassa Virus Infection of Human Dendritic Cells and Macrophages Is Productive but Fails to Activate Cells. *The Journal of Immunology*. 2004;172(5):2861-2869. doi:10.4049/JIMMUNOL.172.5.2861
92. Shieh WJ, Demby A, Jones T, et al. Pathology and Pathogenesis of Lassa Fever: Novel Immunohistochemical Findings in Fatal Cases and Clinico-pathologic Correlation. *Clinical Infectious Diseases*. Published online 2021. doi:10.1093/CID/CIAB719
93. Kunz S, de la Torre JC. Breaking the Barrier: Host Cell Invasion by Lujo Virus. *Cell Host & Microbe*. 2017;22(5):583-585. doi:10.1016/J.CHOM.2017.10.014
94. Oldenburg J, Reignier T, Flanagan ML, Hamilton GA, Cannon PM. Differences in tropism and pH-dependence for glycoproteins from the Clade B1 arenaviruses: implications for receptor usage and pathogenicity. *Virology*. 2007;364(1):132. doi:10.1016/J.VIROL.2007.03.003
95. Ganaie SS, Schwarz MM, McMillen CM, et al. Lrp1 is a host entry factor for Rift Valley fever virus. *Cell*. 2021;184(20):5163-5178.e24. doi:10.1016/j.cell.2021.09.001
96. Wright D, Kortekaas J, Bowden TA, Warimwe GM. Rift valley fever: Biology and epidemiology. *Journal of General Virology*. 2019;100(8):1187-1199. doi:10.1099/JGV.0.001296/CITE/REFWORKS
97. Charrel RN, Gallian P, Navarro-Marí JM, et al. Emergence of Toscana Virus in Europe. *Emerging Infectious Diseases*. 2005;11(11):1657. doi:10.3201/EID1111.050869
98. Chiang CF, Flint M, Lin JMS, Spiropoulou CF. Endocytic Pathways Used by Andes Virus to Enter Primary Human Lung Endothelial Cells. *PLOS ONE*. 2016;11(10):e0164768. doi:10.1371/JOURNAL.PONE.0164768
99. Mayor J, Torriani G, Rothenberger S, Engler O. T-cell immunoglobulin and mucin (TIM) contributes to the infection of human airway epithelial cells by pseudotype viruses containing Hantaan virus glycoproteins. *Virology*. 2020;543:54-62. doi:10.1016/J.VIROL.2020.02.002
100. Hallam SJ, Koma T, Maruyama J, Paessler S. Review of Mammarenavirus Biology and Replication. *Frontiers in Microbiology*. 2018;9(AUG):1751. doi:10.3389/FMICB.2018.01751
101. Jangra RK, Herbert AS, Li R, et al. Protocadherin-1 is essential for cell entry by New World hantaviruses. *Nature*. 2018;563(7732):559-563. doi:10.1038/S41586-018-0702-1
102. Lambert AJ, Hughes HR. Clinically Important Phleboviruses and Their Detection in Human Samples. *Viruses 2021, Vol 13, Page 1500*. 2021;13(8):1500. doi:10.3390/V13081500
103. Mittler E, Dieterle ME, Kleinfelter LM, Slough MM, Chandran K, Jangra RK. Hantavirus entry: Perspectives and recent advances. *Advances in virus research*. 2019;104:185-224. doi:10.1016/BS.AIVIR.2019.07.002

104. Noack D, Goeijenbier M, Reusken CBEM, Koopmans MPG, Rockx BHG. Orthohantavirus Pathogenesis and Cell Tropism. *Frontiers in cellular and infection microbiology*. 2020;10:399. doi:10.3389/FCIMB.2020.00399/BIBTEX
105. Odendaal L, Clift SJ, Fosgate GT, Davis AS. Lesions and Cellular Tropism of Natural Rift Valley Fever Virus Infection in Adult Sheep. *Veterinary pathology*. 2019;56(1):61-77. doi:10.1177/0300985818806049
106. Brouillette RB, Phillips EK, Patel R, et al. TIM-1 Mediates Dystroglycan-Independent Entry of Lassa Virus. *Journal of Virology*. 2018;92(16). doi:10.1128/JVI.00093-18
107. Fedeli C, Moreno H, Kunz S. The Role of Receptor Tyrosine Kinases in Lassa Virus Cell Entry. *Viruses*. 2020;12(8). doi:10.3390/V12080857
108. Raaben M, Jae LT, Herbert AS, et al. NRP2 and CD63 Are Host Factors for Lujo Virus Cell Entry. *Cell host & microbe*. 2017;22(5):688-696.e5. doi:10.1016/J.CHOM.2017.10.002
109. Saito T, Hattori T, Okuya K, et al. Molecular Mechanisms Underlying the Cellular Entry and Host Range Restriction of Lujo Virus. *mBio*. 2022;13(1). doi:10.1128/MBIO.03060-21
110. Radoshitzky SR, Abraham J, Spiropoulou CF, et al. Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. *Nature*. 2007;446(7131):92. doi:10.1038/NATURE05539
111. Hackett BA, Yasunaga A, Panda D, et al. RNASEK is required for internalization of diverse acid-dependent viruses. *Proceedings of the National Academy of Sciences of the United States of America*. 2015;112(25):7797-7802. doi:10.1073/PNAS.1424098112
112. Choi Y, Kwon YC, Kim SI, Park JM, Lee KH, Ahn BY. A hantavirus causing hemorrhagic fever with renal syndrome requires gC1qR/p32 for efficient cell binding and infection. *Virology*. 2008;381(2):178-183. doi:10.1016/J.VIROL.2008.08.035
113. Albornoz A, Hoffmann AB, Lozach PY, Tischler ND. Early Bunyavirus-Host Cell Interactions. *Viruses*. 2016;8(5). doi:10.3390/V8050143
114. de Boer SM, Kortekaas J, de Haan CAM, Rottier PJM, Moormann RJM, Bosch BJ. Heparan sulfate facilitates Rift Valley fever virus entry into the cell. *Journal of virology*. 2012;86(24):13767-13771. doi:10.1128/JVI.01364-12
115. Cao W, Henry MD, Borrow P, et al. Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science (New York, NY)*. 1998;282(5396):2079-2081. doi:10.1126/SCIENCE.282.5396.2079
116. Fedeli C, Torriani G, Galan-Navarro C, et al. Axl Can Serve as Entry Factor for Lassa Virus Depending on the Functional Glycosylation of Dystroglycan. *Journal of Virology*. 2018;92(5):1613-1630. doi:10.1128/JVI.01613-17

117. Goncalves AR, Moraz ML, Pasquato A, Helenius A, Lozach PY, Kunz S. Role of DC-SIGN in Lassa virus entry into human dendritic cells. *Journal of virology*. 2013;87(21):11504-11515. doi:10.1128/JVI.01893-13
118. Hulswit RJG, Paesen GC, Bowden TA, Shi X. Recent Advances in Bunyavirus Glycoprotein Research: Precursor Processing, Receptor Binding and Structure. *Viruses*. 2021;13(2). doi:10.3390/V13020353
119. Jae LT, Raaben M, Herbert AS, et al. Virus entry. Lassa virus entry requires a trigger-induced receptor switch. *Science (New York, NY)*. 2014;344(6191):1506-1510. doi:10.1126/SCIENCE.1252480
120. Koch J, Xin Q, Tischler ND, Lozach PY. Entry of Phenuiviruses into Mammalian Host Cells. *Viruses*. 2021;13(2). doi:10.3390/V13020299
121. Phoenix I, Nishiyama S, Lokugamage N, et al. N-Glycans on the Rift Valley Fever Virus Envelope Glycoproteins Gn and Gc Redundantly Support Viral Infection via DC-SIGN. *Viruses*. 2016;8(5). doi:10.3390/V8050149
122. Shimojima M, Ströher U, Ebihara H, Feldmann H, Kawaoka Y. Identification of Cell Surface Molecules Involved in Dystroglycan-Independent Lassa Virus Cell Entry. *Journal of Virology*. 2012;86(4):2067. doi:10.1128/JVI.06451-11
123. Torriani G, Galan-Navarro C, Kunz S. Lassa Virus Cell Entry Reveals New Aspects of Virus-Host Cell Interaction. *Journal of Virology*. 2017;91(4). doi:10.1128/JVI.01902-16/ASSET/C13350D0-860D-45C4-A767-04ABF5FE647E/ASSETS/GRAPHIC/ZJV9991823330001.JPEG
124. To KF, Tong JHM, Chan PKS, et al. Tissue and cellular tropism of the coronavirus associated with severe acute respiratory syndrome: an in-situ hybridization study of fatal cases. *The Journal of Pathology*. 2004;202(2):157. doi:10.1002/PATH.1510
125. Han Y, Yang L, Lacko LA, Chen S. Human organoid models to study SARS-CoV-2 infection. *Nature methods*. 2022;19(4):418-428. doi:10.1038/S41592-022-01453-Y
126. Wong DWL, Klinkhammer BM, Djudjaj S, et al. Multisystemic cellular tropism of sars-cov-2 in autopsies of covid-19 patients. *Cells*. 2021;10(8):1900. doi:10.3390/CELLS10081900/S1
127. Chan RWY, Hemida MG, Kayali G, et al. Tropism and replication of Middle East respiratory syndrome coronavirus from dromedary camels in the human respiratory tract: an in-vitro and ex-vivo study. *The Lancet Respiratory Medicine*. 2014;2(10):813. doi:10.1016/S2213-2600(14)70158-4
128. Zhou J, Chu H, Chan JFW, Yuen KY. Middle East respiratory syndrome coronavirus infection: Virus-host cell interactions and implications on pathogenesis. *Virology Journal*. 2015;12(1):1-7. doi:10.1186/S12985-015-0446-6/METRICS
129. Chu H, Chan JFW, Yuen TTT, et al. Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations,

- transmissibility, and laboratory studies of COVID-19: an observational study. *The Lancet Microbe*. 2020;1(1):e14-e23. doi:10.1016/S2666-5247(20)30004-5
130. Hui KPY, Cheung MC, Perera RAPM, et al. Tropism, replication competence, and innate immune responses of the coronavirus SARS-CoV-2 in human respiratory tract and conjunctiva: an analysis in ex-vivo and in-vitro cultures. *The Lancet Respiratory Medicine*. 2020;8(7):687. doi:10.1016/S2213-2600(20)30193-4
  131. Najafi Fard S, Petrone L, Petruccioli E, et al. In Vitro Models for Studying Entry, Tissue Tropism, and Therapeutic Approaches of Highly Pathogenic Coronaviruses. *BioMed research international*. 2021;2021. doi:10.1155/2021/8856018
  132. Heurich A, Hofmann-Winkler H, Gierer S, Liepold T, Jahn O, Pöhlmann S. TMPRSS2 and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein. *Journal of virology*. 2014;88(2):1293-1307. doi:10.1128/JVI.02202-13
  133. Hoffmann M, Hofmann-Winkler H, Smith JC, et al. Camostat mesylate inhibits SARS-CoV-2 activation by TMPRSS2-related proteases and its metabolite GBPA exerts antiviral activity. *EBioMedicine*. 2021;65. doi:10.1016/J.EBIOM.2021.103255
  134. Huang IC, Bosch BJ, Li F, et al. SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. *The Journal of biological chemistry*. 2006;281(6):3198-3203. doi:10.1074/JBC.M508381200
  135. Kawase M, Shirato K, van der Hoek L, Taguchi F, Matsuyama S. Simultaneous treatment of human bronchial epithelial cells with serine and cysteine protease inhibitors prevents severe acute respiratory syndrome coronavirus entry. *Journal of virology*. 2012;86(12):6537-6545. doi:10.1128/JVI.00094-12
  136. Li W, Moore MJ, Vasllieva N, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature*. 2003;426(6965):450-454. doi:10.1038/NATURE02145
  137. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, Taguchi F. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *Journal of virology*. 2010;84(24):12658-12664. doi:10.1128/JVI.01542-10
  138. Yang ZY, Huang Y, Ganesh L, et al. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. *Journal of virology*. 2004;78(11):5642-5650. doi:10.1128/JVI.78.11.5642-5650.2004
  139. Yu YTC, Chien SC, Chen IY, et al. Surface vimentin is critical for the cell entry of SARS-CoV. *Journal of Biomedical Science*. 2016;23(1):1-10. doi:10.1186/S12929-016-0234-7/FIGURES/8
  140. Amraei R, Xia C, Olejnik J, et al. Extracellular vimentin is an attachment factor that facilitates SARS-CoV-2 entry into human endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2022;119(6). doi:10.1073/PNAS.2113874119/SUPPL\_FILE/PNAS.2113874119.SAPP.PDF

141. Belouzard S, Madu I, Whittaker GR. Elastase-mediated Activation of the Severe Acute Respiratory Syndrome Coronavirus Spike Protein at Discrete Sites within the S2 Domain. *The Journal of Biological Chemistry*. 2010;285(30):22758. doi:10.1074/JBC.M110.103275
142. Garcia-Dorival I, Cuesta-Geijo MÁ, Barrado-Gil L, et al. Identification of NPC1 as a novel SARS-CoV-2 intracellular target. *bioRxiv*. Published online 2020:2020.12.19.423584. doi:10.1101/2020.12.19.423584
143. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*. 2020;181(2):271-280.e8. doi:10.1016/J.CELL.2020.02.052
144. Mori Y, Fink C, Ichimura T, et al. KIM-1/TIM-1 is a Receptor for SARS-CoV-2 in Lung and Kidney. *medRxiv*. Published online January 11, 2022:2020.09.16.20190694. doi:10.1101/2020.09.16.20190694
145. Suprewicz Ł, Swoger M, Gupta S, et al. Extracellular Vimentin as a Target Against SARS-CoV-2 Host Cell Invasion. *Small (Weinheim an der Bergstrasse, Germany)*. 2022;18(6). doi:10.1002/SMLL.202105640
146. Tang X, Yang M, Duan Z, et al. Transferrin receptor is another receptor for SARS-CoV-2 entry. *bioRxiv*. Published online 2020:2020.10.23.350348. doi:10.1101/2020.10.23.350348
147. Wang K, Chen W, Zhang Z, et al. CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. *Signal transduction and targeted therapy*. 2020;5(1). doi:10.1038/S41392-020-00426-X
148. Wang S, Qiu Z, Hou Y, et al. AXL is a candidate receptor for SARS-CoV-2 that promotes infection of pulmonary and bronchial epithelial cells. *Cell Research* 2021 31:2. 2021;31(2):126-140. doi:10.1038/s41422-020-00460-y
149. Xia S, Lan Q, Su S, et al. The role of furin cleavage site in SARS-CoV-2 spike protein-mediated membrane fusion in the presence or absence of trypsin. *Signal Transduction and Targeted Therapy*. 2020;5(1). doi:10.1038/S41392-020-0184-0
150. Zang R, Castro MFG, McCune BT, et al. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. *Science immunology*. 2020;5(47). doi:10.1126/SCIIMMUNOL.ABC3582
151. Zhang L, Jackson CB, Mou H, et al. The D614G mutation in the SARS-CoV-2 spike protein reduces S1 shedding and increases infectivity. *bioRxiv*. Published online 2020. doi:10.1101/2020.06.12.148726
152. Bertram S, Glowacka I, Müller MA, et al. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *Journal of virology*. 2011;85(24):13363-13372. doi:10.1128/JVI.05300-11

153. Fuentes-Prior P. Priming of SARS-CoV-2 S protein by several membrane-bound serine proteinases could explain enhanced viral infectivity and systemic COVID-19 infection. *The Journal of Biological Chemistry*. 2021;296:100135-100136. doi:10.1074/JBC.REV120.015980
154. Magrone T, Magrone M, Jirillo E. Focus on Receptors for Coronaviruses with Special Reference to Angiotensin- Converting Enzyme 2 as a Potential Drug Target - A Perspective. *Endocrine, metabolic & immune disorders drug targets*. 2020;20(6):807-811. doi:10.2174/1871530320666200427112902
155. Masre SF, Jufri NF, Ibrahim FW, Abdul Raub SH. Classical and alternative receptors for SARS-CoV-2 therapeutic strategy. *Reviews in Medical Virology*. 2021;31(5):1-9. doi:10.1002/RMV.2207
156. Millet JK, Jaimes JA, Whittaker GR. Molecular diversity of coronavirus host cell entry receptors. *FEMS microbiology reviews*. 2021;45(3). doi:10.1093/FEMSRE/FUAA057
157. Murgolo N, Therien AG, Howell B, et al. SARS-CoV-2 tropism, entry, replication, and propagation: Considerations for drug discovery and development. *PLoS pathogens*. 2021;17(2). doi:10.1371/JOURNAL.PPAT.1009225
158. Shang J, Ye G, Shi K, et al. Structural basis of receptor recognition by SARS-CoV-2. *Nature*. 2020;581(7807):221-224. doi:10.1038/S41586-020-2179-Y
159. Simmons G, Zmora P, Gierer S, Heurich A, Pöhlmann S. Proteolytic activation of the SARS-coronavirus spike protein: Cutting enzymes at the cutting edge of antiviral research. *Antiviral Research*. 2013;100(3):605. doi:10.1016/J.ANTIVIRAL.2013.09.028
160. Lin JY, Shih SR. Cell and tissue tropism of enterovirus 71 and other enteroviruses infections. *Journal of Biomedical Science*. 2014;21(1):18. doi:10.1186/1423-0127-21-18
161. Majer A, McGreevy A, Booth TF. Molecular Pathogenicity of Enteroviruses Causing Neurological Disease. *Frontiers in Microbiology*. 2020;11:540. doi:10.3389/FMICB.2020.00540/BIBTEX
162. Sarmiento L, Frisk G, Anagandula M, et al. Echovirus 6 Infects Human Exocrine and Endocrine Pancreatic Cells and Induces Pro-Inflammatory Innate Immune Response. *Viruses*. 2017;9(2). doi:10.3390/V9020025
163. Garmaroudi FS, Marchant D, Hendry R, et al. Coxsackievirus B3 replication and pathogenesis. *Future Microbiology*. 2015;10(4):629-652. doi:10.2217/FMB.15.5/ASSET/IMAGES/LARGE/FIGURE2.JPEG
164. Harvala H, Kalimo H, Dahllund L, et al. Mapping of tissue tropism determinants in coxsackievirus genomes. *The Journal of general virology*. 2002;83(Pt 7):1697-1706. doi:10.1099/0022-1317-83-7-1697
165. Nomoto A, Koike S, Aoki J. Tissue tropism and species specificity of poliovirus infection. *Trends in Microbiology*. 1994;2(2):47-51. doi:10.1016/0966-842X(94)90125-2

166. Freeman MC, Wells AI, Ciomperlik-Patton J, et al. Respiratory and intestinal epithelial cells exhibit differential susceptibility and innate immune responses to contemporary EV-D68 isolates. *eLife*. 2021;10. doi:10.7554/ELIFE.66687
167. Smura T, Ylipaasto P, Klemola P, et al. Cellular tropism of human enterovirus D species serotypes EV-94, EV-70, and EV-68 in vitro: Implications for pathogenesis. *Journal of Medical Virology*. 2010;82(11):1940-1949. doi:10.1002/JMV.21894
168. Jacobs SE, Lamson DM, Kirsten S, Walsh TJ. Human Rhinoviruses. *Clinical Microbiology Reviews*. 2013;26(1):135. doi:10.1128/CMR.00077-12
169. Schreiber MT. Determinants of Human Rhinovirus Cellular Tropism in Monocyte-Lineage Cells. Published online 2016. doi:10.7916/D8CC10HB
170. Andréoletti L, Renois F, Jacques J, Lévêque N. [Human enteroviruses and respiratory infections]. *Medecine sciences : M/S*. 2009;25(11):921-929. doi:10.1051/MEDSCI/20092511921
171. Chen BS, Lee HC, Lee KM, Gong YN, Shih SR. Enterovirus and Encephalitis. *Frontiers in Microbiology*. 2020;11. doi:10.3389/FMICB.2020.00261
172. Filipe IC, Guedes MS, Zdobnov EM, Tapparel C. Enterovirus D: A Small but Versatile Species. *Microorganisms*. 2021;9(8). doi:10.3390/MICROORGANISMS9081758
173. Tuthill TJ, Groppelli E, Hogle JM, Rowlands DJ. Picornaviruses. *Current topics in microbiology and immunology*. 2010;343:43. doi:10.1007/82\_2010\_37
174. Wells AI, Coyne CB. Enteroviruses: A Gut-Wrenching Game of Entry, Detection, and Evasion. *Viruses*. 2019;11(5). doi:10.3390/V11050460
175. Ylipaasto P, Klingel K, Lindberg AM, et al. Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia*. 2004;47(2):225-239. doi:10.1007/S00125-003-1297-Z/FIGURES/9
176. Vandesande H, Laajala M, Kantoluoto T, Ruokolainen V, Lindberg AM, Marjomäki V. Early Entry Events in Echovirus 30 Infection. *Journal of Virology*. 2020;94(13). doi:10.1128/JVI.00592-20
177. Excoffon KJDA. The coxsackievirus and adenovirus receptor: virological and biological beauty. *FEBS letters*. 2020;594(12):1828-1837. doi:10.1002/1873-3468.13794
178. Heikkilä O, Susi P, Tevaluoto T, et al. Internalization of Coxsackievirus A9 Is Mediated by  $\beta$ 2-Microglobulin, Dynamin, and Arf6 but Not by Caveolin-1 or Clathrin. *Journal of Virology*. 2010;84(7):3666. doi:10.1128/JVI.01340-09
179. Shafren DR, Bates RC, Agrez MV, Herd RL, Burns GF, Barry RD. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. *Journal of virology*. 1995;69(6):3873-3877. doi:10.1128/JVI.69.6.3873-3877.1995

180. Elrick MJ, Pekosz A, Duggal P. Enterovirus D68 molecular and cellular biology and pathogenesis. *The Journal of biological chemistry*. 2021;296. doi:10.1016/J.JBC.2021.100317
181. Baggen J, Thibaut HJ, Strating JRPM, Van Kuppeveld FJM. The life cycle of non-polio enteroviruses and how to target it. *Nature reviews Microbiology*. 2018;16(6):368-381. doi:10.1038/S41579-018-0005-4
182. Wang K, Zhu L, Sun Y, et al. Structures of Echovirus 30 in complex with its receptors inform a rational prediction for enterovirus receptor usage. *Nature Communications*. 2020;11(1). doi:10.1038/S41467-020-18251-9
183. Wen X, Sun D, Guo J, et al. Multifunctionality of structural proteins in the enterovirus life cycle. *Future microbiology*. 2019;14(13):1147-1157. doi:10.2217/FMB-2019-0127
184. Escaffre O, Borisevich V, Rockx B. Pathogenesis of Hendra and Nipah virus infection in humans. *Journal of infection in developing countries*. 2013;7(4):308-311. doi:10.3855/JIDC.3648
185. Wong KT. Emerging epidemic viral encephalitides with a special focus on henipaviruses. *Acta Neuropathologica*. 2010;120(3):317-325. doi:10.1007/S00401-010-0720-Z/FIGURES/2
186. Eaton BT, Broder CC, Middleton D, Wang LF. Hendra and Nipah viruses: different and dangerous. *Nature Reviews Microbiology 2006 4:1*. 2006;4(1):23-35. doi:10.1038/nrmicro1323
187. Pernet O, Wang YE, Lee B. Henipavirus Receptor Usage and Tropism. *Current topics in microbiology and immunology*. 2012;359:59. doi:10.1007/82\_2012\_222
188. Negrete OA, Wolf MC, Aguilar HC, et al. Two Key Residues in EphrinB3 Are Critical for Its Use as an Alternative Receptor for Nipah Virus. *PLoS Pathogens*. 2006;2(2):0078-0086. doi:10.1371/JOURNAL.PPAT.0020007
189. Bonaparte MI, Dimitrov AS, Bossart KN, et al. From the Cover: Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(30):10652. doi:10.1073/PNAS.0504887102
190. Negrete OA, Levroney EL, Aguilar HC, et al. EphrinB2 is the entry receptor for Nipah virus, an emergent deadly paramyxovirus. *Nature 2005 436:7049*. 2005;436(7049):401-405. doi:10.1038/nature03838



# General Discussion and Perspectives

The aim of my PhD was to characterize the infection of the human semen and testis by two emergent viruses, ZIKV and SARS-CoV-2, and to implement an analysis of the expression of putative receptors for a range of emerging RNA viruses in the testis.

For this, I used a mix of approaches, spanning from organ and cell culture (experimental approaches) to literature search and data mining in RNA-seq data bases (in silico approaches).

I discuss below the advantages and limits of these approaches as well as the perspectives they offer.

## I. Experimental approaches

### A. Cell lines and isolated primary cells

Cell lines have the advantage of being a cost-effective, easily accessible material which allows experimentations in a controlled environment with good reproducibility. In particular, it allows at single cell type level the characterization of molecular mechanisms which could not be studied in a multicellular environment. However, immortalized cells have usually been passed many times, and often present distinct characteristics to their primary cell's counterpart. In turn they frequently fail to accurately reproduce surface proteins or the innate immune system<sup>372,373</sup>.

Primary cells, while keeping their physiological characteristics, can be challenging in long-term cultures. Indeed, when isolated, Leydig cells rapidly lose their steroidogenic capacities<sup>374</sup>. Yet, they can be maintained up to a month when co-cultured with Sertoli cells<sup>375</sup>.

For primary germ cells, a major challenge for their study is their low survival rate outside the testicular environment, as they require physical contacts and paracrine exchange with feeder somatic cells for their maintenance and development. To overcome this challenge, we use a specific StemPro-34 medium supplemented with vitamins, antibiotics and growth factors, allowing their maintenance in culture up to 15 weeks<sup>376</sup>.

The isolation and short-term culture of TGC enabled us to assess their permissiveness to SARS-CoV-2. Indeed, while a few TGC expressed ACE2 and were infected in testis explants, isolated cell cultures

confirmed they did not sustain productive infection. On the contrary, Leydig and Sertoli cells were the main cells infected in testis explants. It would be important to assess their respective innate immune responses and ability to produce infectious virions in future experiments, in order to determine their contribution to the testis explants viral titer and in the decrease in infectious virions observed at the end of the 9 days culture period. As a first approach we could (i) compare the viral replication dynamics by RTqPCR and plaque assay in these cells separately; (ii) compare the innate immune responses triggered by infection, using RTqPCR and Western Blot. In order to further characterize potential differences, a single cell RNA sequencing (scRNA-seq) approach could be implemented in order to decipher the modulation of host factors potentially implicated in the restriction of viral replication in each cell type.

SARS-CoV-2 receptor, ACE2, is part of the Renin-Angiotensin System (RAS). It converts Angiotensin (Ang) II into Ang(1-7), which interacts with the Mas receptor to regulate blood pressure<sup>377,378</sup>. While the role of RAS in the testis is unclear, ACE2 (as we showed) and Mas are expressed by human Leydig cells<sup>379</sup>. The deletion of Mas in mice affected the expression of steroidogenic enzymes such as STAR, which was downregulated upon infection in our experiments<sup>380</sup>. In men, the downregulation of ACE2 in Leydig cells correlated with spermatogenesis impairment<sup>379</sup>, suggesting the binding of SARS-CoV-2 to ACE2 on Leydig cells could be involved in the alterations of testicular functions reported in men with Covid-19. Since ACE2 is internalized upon infection by SARS-CoV-2<sup>381</sup>, infection of Leydig cells could impact ACE2 expression at the cell surface, in turn impairing Ang(1-7) conversion and Mas activation. This would result in steroidogenesis inhibition, and ultimately lead to spermatogenesis impairment. Our RT-qPCR and IHC experiments on testicular explants cannot assess differences in the intracellular localization of ACE2 during infection. Experiments on isolated Leydig cells would allow us to perform confocal microscopy upon ACE2 immunofluorescent staining (i.e. immunocytochemistry) to follow the potential receptor internalization at the cellular level upon infection, and verify its potential downregulation by Western Blot. The cell-specific dynamic of ACE2 expression at the cell surface might indeed explain the decrease in the expression of steroidogenic enzymes. The implication of ACE2 downregulation in the steroidogenesis inhibition via the RAS could also be explored in isolated Leydig cells through the knockdown of ACE2 by a silencing RNA (siRNA). This way we could check the impact of ACE2 downregulation on the transcription of steroidogenic enzymes and testosterone production.

Another aspect that would require further investigations in isolated cells is that of the impact of SARS-CoV-2 on the blood testis barrier. When polarized, cell lines and their primary counterparts can reproduce epithelial and endothelial barriers *in vitro*. This way, SARS-CoV-2 was shown to cross the human blood-brain barrier via infection of Brain Microvascular Endothelial Cells (BMEC) without disruption of the tight junctions<sup>382</sup>. Accordingly, using a human Sertoli cell line, ZIKV was reported to cross the Sertoli Cell Barrier (SCB). Its permeability was disturbed by inflammatory mediators released by co-cultured infected blood-derived macrophages<sup>383</sup>. In the case of SARS-CoV-2, we showed that Sertoli cells are infected by the virus. SARS-CoV-2 might be released in the seminiferous tubules through the polarized excretion of virions at the adluminal side of Sertoli cells. A polarized culture of infected Sertoli cells would help to determine whether SARS-CoV-2 excretion takes place on the apical and/or basal side of these cells, providing it occurs. Using RT-qPCR on Sertoli cell markers and IHC against ZO-1, the Sertoli Cell Barrier (SCB) did not appear to be disturbed by infection during our culture time frame. However, as undertaken for ZIKV, an *in vitro* SCB model would benefit the investigation of the Sertoli cell tight junctions upon SARS-CoV-2 infection, and the risk for leukocytes to infiltrate the seminiferous tubule and attack the antigenic TGC. Indeed, leukocyte infiltration is believed to be an essential aspect of the adaptive immune response in SARS-CoV-2 pathology<sup>384</sup>. In our experiments, their attraction to the testis can only be hypothesized based on the analysis of the chemokine expression by testis explants upon infection. In that matter, innate proinflammatory responses were restricted to CXCL10 upregulation. This chemokine is involved in the attraction of leukocytes, such as macrophages and NK cells<sup>385</sup> which, upon activation, secrete several effector molecules implicated in the disturbance of the SCB<sup>383</sup>. Therefore, a SCB model, in a Boyden chamber for example, could help us in two ways. Firstly, to better characterize the permeability of the barrier, by measuring its transepithelial electrical resistance upon infection or in coculture with blood-derived leukocytes. Secondly, to explore the potential chemotaxis of blood-derived leukocytes across the SCB. By co-culturing a polarized SCB with leukocytes in the upper chamber and filling the down chamber with medium containing the chemokines of interest, the transmigration of leukocytes could then be explored by immunofluorescence techniques for example. However, the culture of primary human Sertoli cells is not established in the team and only work in a commercial cell line has been published by other authors. This cell line may not accurately reflect Sertoli cells in their tissue environment. For instance, commercial Sertoli cells were readily infected by ZIKV<sup>383</sup>, whereas we only observed poor infection of Sertoli cells in the testis explants.

## B. Organotypic cultures

Unlike isolated cells, organotypic cultures better mimic the physiological context, since the organs keep their tissue architecture and the cells bath in their microenvironment influenced by cell-cell interactions and secretions. This model is of particular interest when accessibility to organs via autopsy is limited (e.g. with ZIKV infection). Also, it is advantageous when the disease studied includes systemic inflammation, intrinsically triggering multi organs failure (e.g. with severe SARS-CoV-2 infection). Indeed, with the goal of studying the direct impact of viral infection on the organ, *ex vivo* models overcome the systemic influences of fever or disturbance of the hypothalamic-pituitary axis for instance, which both impair testicular functions <sup>366</sup>.

However, while infection parameters are controlled for reproducibility, it cannot fully reproduce the physiological conditions of viral load and penetration through the vasculature into the organ, nor the role of the acquired immunity in the tissue disruption. Viruses coming from the vascular system have to pass several physiological barriers before infecting their target cells (i.e the endothelial BTB followed by the actual SCB in the case of the testis). In contrast, the explants are “openly exposed” tissues which cannot resume the actual course of infection. Additionally, while being free from systemic influence can be an advantage in the investigation of direct impact of infection on the organ, it can also be seen as a downside since the overall physiopathology of the disease is not resumed. As mentioned earlier, leukocyte infiltration is most probably an important aspect of SARS-CoV-2 pathology.

Another limitation of organ explant is that the tissue integrity is only preserved for a limited duration, here up to 2 weeks for testis explants, after which TGC start to degenerate <sup>386</sup>. As a consequence, we could not pursue the observation of the effect of SARS-CoV-2 infection on Leydig cells, as the first significative alterations of the steroidogenesis (i.e. the significant decrease of STAR and CYP11A1, involved in the first steps of the cascade) were only observed at the end of the culture at 9 dpi. This observation was accompanied by a tendency for decreasing expression of downstream actors of the steroidogenesis and decreasing testosterone secretion. However, the causality link between these observations could not be confirmed in later times. Therefore, testosterone production impairment by infection can only be hypothesized upon the observation we made on a patient’s cohort. In order to link the decreased expression of STAR and Cyp11A1 to an upcoming decreased of testosterone production, the precursors (e.g. pregnenolone, progesterone etc.) of the testosterone in the steroidogenesis cascade could be examined.

To this end, our model would benefit from an extended culture time. Experiments based on the use of microfluidic devices are currently undergoing at the lab. Komeya et al. reported in 2016 the development of a microfluidic system mimicking blood flow. This system maintained the integrity and spermatogenesis of mice testicular explants in culture for up to 6 months<sup>387</sup>. Therefore, this system could help investigate the long-term effect of infection on steroidogenesis and possibly spermatogenesis. Moreover, it might enable to analyze the chemoattraction of leukocytes to the testis by adding these cells to the mimicking blood flow and examine their infiltration into the explants by IHC.

## C. Animal models

Animal models represent a chance to gather the controlled experimental design, organ accessibility, and systemic physiopathology criteria. By a controlled inoculum in a controlled environment, these models allow the study of the early steps of infection, its evolution and spread, and the establishment of immune responses; all of which are hardly possible during natural infection. For these reasons, they are also used as models for drugs and vaccine testing, as not being subject to external inputs which might interfere with the treatments.

However, animal models such as NHP, which are physiologically the closest to humans, are expensive and interindividual genetic differences also occur, so that a sufficient number of individuals are needed to reach statistical significance, which raises ethical concerns. Animal models including NHP also generally fail to fully reproduce the pathogenesis spectrum and the virus transmission routes and virus tropism for human organs and cells.

Differences with humans can occur at several levels:

Entry factors may differ between animals and humans. As an example, until the N501Y mutation of the last SARS-CoV-2 variants<sup>253,388</sup>, the previous strains had low affinity to mouse ACE2, and mice could not be efficiently infected by wild type SARS-CoV-2. Transgenic humanized mice, genetically engineered to express the human ACE2, had to be used to reproduce infection and amount the pathological severity observed in patients<sup>389,390</sup>. However, ACE2 expression in these transgenic mice is driven by a specific promoter (eg CK18 for expression in epithelial cells) and thus does not reflect natural expression in various cell types.

Immune responses and viral counteractions are not the same either. For instance, wild-type mice are normally resistant to ZIKV, the replication of which is efficiently inhibited by murine type I IFN. In humans, type I IFN signaling is counteracted by the virus NS5 protein <sup>391</sup>. As a consequence, productive infection can only be resumed in IFNAR<sup>-/-</sup> mice in which the receptor for IFN has been knocked out. This model thus cannot recapitulate the overall immune response one might be seeking in an animal model.

Pathogenesis is also frequently different. For example, while NHP models display an overall similarity in ZIKV susceptibility and immunological responses <sup>392,393</sup> ZIKV viremia is lower than in humans and the course of disease is milder. The virus is only occasionally detected at low level in the testes <sup>366</sup>. Additionally, infection of the IFNAR<sup>-/-</sup> model with ZIKV leads to high levels of systemic inflammation and to the death of the animals upon very high levels of replication. A broad testicular pro-inflammatory response and extended damages in the testis (atrophy, orchitis, leukocyte infiltration) is observed in IFNAR<sup>-/-</sup> mice, which were never reported in clinical cases <sup>366,368</sup>. Besides, mice death upon infection was commonly associated with viral neuro-dissemination, which was not observed in human <sup>394</sup>

Lately, other rhesus and cynomolgus macaque models harbored ZIKV RNA and infectious particles in the MGT until the end of the study protocol at 60 dpi. ZIKV was found in TGC and Sertoli cells, epididymal duct epithelium and glandular epithelia of the seminal vesicle and prostate gland. Infectious particles mostly persisted in the epididymis and seminal vesicles <sup>395</sup>.

When exploring the infected cells in the semen of ZIKV patients, we evidenced infected exfoliated epithelial cells and leukocytes, which tissue origin is unknown <sup>396</sup>. NHP models could contribute to the understanding of their source in the MGT. In that respect, the team previously undertook a phylogenetic comparison of the strains present in semen and MGT organs from six SIV-infected animals, which revealed that the epididymal and seminal vesicles are a source of free virions and infected cells shed in semen. However this tissue source varied among individuals, and thus does not exclude the implication of additional MGT organs in other individuals <sup>397</sup>.

Additionally, the downregulation of ACE2 we hypothesize in Leydig cells upon SARS-CoV-2 infection would also trigger an imbalanced blood pressure. This might increase the testis capillaries permeability <sup>398,399</sup> and participate in leukocytes infiltration. Added to the decrease in testosterone

production, it would trigger testis inflammation and threaten the TGC survival. This comprehensive model cannot be implemented in cell or organotypic culture and animal models are required to explore this aspect.

## D. Clinical studies

Clinical studies are, obviously, the most accurate in terms of physiological reality. They allow a comprehensive overview of a virus dissemination in its host, its pathology, the immune responses triggered and its transmission routes across human populations.

The access to semen from ZIKV infected men during the acute stage of infection and up to 160 days post-symptoms allowed us to validate our *ex vivo* findings of ZIKV<sup>368</sup> and SARS-CoV-2 infection of the testis, and to delineate the testicular origin of cells sustaining prolonged ZIKV infection in semen<sup>396</sup>. It also enabled the study of semen parameters in ZIKV patients<sup>400</sup>. However, while some fluids (blood, saliva, semen...) are easily sampled to detect viral infections and shedding, *in situ* detection in organs of patients generally requires biopsy (which is an invasive, not essential procedure) or autopsy, which is possible only in case of a deadly disease. Autopsy enables the assessment of specific organs infection and pathology. However, it is representative of the overall course of the disease, which consequences can be driven by infection itself, systemic inflammation and fever, as well as co-infection and comorbidities. Medications can also affect the organs functions and morphology. Analgesics (e.g. ibuprofen) for example, were showed in our laboratory to exert antiandrogen effects, through the direct repression at the transcription level of the steroidogenic cascade in Leydig cells<sup>401</sup>.

Blood sampling allows the follow up of viremia as well as several health parameters including proinflammatory cytokines, circulating immune cells, and sexual hormones. By measuring the hormonal blood levels of 7 SARS-CoV-2 patients without co-morbidities, we could witness decreased testosterone levels when compared to European standards<sup>402</sup> at times corresponding to 3-10 days post symptoms onset (dps), for an average 8-15 days post infection (dpi) if calculated with a 5 days average incubation period. This testosterone decrease was accompanied by LH levels within published normal ranges, indicating a specific affection of testosterone-producing Leydig cells rather than a deregulation of the hypothalamic-pituitary-gonadal axis by systemic inflammation or fever. These *in vivo* results thus correlate our *ex vivo* findings and allow us to extrapolate at later times the tendency for decreased testosterone production we observed upon testis infection. This hormonal

dysregulation was transient, as testosterone blood levels increased 6-9 days later, which might suggest transient infection of the organ. However, while these findings resume the ones reported by Enikeev et al.<sup>369</sup>, our study presents several flaws.

Indeed, with the aim of overcoming comorbidity bias, we only selected previously healthy patients from the French COVID cohort, ultimately representing a very small sample of the population, which will require confirmation studies in more patients. Also, this patient's cohort did not comprise non-infected controlled individuals to which hormonal levels could be compared. While we sought to compare our results to literature “reference ranges” of population resembling our patients in age and ethnicity, all inclusion criteria (e.g. drug history, BMI) might not be equivalent. Also, a direct effect on the testis of fever or medication for instance cannot be ruled out.

SARS-CoV-2 RNA, even though only detected in the semen of patients in a subset of studies, was detected in the seminal plasma rather than the cellular fractions (i.e. spermatozoa pellet)<sup>403</sup>. Its origin in this fluid is unclear and some authors have casted doubts on its MGT provenance. Nonetheless, our analysis of a semen sample from a man acutely but mildly infected, revealed the presence of infected Sertoli cells. This result confirms the *in vivo* infection of the human testis by SARS-CoV-2 as well as its ability to be shed in semen early in the infection dynamic.

To date there is no evidence of SARS-CoV-2 persistence in the MGT. However, since most studies focused on the acute phase of the disease<sup>340</sup>, the possibility of intermittent and prolonged shedding in the semen of a subset of men deserves further investigations and is currently under investigation. Nevertheless, no infectious virus could be rescued in the attempts from a couple of studies<sup>340</sup>. This, together with the low rate of detection of SARS-CoV-2 in semen, makes sexual transmission of SARS-CoV-2 unlikely or at very low incidence.

## E. Contribution of the conventional approaches

Altogether, our *ex vivo* and *in vivo* complementary approaches revealed the testis tropism of ZIKV and SARS-CoV-2. We also confirmed ZIKV prolonged shedding in semen and characterized the nature of the infected cells (e.g. germ cells, epithelial cells, monocytes and macrophages) and the duration of their infection in patients. Regarding SARS-CoV-2, we unveiled its primary testicular target cells, ie Leydig and Sertoli cells; as well as the probable receptor involved in their infection (i.e ACE2). We evaluated the direct impact of infection on Leydig cells function and link it with patients impaired steroidogenesis. Our results suggest that the infection is transient, possibly dampened by the



antiviral response triggered in the testis and/or by intrinsic restriction factors or cell deficiency impairing the release of infectious virions. As detailed above, further experimental approaches will help to refine the mechanisms involved during testis infection and their consequences for the organ homeostasis.

## **F. Limits of the conventional approaches**

In order to decipher the mechanism underlying the infection of ZIKV main target cells in the testis (i.g germ cells and macrophages), during my PhD I aimed to determine the availability of host receptor(s) for ZIKV entry. Using flow cytometry on dissociated cells from human testis explants, I explored the expression of ZIKV main receptors, namely the TAM and C-type lectins families. While AXL, MERTK and DC-SIGN (CD209) proteins were detected in testicular macrophages, none were detected in TGC. In these cells, our initial experimental approach thus failed to unveil entry factors.

In this context, we aimed at uncovering candidate receptors for ZIKV in TGC and predicting new receptors potentially involved in infection by other viruses, using *in silico* approaches.

## **II. In Silico approaches**

### **A. Unveiling viral entry factors expression in the testis through scRNA-seq analysis**

With the initial goal to extend the list of potential receptors for ZIKV, we searched for putative receptors reported in the literature and for receptors used by other viruses of the Flavivirus genus. The scRNA-seq approach evidenced the transcription in the testis of newly discovered receptors for ZIKV (e.g. NCAM1, EGFR, Integrin  $\alpha V\beta 5$ ) as well as receptors involved in other Flaviviruses viral entry (e.g. MRC1, CLEC4M, PHB etc.) that we later investigated.

While exploring SARS-CoV-2 infection in the testis, we found that the virus could infect peritubular myoid cells, albeit at a low frequency. These cells did not express ACE2 as per IHC experiment. ScRNA-seq data analysis helped hypothesizing the putative receptors involved in the infection of these cells (i.e. AXL, BSG, VIM, TRFC, NRP1).

To extent the benefit of this approach, we used it as a tool to predict the potential testis susceptibility to emerging viruses. This approach aims at giving a global in silico picture of putative entry factors expressed by the different testicular cell populations for a wide range of viruses from distinct families. The human testis being a source of prolonged infection and seminal shedding, this entry factor mapping is a first step towards the prediction and in turn the anticipation of emerging viruses' infection in the testis and seminal excretion. Using this approach, we uncovered several receptors potentially involved in the infection of the different testicular cell types by a variety of viruses.

Interestingly, when analyzing the receptors most broadly expressed in the human testis, a striking result was the transcription of the majority of Enteroviruses receptors. Only few reports isolated Coxsackie viruses (CV-A6 and CV-B5) in patients' testis and epididymal fluid <sup>366,404</sup>, while Poliovirus efficiently replicated in human testicular tissue in vitro <sup>405</sup>. Otherwise, Enteroviruses do not typically present a genital tropism <sup>406</sup>. In the same way, the receptors of neurotropic Flaviviruses, MAYV, WEEV, LCMV and SARS-2 are also well expressed in the testis and many viruses reported in the human MGT are neurotropic <sup>366</sup>. Viral trafficking between this system and the central nervous system via nerves has been described <sup>112,407</sup>. Also, a recent study reported 81% overlapping in the brain and testis proteome, reporting proteins involved in spermatogenesis, neuronal development as well as peripheral nervous system maintenance and transmission <sup>408</sup>. These observation raises the question of a potential viral trafficking between the CNS and testis, both immune-privileged organs, and whether this trafficking might be involved in the persistence of neuro-tropic viruses in the human body, as is the case for Herpesviruses <sup>366,409–411</sup>.

However, this approach, while being a great source of information for further research, also presents several gaps and flaws.

## **B. Gaps and flaws of the scRNA-seq approach**

Thus, a number of issues can be raised:

- 1) The restriction of our research to medically important viruses is leaving aside animal viruses, of which more than 30 are already known to infect their host MGT <sup>366</sup>. Knowing that 75% of emergent viruses are zoonotic, some of them could soon cross the species barrier and potentially infect human testis too.

2) Working on emerging viruses implies they are, for most, understudied. Thus, information on their tropism, receptor usage and mechanism of infection are often incomplete or poorly understood.

3) We sought to integrate to this study only the entry factors which were proven to be used by a given virus via gain and loss of function experiments, resulting in an increased or decreased viral entry into target cells. This method thus excludes entry factors that were only proven to have binding abilities to viral proteins (e.g. GRP78 with SARS-CoV-2 Spike <sup>412</sup>) or which only belongs to the family of proteins involved in the virus entry (e.g. the TAM receptor MERKT and ZIKV <sup>413</sup>). While seeking to be accurate, this method could be considered too stringent for an initial approach, which intrinsically requires further confirmations of entry factor usage and involves understudied viruses. Besides, some published information might also have been missed during our literature review.

4) The study of entry factors transcription was based on a single chosen dataset. While this dataset has already been used to describe the expression of ACE2 entry factors in Sertoli cells <sup>414</sup>, as for every dataset, some discrepancies with other published datasets exist. For instance, while studies using Guo et al. <sup>415</sup> (used in Stanley et al., 2020<sup>416</sup> and Hikmet et al., 2020<sup>417</sup>) or Hermann et al. <sup>418</sup> (used in Zhou et al., 2020<sup>419</sup> and Wang et al., 2020<sup>420</sup>) datasets showed a good expression of ACE2 in Leydig cells and spermatogonia (SPG), the Wang et al. <sup>421</sup> dataset used in our study did not reproduce this result. However, the difference in ACE2 expression among different datasets could be explained by the fact that most studies used data merging Sertoli and Leydig cells for their interpretation <sup>417,419,420</sup>.

Therefore, this approach would greatly benefit from a participative mechanism in order to be continuously updated by the scientific community, as new entry factors use are continuously confirmed, and new scRNA-seq data become available. In this regard, a website similar to the Reprogenomic Viewer <sup>422,423</sup> (<https://rgv.genouest.org/>), for genes involved in reproduction, would be a long-term goal to achieve in order to propose an up to date and ergonomic (easy to read) source of information. In this regard, to complete the interpretation of the testis cells susceptibility to our list of emergent viruses, we are currently working on a virus-cell interaction network figure which would display an overview of all viruses possibly infecting one cell, and all the cells potentially susceptible to one virus at the same time.

While this approach initiates the prediction of a cell susceptibility based on the expression of entry receptors in a given testicular cell type, studies have shown that some entry factors could work “in

*trans*” from a cell to another. For instance, in the case of SARS-CoV-1, “effector” cells bearing the S protein and HAT at their surface can prime S “in cis” and thus fusion with ACE2-bearing “target cells”. This mechanism mimicked cell-to-cell infection<sup>424</sup>. Thus, in a tissue where close interactions between cells exist, as in the testis, one should remember that the susceptibility of a cell might not depend solely on its expression of entry factors, but also on the surrounding cells’.

In conclusion, this in silico analysis is only a first step in the characterization of the testis susceptibility, which requires several complementary experiments.

## C. Complementary experiments to infer tropism

### C.1. Confirmation of the entry factor protein expression

As there can be discrepancies between RNA and protein levels<sup>419</sup>, the confirmation of the entry factors’ protein expression in the tissue and cells of interest are required. In that respect, while our scRNA-seq dataset suggests that only Sertoli cells express ACE2, we demonstrated by IHC on testis explants that ACE2 protein was highly expressed in Sertoli, but also in Leydig cells and in rare TGC, which was expected from others scRNA-seq datasets results<sup>416,417</sup>. Furthermore, while TMPRSS2 mRNA was detected in SSC, Spermatids and Leydig cells in our dataset and others<sup>416</sup>, we failed to detect protein expression by IHC.

With this in mind, I started to investigate the protein expression of ZIKV estimated potential receptors. Using flow cytometry on isolated TGC from testis explants, preliminary results endorsed the expression of MRC1 and Integrin  $\alpha V\beta 5$ .

### C.2. Confirmation of the entry factor use

Once the protein expression is confirmed, the validation of its involvement in the viral entry should be explored. Indeed, and as an example, while the TAM receptor family are considered as the main receptors for ZIKV entry, some studies inferred they were not necessary for infection of certain cell types, including human Leydig cells<sup>425</sup>.

In that respect, I have initiated binding competition experiments in order to decipher the cellular receptor(s) implicated in ZIKV infection of TGC. Our preliminary results in primary TGC and in TCam-2 (a germ cell line derived from a testicular seminoma and resembling SPG) showed that competitive molecules such as Heparin and Mannan could partly inhibit the attachment of ZIKV to Heparan

Sulfate Proteoglycans (HSPG) and MRC1 respectively. These results need to be confirmed in more donors and the investigation extended to putative entry receptors, using RNA silencing or CRISPR/Cas 9 approaches to more specifically target the gene of interest. Methodological issues will first need to be resolved, as efficient transfection of TGC is not achieved using conventional approaches (e.g. lipofectamine or VSV-G pseudotyped lentiviral vectors).

Additionally, when predicting the susceptibility of the organ upon expression of viral receptors, validation of infection by prototypic viruses of interest would be necessary to analyze the solidity of the model. In that respect, while ZIKV infection of the human testis and seminal shedding has been demonstrated through a number of case reports and cohorts' studies, only isolated case reports have described the seminal shedding of YFV and DENV. Two cases of sexually transmitted DENV were reported and WNV suspected to be sexually transmitted once. Nevertheless, JEV is shed in the semen of pigs <sup>366</sup>. Based on these observations and the fact that many receptors are shared among Flaviviruses (and broadly transcribed in the testis according to our scRNA-seq analysis), our team is currently investigating the infection of human testis explants by these viruses. The preliminary results show differences in the dynamic of infection and viral loads between each other and with ZIKV.

These differences could be explained, as a first step, by differential entry mechanisms, generally involving structural proteins. In that respect, and with the aim to identify the viral proteins involved in the testicular infection disparity; we started to develop chimeric viruses by substituting the structural proteins (i.e. prM-E and C-NS1 generally involved in entry of Flavivirus into cell) of ZIKV (having a broad cellular tropism in the testis) with the ones of the other Flaviviruses. The intention is to use these viral chimeras for infection of our tissue and cells of interest in order to determine the involvement of the structural protein-receptor interaction, involved in the differential tropism of Flaviviruses for the testis.

### C.3. Investigation of the replication and restriction factors

Viral tropism is defined as the ability of a virus to infect a distinct group of cells in the host <sup>371</sup> .

It is determined in a sequential manner by the availability of viral receptor(s) on the target cell, defining the entry factors, and by the capacity to complete a viral replication cycle, which depends on the balance between factors that support replication or restrict it. Therefore, besides entry factors, tropism depends on the presence of facilitating and restricting cellular factors that interact with the viral cycle.

Regarding SARS-CoV-2, the Endosomal Sorting Complex Required for Transport (ESCRT) machinery involving many proteins (CHMP3, CHMP5, CHMP1A, VPS37B) is important for the virus replication cycle and allows an efficient production of viral particles in type II alveolar cells during the initial step of human infection <sup>426</sup>. The expression of this factor in testicular cells will require investigations.

The ability of a given virus to counteract the immune response is another important parameter for efficient replication and is generally mediated by nonstructural proteins (NSP). The functions of NSP diverge among Flaviviruses. As an example, ZIKV, DENV, WNV and JEV but not YFV NS2B3 cleave the STING protein thus impairing the infected cell ability to induce ISG through this pathway <sup>427</sup>. Furthermore, while WNV uses the same receptors as DENV, of which some (MERTK, TYRO3, CLECL4M) are well transcribed in Sertoli cells, only WNV seems to replicate efficiently in this cell type <sup>383</sup> . Therefore, the investigation of the nonstructural proteins of Flaviviruses involved in tropism is warranted and we seek to additionally produce NSP chimeras. These would help determine the involvement of the NSP-host factors interaction, at the replication step, in the differential tropism.

Finally, the scRNA-seq approach can be complementary used to determine the facilitating and restricting host factors expressed in each cell type. Indeed, the team recently showed cell-specific differences in the innate immune responses to infection of the testis (Kuassivi et al, submitted). Thus, human peritubular cells were able to mount a strong innate immune response to a range of viruses and stimuli, whereas TGC were devoid of antiviral responses. A scRNA-seq characterization of the different cell types' host factors, in a baseline manner and upon infection with viruses, would help deciphering the molecular signatures associated with viral clearance or persistence in a given cell type.

Alternatively, the use of scRNA-seq would also help drawing the transcription profiles of the infected cells in semen. The comparison with the one of equivalent cell types within the different MGT organs would help inferring the source of shedding. The same could be done on the virus, with the phylogenetic comparison of the seminal strains with the ones retrieved in the different organs <sup>397</sup> .

Overall the scRNA-seq approach provides an overlook on the human testicular cell types susceptibility to a range of emergent viruses. It represents an informative database, which goal is to orientate further research on the putative infection and associated entry factors. It thus constitutes an initial step in the characterization of viral infections of the testis, which requires not only viral entry but also subsequent replication steps in order to lead to a productive viral replication. A comprehensive characterization therefore requires the input of in silico analysis and complementary experiments to confirm predictions and integrate the whole scale of host factors implicated in tropism and viral persistence

# Conclusion

In conclusion, the work undertaken during my PhD contributed to characterize the prolonged excretion of ZIKV in semen and the tropism of SARS-CoV-2 for the human testis. Thus we demonstrated that the testis is a reservoir for ZIKV in semen. Importantly also, we detected prolonged shedding of infected epithelial cells and leukocytes, which indicates that other MGT organs harbor ZIKV for extended duration. The exact source(s) of these cells will need to be determined.

Using an *ex vivo* culture of human testis explants, we revealed the replication of SARS-CoV-2 in this organ (i.e. Leydig and Sertoli cells mainly). Although infection appeared to impact Leydig cells steroidogenesis, viral replication declined over time, suggesting that the testicular infection may not be sustained. The COVID-19 patients analyzed here presented a transient primary hypogonadism, which is in support of a direct impact of SARS-CoV-2 infection onto the testis. Furthermore, infected Sertoli cells were detected in the semen of a patient, confirming SARS-CoV-2 tropism for the human testis *in vivo*.

Besides, I initiated the analysis of the expression of emerging viruses entry factors in the testis. Using a single cell RNA sequencing approach, we sought to predict the susceptibility of the different testicular cells to a range of viruses. Complementary analyses are needed to validate the relevance of these findings and determine the expression of facilitating and restricting factors of infection. Nevertheless, this work constitutes an initial step towards the characterization of the testicular tropism of emergent viruses, and an informative database to guide future research.

Overall this work contributed to a better understanding of the human testis infection and susceptibility to emergent viruses. As evidences accumulate on their ability to be shed in semen, and to be sexually transmitted, predicting and characterizing the source of shedding is key to control their spreading. The immune-privileged testis appears as an important site for prolonged infection and excretion. Understanding the mechanisms underlying viral entry and prolonged infection of the testis is crucial to prevent and anticipate the consequences of emerging viruses onto men's reproductive health and for viral spreading.



# Annexes

## Relevant publications

- Matusali G, Houzet L, Satie AP, Mahé D, Aubry F, Couderc T, Frouard J, **Bourgeau S**, Bensalah K, Lavoué S, Joguet G, Bujan L, Cabié A, Avelar G, Lecuit M, Le Tortorec A, Dejuqc-Rainsford N. **Zika virus infects human testicular tissue and germ cells**. J Clin Invest. 2018 Oct 1;128(10):4697-4710. doi: 10.1172/JCI121735. PMID: 30063220.
- Mahé D, **Bourgeau S**, Frouard J, Joguet G, Pasquier C, Bujan L, Dejuqc-Rainsford N. **Long-term Zika virus infection of non-sperm cells in semen**. Lancet Infect Dis. 2020 Dec;20(12):1371. doi: 10.1016/S1473-3099(20)30834-3. PMID: 33248032.
- Li T, Zhou B, Li Y, Huang S, Luo Z, Zhou Y, Lai Y, Gautam A, **Bourgeau S**, Wang S, Bao J, Tan J, Lavillette D, Li D. **Isolation, characterization, and structure-based engineering of a neutralizing nanobody against SARS-CoV-2**. Int J Biol Macromol. 2022 Apr 20;209(Pt A):1379-1388. doi: 10.1016/j.ijbiomac.2022.04.096. PMID: 35460753.
- Li T, Zhou B, Luo Z, Lai Y, Huang S, Zhou Y, Li Y, Gautam A, **Bourgeau S**, Wang S, Bao J, Tan J, Lavillette D, Li D. **Structural Characterization of a Neutralizing Nanobody With Broad Activity Against SARS-CoV-2 Variants**. Front Microbiol. 2022 Jun 2;13:875840. doi: 10.3389/fmicb.2022.875840. PMID: 35722331.

## Relevant communications

- Mechanisms of human testis infection and sexual transmission by Zika Virus. **Scientific days 2020, Ecole doctorale Biologie Santé Bretagne-Loire**, December 10th 2020  
- *Best Presentation Award*
- Mechanisms of human testis infection by Zika virus and SARS-CoV-2. **4ème Journées Reprosiences 2021, GdR Repro 3606**, April 29th 2021

# Zika virus infects human testicular tissue and germ cells

Giulia Matusali,<sup>1</sup> Laurent Houzet,<sup>1</sup> Anne-Pascale Satie,<sup>1</sup> Dominique Mahé,<sup>1</sup> Florence Aubry,<sup>1</sup> Thérèse Couderc,<sup>2,3</sup> Julie Frouard,<sup>1</sup> Salomé Bourgeau,<sup>1</sup> Karim Bensalah,<sup>4</sup> Sylvain Lavoué,<sup>5</sup> Guillaume Joguet,<sup>6</sup> Louis Bujan,<sup>7</sup> André Cabié,<sup>8</sup> Gleide Avelar,<sup>9</sup> Marc Lecuit,<sup>2,3,10</sup> Anna Le Tortorec,<sup>1</sup> and Nathalie Dejcq-Rainsford<sup>1</sup>

<sup>1</sup>Université de Rennes, Inserm, École des hautes études en santé publique (EHESP), Institut de recherche en santé, environnement et travail (Irset) – UMR\_S1085, Rennes, France. <sup>2</sup>Institut Pasteur, Biology of Infection Unit, Paris, France. <sup>3</sup>Inserm U1117, Paris, France. <sup>4</sup>Service d'Urologie, Centre Hospitalier Universitaire de Rennes, Rennes, France. <sup>5</sup>Unité de coordination hospitalière des prélèvements d'organes et de tissus, Centre Hospitalier Universitaire de Rennes, Rennes, France. <sup>6</sup>Centre Caribéen de Médecine de la Reproduction-CECOS CHU de Pointe-à-Pitre, Pointe-à-Pitre, France. <sup>7</sup>Research Group on Human Fertility EA 3694, University Paul Sabatier Toulouse III – CECOS, Hôpital Paule de Viguier, CHU Toulouse, Toulouse, France. <sup>8</sup>Inserm Centre d'Investigation Clinique 1424, Centre Hospitalier Universitaire de Martinique, and Service de maladies infectieuses, Centre Hospitalier Universitaire de Martinique, Fort de France, France. <sup>9</sup>Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Brazil. <sup>10</sup>Paris-Descartes University, Department of Infectious Diseases and Tropical Medicine, Necker-Enfants Malades University Hospital, Paris, France.

**Zika virus (ZIKV) is a teratogenic mosquito-borne flavivirus that can be sexually transmitted from man to woman. The finding of high viral loads and prolonged viral shedding in semen suggests that ZIKV replicates within the human male genital tract, but its target organs are unknown. Using ex vivo infection of organotypic cultures, we demonstrated here that ZIKV replicates in human testicular tissue and infects a broad range of cell types, including germ cells, which we also identified as infected in semen from ZIKV-infected donors. ZIKV had no major deleterious effect on the morphology and hormonal production of the human testis explants. Infection induced a broad antiviral response but no IFN upregulation and minimal proinflammatory response in testis explants, with no cytopathic effect. Finally, we studied ZIKV infection in mouse testis and compared it to human infection. This study provides key insights into how ZIKV may persist in semen and alter semen parameters, as well as a valuable tool for testing antiviral agents.**

## Introduction

Zika virus (ZIKV) is a teratogenic arthropod-borne flavivirus that recently emerged in the Pacific Islands (2007), Oceania (2013), and the Americas (2015). While ZIKV's primary mode of transmission is through mosquito bites, male-to-female sexual transmission has been reported by cohort studies (1, 2) and by case reports in non-endemic countries (3). Importantly, male-to-female sexual transmission in animal models was found to enhance viral dissemination in the female genital tract and transmission to the fetus (4–8). In humans, high viral loads and prolonged shedding of viral RNA (vRNA) and infectious virus (up to 1 year and 69 days, respectively) in the absence of viremia have been found in semen (9–11), strongly suggesting tropism of ZIKV for the male genital tract. Studies in immunodeficient mice evidenced high levels of ZIKV infection within the testis, leading to orchitis and impaired testosterone and sperm production (12–15). However, these mouse models do not reflect the pathophysiology in humans: unlike humans, mice only become infected following abrogation of type I IFN signaling and die of infection in most cases. This defective antiviral response may enhance the susceptibility and pathogenicity of ZIKV. In sharp contrast, ZIKV infection in macaque models either spared the testis or led to moderate infection, with no deleterious effects observed (16–18). Interestingly, a recent study on 15 ZIKV-infected men reported a lower total sperm count on day 30 after

onset of symptoms compared with day 7, suggesting an effect of the infection on the testis or epididymis (19).

Here, by infecting with ZIKV testicular tissue explants from healthy donors, we show that ZIKV replicates and produces infectious viral particles in human testis. We found evidence of infection of a broad range of testicular cell types, including resident macrophages and the germ cell line, and confirmed the latter in patients' semen. Infection had no effect on basal testosterone and inhibin B production or overall cell viability ex vivo. ZIKV triggered a wide range of antiviral genes in human testes, but upregulation of types I, II, and III IFN was not observed, and proinflammatory response was minimal. Finally, our data on IFNAR<sup>-/-</sup> mice points to similarities and differences between mouse and human testis in response to ZIKV infection.

## Results

**ZIKV replicates in human testicular tissue.** Testis explants from 8 uninfected donors were exposed to ZIKV ex vivo and maintained in culture medium as previously described (20). We first assessed the replication rate of a ZIKV strain derived from the 2015 outbreak in the Americas by measuring viral release over 3-day culture periods on days 3, 6, and 9 after infection (p.i.). A significant increase in vRNA release rate was observed between days 3 and 6 (median  $5.85 \times 10^7$  copies/ml) and 6 and 9 (median  $8.28 \times 10^7$  copies/ml) compared with days 0–3 p.i. (median  $5.29 \times 10^6$  copies/ml) (Figure 1A), while vRNA was below the detection threshold in mock-infected testes (data not shown).

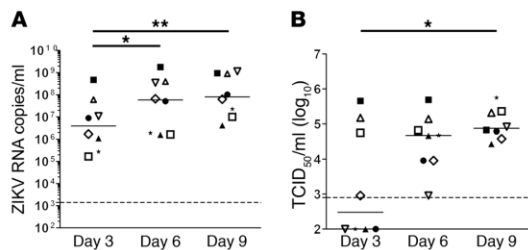
The ability of testes to produce infectious ZIKV particles was tested on reporter VeroE6 cells. A significant increase in supernatant infectivity was observed between days 0 and 3 (median  $3 \times 10^2$

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Submitted:** April 19, 2018; **Accepted:** July 24, 2018.

**Reference information:** *J Clin Invest.* 2018;128(10):4697–4710.

<https://doi.org/10.1172/JCI121735>.



**Figure 1. ZIKA virus replicates in human testicular tissue.** Human testis explants from 8 donors were ex vivo infected overnight with  $10^5$  TCID<sub>50</sub> (corresponding to  $2.2 \times 10^7$  to  $2.9 \times 10^7$  vRNA copies) from a low-passage ZIKV strain isolated in 2015 in the French Caribbean (MRS\_OPY\_Martinique\_PaRi-2015). Explants were thoroughly washed and cultured on inserts in 1 ml medium/well for 9 days, with media fully removed and changed every 3 days. Each of the time points (days 3, 6, 9) represents de novo viral release over a 3-day culture period. (A) ZIKV RNA release over a 3-day culture period on days 3, 6, and 9 detected by RT-qPCR. (B) Viral titers determined by infectivity assay of 3-day culture period tissue supernatants on VeroE6 cells. Each symbol represents a different donor (same symbol/donor throughout the figures). Dotted lines represent the detection limit of the assays. Mock-infected explants were always below detection level. Bars represent median. \* $P < 0.05$ , \*\* $P < 0.01$  (Friedman-Dunn nonparametric comparison).

TCID<sub>50</sub> [50% tissue culture infective dose]/ml) and days 6 and 9 p.i. (median  $7.50 \times 10^4$  TCID<sub>50</sub>/ml), demonstrating the infectivity of viral progeny (Figure 1B). The highest cumulative titer on day 9 (i.e., reflecting infectious viral production throughout culture) was  $2 \times 10^6$  TCID<sub>50</sub>/ml (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/JCI121735DS1>), with a median of  $3.16 \times 10^5$  TCID<sub>50</sub>/ml. Similarly, vRNA and infectious virion release rates increased during the culture of testis explants exposed to another ZIKV strain isolated during the 2013 outbreak in French Polynesia (Supplemental Figure 2).

Altogether, these data demonstrate that ZIKV efficiently infects and replicates in the human testis ex vivo, producing infectious viral particles.

**ZIKV infects somatic and germ cells in human testis explants.** To determine ZIKV's target cells in the human testis, we submitted mock- or ZIKV-infected testis explants to RNAscope in situ hybridization (ISH) using probes specific for ZIKV RNA (Figure 2, A-H; controls in Figure 2A and Supplemental Figure 3) and to IHC using an antibody against the nonstructural NS1 viral protein (Figure 2, I-M). Infected testes showed strong vRNA staining of the interstitial tissue cells and within the extracellular matrix bordering the seminiferous tubules, along with more diffuse staining in some interstitial areas (Figure 2, B-F). A weaker spotty staining was also observed inside a few seminiferous tubules (Figure 2, G and H), suggestive of association of the ZIKV with germ cells (Figure 2G) and Sertoli cells (Figure 2H). NS1 antibody (Figure 2, I-M) similarly labeled cells within the seminiferous tubule wall (Figure 2I) and the interstitium (Figure 2J), demonstrating ZIKV replication in these target cells. Within the tubules, different germ cell categories including spermatogonia (identified based on their position in the seminiferous epithelium, nucleus size, and distinctive morphological features) (Figure 2K) and a few Sertoli cells (identified based

on distinctive nucleus shape) (Figure 2L) stained positive for NS1. Infected cells (vRNA<sup>+</sup> or NS1<sup>+</sup>) displayed similar localization at the different time points of infection (days 3, 6, and 9) for the 2 ZIKV strains tested (Supplemental Figure 4 and data not shown).

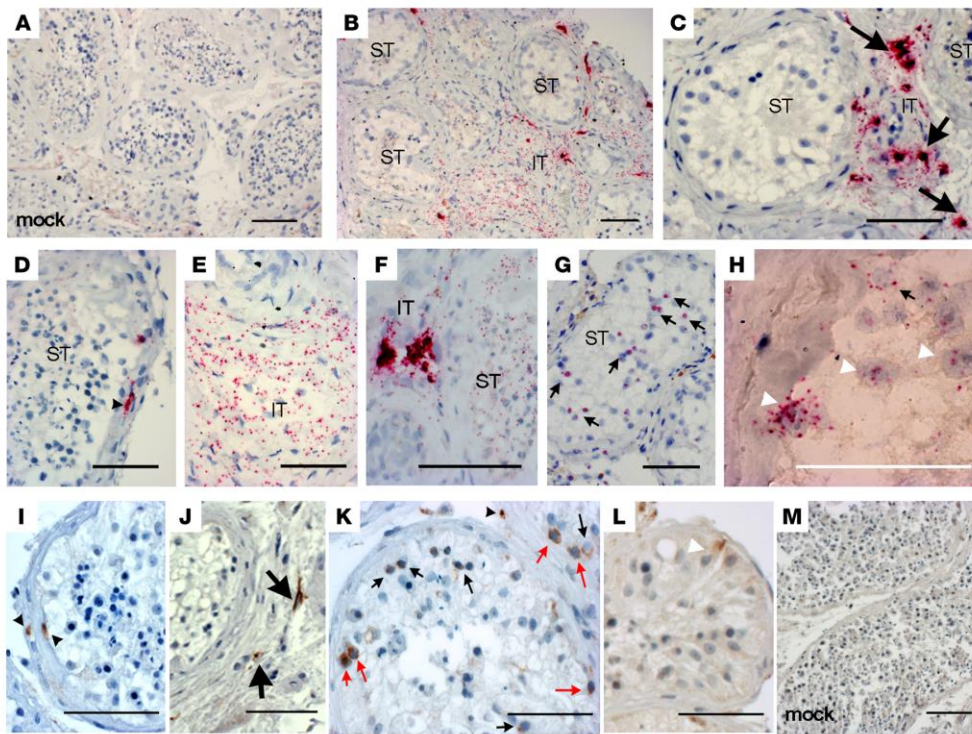
To further identify the nature of the infected cells, we combined ISH for vRNA with fluorescence immunolabeling for specific cell markers and undertook quantification of infected cells in testicular tissue from 4 donors. Interstitial infected cells were primarily CD68/CD163<sup>+</sup> testicular macrophages (median 12.7 cells/mm<sup>2</sup>), and to a lesser extent CYP11A1<sup>+</sup> Leydig cells (median 3 cells/mm<sup>2</sup>) (Figure 3, A, B, and G). Staining for  $\alpha$ -smooth actin ( $\alpha$ -SMA) demonstrated the infection of myoid peritubular cells bordering the seminiferous tubules (median 10 cells/mm<sup>2</sup>) (Figure 3, C and G). Within the tubules, spotty fluorescent ZIKV staining close to the lumen histologically colocalized with late germ cells (Figure 3D). Such staining was also present at the base of the tubules, where colabeled DDX4<sup>+</sup> early germ cells were evidenced (DDX4 being a specific marker expressed by most of the germ cells) (Figure 3E). Staining was not observed when a vRNA probe was used on mock-infected negative controls (Figure 3F). Infected cells in seminiferous tubules were mostly germ cells (median 11 cells/mm<sup>2</sup>), while infected Sertoli cells represented a median of 3.5 cells/mm<sup>2</sup> (Figure 3G).

Collectively these data indicate that ZIKV has tropism for germ cells and somatic cells within the human testis.

**ZIKV replicates in human testicular germ cells in vitro and in vivo.** We exposed freshly isolated seminiferous tubule cells to ZIKV to investigate their ability to produce infectious viral particles that might be released into semen. In 3 independent primary cultures, vRNA increased in cells from a median of  $2.82 \times 10^3$  to  $2.09 \times 10^7$  copies/ $\mu$ g total RNA between 6 and 120 hours p.i. (Figure 4A). ZIKV RNA in culture supernatants significantly increased about 4 log<sub>10</sub> between 6 and 120 hours p.i. (median values of  $1.26 \times 10^4$  and  $5.01 \times 10^7$  copies/ml, respectively) (Figure 4B). Infectious virus titers also rose between 48 and 120 hours, reaching a median of  $4 \times 10^5$  TCID<sub>50</sub>/ml and maximum titer of  $4 \times 10^6$  TCID<sub>50</sub>/ml (Figure 4C). ZIKV replicated in DDX4<sup>+</sup> germ cells, FSH receptor<sup>+</sup> Sertoli cells, and  $\alpha$ -SMA<sup>+</sup> peritubular cells (Figure 4D). ZIKV envelope (ZIKV-E) was detected in undifferentiated spermatogonia (MAGEA-4<sup>+</sup>Stra8<sup>+</sup>) and in MAGEA-4<sup>+</sup>STRA8<sup>+</sup> cells, corresponding to differentiated spermatogonia up to the preleptotene spermatocytes stage (Figure 4D).

To further explore the germ cells' productive infection and since primary testicular germ cells cannot be cultured without somatic support, we used the seminoma-derived germ cell line T-cam2, which displays characteristics of fetal germ cells (21). In 3 independent experiments, vRNA levels in T-cam2 cells rose from below detection at 6 hours to a median of  $6.31 \times 10^5$  copies/ $\mu$ g total RNA at 72 hours p.i., reaching a maximum of  $1 \times 10^6$  copies/ml in culture supernatants (Supplemental Figure 5, A and B). ZIKV-E was evidenced in T-cam2 by immunofluorescence (Supplemental Figure 5C). The production of infectious viral particles was evidenced in the 2 cultures showing the highest viral loads, with a maximum titer of  $8.2 \times 10^3$  TCID<sub>50</sub>/ml (Supplemental Figure 5D).

These findings were corroborated in vivo by analysis of the semen cell smear from 2 ZIKV-infected donors, in which we revealed the presence of ZIKV-E<sup>+</sup> or NS1<sup>+</sup> germ cells exfoliated from



**Figure 2. ZIKV infects somatic and germ cells in human testis explants.** (A–H) Representative images of RNAscope ISH for ZIKV RNA in control mock-infected (A) and ZIKV-infected testis explants ( $n = 8$  independent donors) after 6 days of culture (B–H). ZIKV RNA labeling was observed in the interstitial tissue (IT) of testis explants (B, C, E, and F), in cells bordering the seminiferous tubules (ST) (B and D), and within seminiferous tubules (F–H). (I–M) Representative images of IHC staining of NS1-ZIKV performed on ZIKV-infected (I–L) and mock-infected (M) testis explants in culture for 6 days ( $n = 8$  independent donors). Black arrowheads indicate infected cells in the extracellular matrix surrounding the seminiferous tubules. Thick arrows indicate infected cells in the interstitial tissue. Thin black arrows indicate infected germ cells. Thin red arrows indicate infected spermatogonia. White arrowheads indicate Sertoli cell nuclei. Black scale bars: 100  $\mu\text{m}$ ; white bar: 50  $\mu\text{m}$ .

the testis 7 and 11 days after symptoms onset (Figure 4E). A subset of spermatozoa also labeled for ZIKV-E (Supplemental Figure 6).

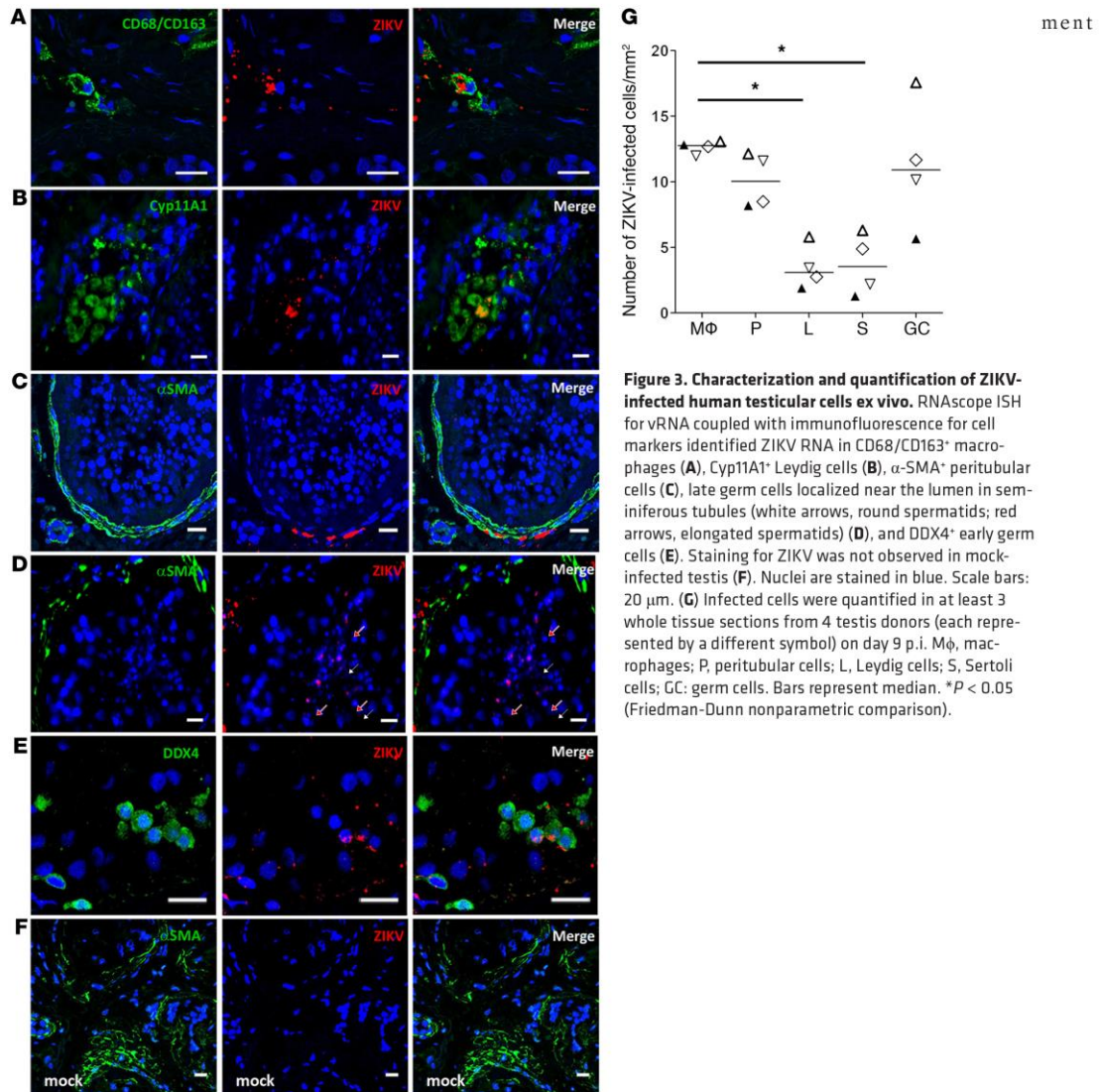
Altogether, these data indicate that ZIKV replicates in human germ cells at different stages of differentiation and infects testicular germ cells in ZIKV-infected men.

*ZIKV infection ex vivo has no major impact on human testis morphology or hormonal production.* We next assessed the impact of ZIKV on human testis morphology, viability, and function during the ex vivo culture time frame. The tissue architecture and histology of infected testes were similar to those of mock-infected testes all throughout the culture period (Figure 5). In both infected and mock-infected testes, we observed conserved interstitial tissue (comprising groups of Leydig cells, mast cells, and blood vessels), seminiferous basement membrane of similar thickness (increased thickness being a sign of injury), and seminiferous tubules encompassing Sertoli cells and early and late germ cells (Figure 5, A and D). Caspase-dependent apoptosis evidenced by cleaved caspase-3 immunostaining was similar in infected and mock-infected testis and, as expected, primarily affected isolated germ cells (Figure 5B). Measurement of lactate dehydrogenase (LDH) release confirmed that the overall viability of the organ was not affected by

the infection (Figure 5C). Testosterone concentrations were not different in infected versus mock-infected testes (Figure 5E), and expression of genes encoding steroidogenic enzymes was unmodified by ZIKV (Supplemental Figure 7A). Sertoli cells positively stained for the tight junction marker protein ZO-1 in both infected and mock-infected testis until day 9 p.i., suggesting an intact barrier (Figure 5F). Inhibin B (a marker of Sertoli cell function) protein and mRNA levels showed no significant differences between infected and mock-infected testis up to day 9 p.i. (Figure 5G and Supplemental Figure 7B). Finally, levels of peritubular cells ( $\text{Acta2}^+$ ) and early meiotic ( $\text{PGK2}^+$ ) and late postmeiotic ( $\text{PRM2}^+$ ) germ cell transcripts were unchanged by the infection (Supplemental Figure 7B).

Overall, although actively replicating within the testis, ZIKV did not appear to affect testis morphology, induce cell death, or trigger any drastic effect on testis functions during the 9-day culture.

*ZIKV triggers a broad antiviral response but no IFN upregulation and a minimal proinflammatory response in human testicular tissue.* To investigate the immune response to ZIKV infection, we assessed the concentration of a panel of antiviral and proinflammatory cytokines (IFN- $\beta$ , IFN- $\alpha$ 2, IFN- $\lambda$ 1, IFN- $\lambda$ 2/3, IFN- $\gamma$ , IL-1 $\beta$ ,



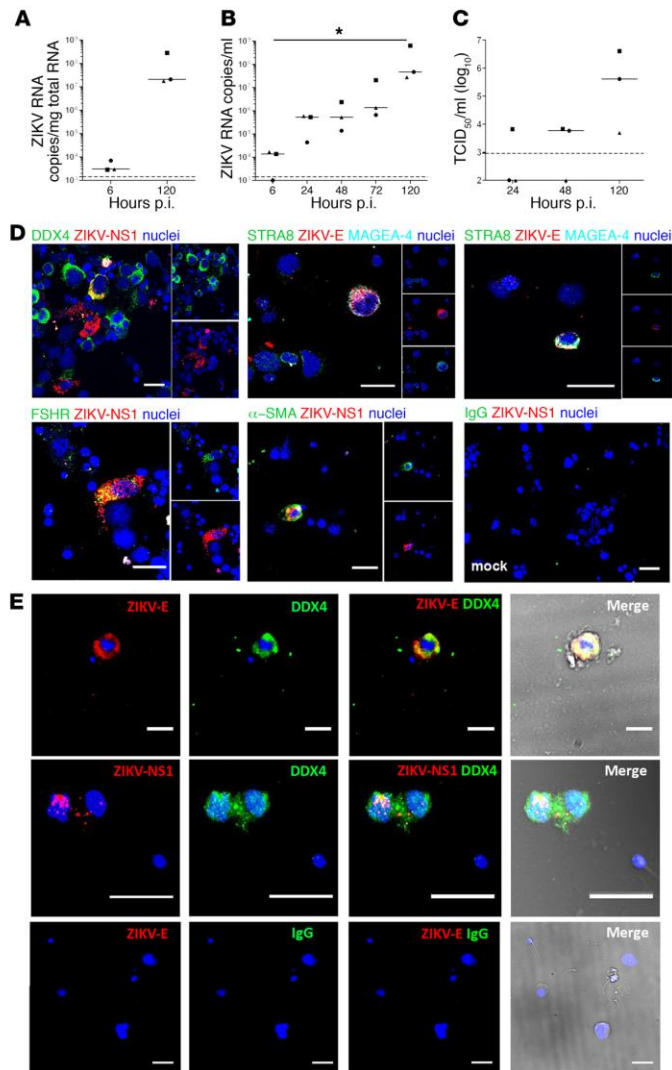
IL-6, TNF- $\alpha$ , IL-8, IL-12p70, CXCL10, IL-10, and GM-CSF) in testis explant supernatants. Type I, II, and III IFN concentrations in testis culture supernatants were unchanged by the infection on days 3 and 6 p.i. in 7 independent testis cultures tested (Figure 6A and Supplemental Figure 8). Among proinflammatory cytokines, only CXCL10 was significantly increased (Figure 6A and Supplemental Figure 8), and its induction positively correlated with vRNA load (Figure 6B).

We then analyzed the transcripts of 11 of these cytokines (IFN- $\beta$ , IFN- $\alpha$ 1, IFN- $\alpha$ 2, IFN- $\alpha$ 4, IFN- $\lambda$ 1, IFN- $\lambda$ 2, IFN- $\lambda$ 3, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and CXCL10) by quantitative real-time PCR (RT-qPCR) on ZIKV-infected versus mock-infected testes. Type I, II, and III IFN transcripts in uninfected testis tissues were below the measure-

threshold irrespective of infection (data not shown), while CXCL10 was increased (median fold change [FC] 43.4 on day 9, range 7.7–227.8) in the testis from 4 of 6 donors (Figure 6C).

Extending the analysis to a wider range of genes involved in pathogen sensing (RIG-I, MDA5), antiviral response (IFN- $\epsilon$ , IFI27, IFIT1, IFITM1, IRF7, ISG15, Mx1, Mx2, OAS1, OAS2, RSAD2), inflammation (CCR7, CD14, CD64, HLA-DR, MCSF), chemotaxis (CCL2, CCL5, CXCL1, CXCL2), and control of inflammation (IL-10, TGF- $\beta$ , CD163, SOCS1, SOCS3), we observed the induction of a broad range of antiviral genes from day 3 onward in testis from 3 of 6 donors and on day 9 in 1 other donor (Figure 6C).

A strong induction of ISG15 (FC 12.2, range 5.2–45.6), IFIT-1 (FC 12.8, range 9.7–29.6), OAS1 (FC 22.9, range 6.4–32.5), OAS2 (FC



**Figure 4.** ZIKV replicates in human testicular germ cells *in vitro* and *in vivo*. (A–C) Primary testicular cells were infected with ZIKV (MOI of 1, corresponding to  $7.15 \times 10^5$  TCID<sub>50</sub> U/ml per 0.5 million cells). ZIKV RNA detected by RT-qPCR in cells (A) and culture supernatants (B). (C) Viral titers determined by infectivity assay of tissue supernatants on VeroE6 cells. Each dot represents an independent donor. Bars represent median values. Dotted lines indicate detection limit. \* $P < 0.05$  (Friedman–Dunn nonparametric comparison). (D) Immunofluorescence against ZIKV NS1 or ZIKV-E proteins combined with cell markers for all germ cells (DDX4) or specific germ cell types (STRA8, MAGEA-4), Sertoli cells (FSHR), and peritubular cells ( $\alpha$ -SMA). Nuclei are stained in blue. (E) Detection of infected germ cells in semen from ZIKV-infected men. Immunofluorescence labeling of semen cell smears from 2 ZIKV-infected patients, one on day 7 (top row) and one on day 11 (middle row) after onset of symptoms. ZIKV-E or NS1 protein colabeled with the germ cell marker DDX4. Bottom panels show semen from a healthy individual stained with anti-ZIKV-E antibody and IgG isotype as a negative control. Nuclei are stained in blue. In the merge panels, brightfield images are included to visualize the cell's morphology. Scale bars: 20  $\mu$ m.

7.1, range 4.2–21.6), Mx1 (FC 9.8, range 5.1–35.1), Mx2 (FC 9.5, range 3.7–25.3), and RSAD2 (FC 31.5, range 3.7–66.8) was measured on day 9 in these 4 donors, along with a more moderate induction of IFI27 (FC 3.1, range 2.2–10.6), IFITM1 (FC 1.8, range 1.5–6.2), IRF7 (FC 2.7, range 1.8–6), MDA5 (FC 1.9, range 1.6–8.5), and RIG1 (FC 2.8, range 1.8–9.4) (Figure 6C).

These genes correlated with one another in FC in expression on day 9, except for IFITM1 and MDA5 (Supplemental Figure 9). The fold increase on day 9 of a number of genes involved in antiviral response (RSAD2, IFIT1, ISG15, OAS1, OAS2, Mx1, Mx2, IFI27, IRF7), pathogen sensing (RIG1), and CXCL10 positively correlated with the level of infection (Figure 6B) in the corresponding testis supernatant on days 3 and 6 (Figure 6E and Supplemental Figure 9).

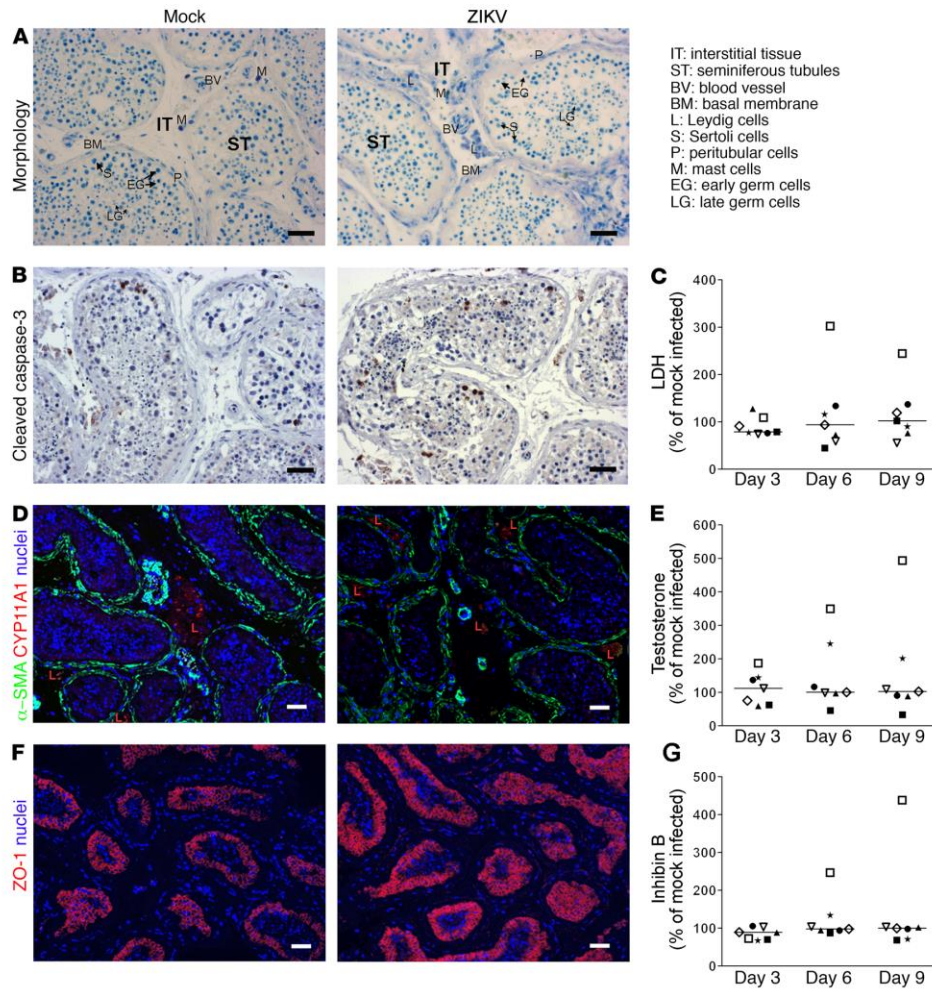
Finally, we assessed expression levels of all transcripts, including type I, II, and III IFN, at earlier time points (4, 18, 48 hours p.i.) in 2 testis explants and did not observe any upregulation (Supplemental Figure 10 and data not shown).

Altogether, these results are consistent with ZIKV infection inducing a broad antiviral and minimal proinflammatory response in the absence of detectable IFN stimulation in human testis explants.

**Innate immune response to ZIKV infection in the testis from *IFNAR*<sup>-/-</sup> mice.** To support our hypothesis of a type I IFN-independent antiviral response induced by ZIKV in testis and compare our findings on ZIKV tropism and initial antiviral/proinflammatory responses in human testis explants with those in a widely used animal model, we analyzed the testis of type I IFN receptor-defective (*IFNAR*<sup>-/-</sup>) mice using similar techniques and viral strain.

ZIKV RNA measurement in testes from *IFNAR*<sup>-/-</sup> mice infected for 5 and 9 days showed high viral loads in this organ (Figure 7A). Despite differences in intensity and sequence of infection, ZIKV tropism in the mouse testis *in vivo* was comparable overall to that in human testis *ex vivo*. On day 5, testicular infection localized primarily within the interstitial tissue and peritubular cells, while seminiferous tubules were spared (Figure 7B). Colabeling of ZIKV RNA with cell markers showed infection of steroidogenic acute regulatory protein-positive (STAR<sup>+</sup>) Leydig cells and F4/80<sup>+</sup> macrophages (Figure 7C). On day 9 p.i. (a time at which death occurred in some mice), strong labeling for vRNA became prominent within Sertoli and germ cells (Figure 7B). We did not observe modifications of testicular morphology at these early time points, in agreement with previous studies (12, 22).

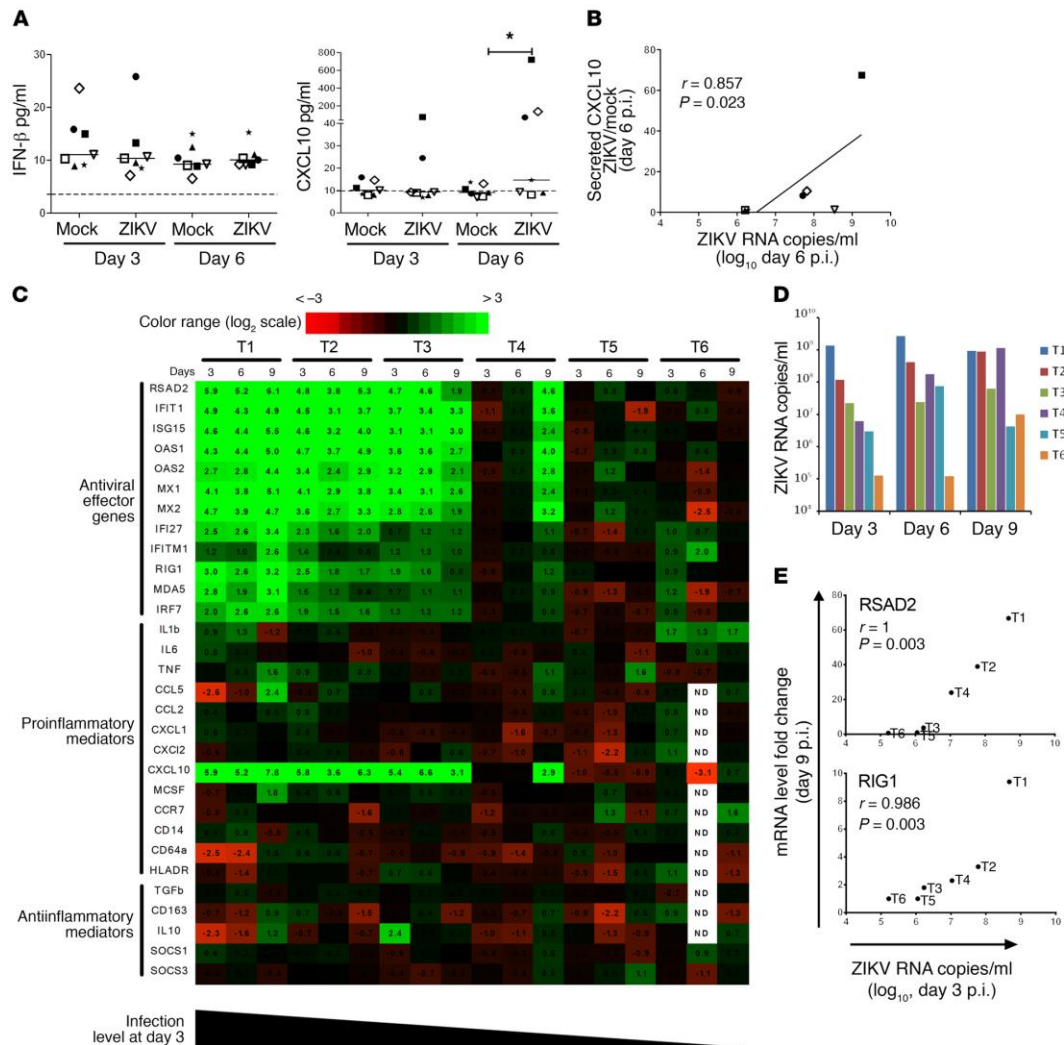
We next examined the induction of genes involved in antiviral response and inflammation in mouse testis (Figure 7D). Similar to human testis explants, and despite a lack of type I IFN signaling, a strong induction of ISG15 (FC 11.7, range 7.7–15.6 on day 5 and FC 7.4, range 4.7–15.1 on day 9), RSAD2



**Figure 5. ZIKV infection ex vivo does not alter human testis explant morphology, cell viability, or hormonal production.** (A) Toluidine histological staining of testis explants, shown here for mock-infected (left) and ZIKV-infected (right) testis explants on day 6 p.i. (B) Cleaved caspase-3 IHC to detect apoptotic cells in mock (left) and ZIKV-infected (right) testis explants, shown here for day 6 p.i. (C) LDH release in testis supernatant expressed as percent of mock-infected explants on the corresponding day of culture. (D) Immunofluorescence colabeling of peritubular ( $\alpha$ -SMA) and Leydig (CYP11A1) cells, shown on tissue sections on day 6 p.i. for mock- (left) and ZIKV-infected (right) explants. Nuclei are stained in blue. (E and G) Testosterone and inhibin B release in testis supernatants expressed as percent of mock-infected explants on the corresponding day of culture. (F) Immunofluorescence labeling of Sertoli cell tight junction-associated protein ZO-1 in tissues sections for mock- (left) and ZIKV-infected (right) explants, shown on day 6 p.i. Nuclei are stained in blue. Scale bars: 50  $\mu$ m. C, E, and G: each symbol represents a different donor; horizontal bars represent median values.

(FC 24.6, range 13.7–45.6 on day 5 and FC 10.1, range 2.21–30.39 on day 9), IFIT1 (FC 36.0, range 19.5–42.6 on day 5 and FC 10.1, range 6.2–27.3 on day 9), and CXCL10 (FC 17.5, range 9.4–39.2 on day 5 and FC 9.0, range 4.2–14.6 on day 9) was detected in infected mice testis (Figure 7D). In contrast to human testis, Mx1, MDA5, and RIG1 were not induced on either day 5 or day 9 (Figure 7D). These results suggest that ZIKV induces a type I IFN signaling-independent antiviral response in both humans and mice. In contrast to human testis, in which CXCL10 was the only proinflammatory gene increased by the infection, TNF- $\alpha$  (median FC 48.0,

range 32.6–122.6 on day 5 and median FC 6.9, range 2.6–12.3 on day 9), IL-1 $\beta$  (median FC 7.7, range 1.8–13.8 on day 5 only), and IL-6 (median FC 10.7, range 4.9–27.5 on day 5 only) were upregulated in infected mouse testis (Figure 7D), while IFN- $\gamma$  (produced by NK and T cells) was maximally increased on day 9 (median FC 11.2, range 6.2–30.8) over day 5 (median FC 6.2, range 4.0–12.7). IFN- $\beta$  was the most dramatically stimulated innate immune gene on day 5 (median 236.7, range 130.2- to 353.4-fold), while IFN- $\alpha$ 1, -2, and -4 genes were modestly and transiently upregulated on day 5, and IFN- $\lambda$ 2/3 mRNA levels were unchanged (Figure 7D). When look-

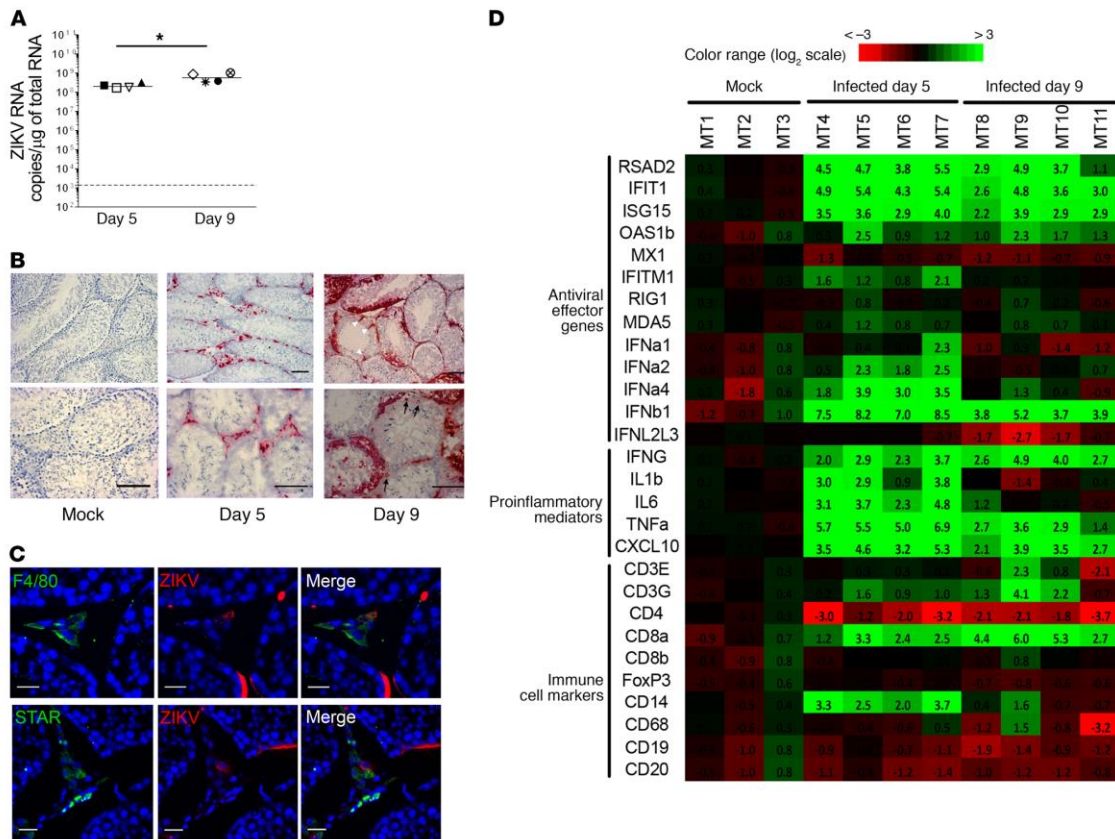


**Figure 6. ZIKV triggers a broad antiviral response but no IFN upregulation and a minimal proinflammatory response in human testicular tissue. (A)** Levels of IFN-β and CXCL10 measured by flow cytometry-based multiplex assay in mock-infected and ZIKV-infected human testis explant supernatants. Each symbol represents a different donor. Bars represent median values. \* $P < 0.05$  (Friedman-Dunn nonparametric comparison). **(B)** Correlation between secreted CXCL10 induction in ZIKV-infected versus mock-infected explants and ZIKV RNA level in culture supernatant on day 6 p.i. (Spearman's nonparametric test). **(C)** Innate immune gene expression determined by RT-qPCR in testis explants from 6 donors (T1-T6) infected with ZIKV for 3, 6, and 9 days (d3, d6, d9). Heatmap shows log<sub>2</sub>-transformed expression ratios between ZIKV-infected and time-matched mock-infected controls. Green indicates upregulation and red downregulation of mRNA compared with controls. Type I and II IFN mRNAs were below the quantification threshold (data not shown). **(D)** Viral loads in supernatants of the testis explants analyzed in C. **(E)** Examples of correlation between gene fold expression on day 9 and the level of infection on day 3 p.i. (Spearman's nonparametric test). Other correlations are shown in Supplemental Figure 9.

ing at markers of immune cell subtypes, we observed a transient increase in transcripts encoding the myeloid cell marker CD14 on day 5, whereas transcripts encoding CD3 (T cell marker) and CD8 (cytotoxic T cell marker) were maximally upregulated on day 9, in line with the IFN-γ expression pattern. The markers for B cells (CD19 and CD20), regulatory T cells (FoxP3), and macrophages (CD68) were unchanged, while CD4 (expressed by T helper and

myeloid cells) was downregulated (Figure 7D). The infiltration of T lymphocytes in infected mouse testis was confirmed by CD3 immunostaining and quantification of positive cells (Supplemental Figure 11), further demonstrating mouse testis inflammation. Overall, the induction of an antiviral response in human and IFNAR<sup>-/-</sup> mice testis supports the existence of a type I IFN signaling-independent response to ZIKV infection in testis.





**Figure 7. Innate immune response to ZIKV infection in testis from IFNAR<sup>-/-</sup> mouse.** (A) vRNA measured by RT-qPCR in testis from mice infected with ZIKV for 5 or 9 days (*n* = 4 animals/group). Each dot represents one animal, and horizontal bars represent the median. The dotted line indicates the limit of detection. Levels in testis from mock-infected mice (*n* = 3) were below the detection threshold (data not shown). \**P* < 0.05 (Mann-Whitney *U* test, nonparametric comparison). (B) Detection of ZIKV RNA by RNA scope ISH in testis tissue sections from mice mock-infected or on day 5 or day 9 after infection. White arrowheads indicate Sertoli cells; thin black arrows indicate germ cells. Scale bars: 100 μm. (C) RNA scope ISH for ZIKV RNA coupled with immunofluorescence for cell markers identified ZIKV RNA in F4/80<sup>+</sup> macrophages and STAR<sup>+</sup> Leydig cells. Nuclei are stained in blue. Scale bars: 20 μm. (D) Expression of a range of innate immune genes and of genes encoding immune cell markers was determined by RT-qPCR in testis from 3 mock-infected mice (mouse testis MT1–MT3) and 4 ZIKV-infected mice at day 5 (MT5–MT7) and day 9 (MT8–MT11) after infection. Fold induction is presented as a heatmap of log<sub>2</sub>-transformed expression ratios to the average expression level in mock-infected mice. On the scale bar, green indicates upregulation and red, downregulation.

Discussion

We show that Asian ZIKV replicates in the human testis ex vivo. Infected somatic cells within testis explants were mostly macrophages and peritubular cells, while smaller numbers of Leydig and Sertoli cells were observed. Considering the relative proportion of these different cell types in human testicular tissue (approximately 1 macrophage for 10 Leydig cells, 36 peritubular cells, 40 Sertoli cells, and 400 germ cells) (23, 24), macrophages are likely the cell type most susceptible to ZIKV infection within the testis. Importantly, we demonstrate that ZIKV replicates within testicular germ cells, from stem cell (spermatogonia) to spermatozoa precursors (spermatids). In agreement with this finding, Robinson et al. recently reported on the infection of male germ cells after 3-day exposure of human seminiferous tubules to African ZIKV (25). Our detection of infected germ cells in semen from ZIKV-

infected men confirms these in vitro and ex vivo findings. The presence of ZIKV in ejaculated spermatozoa adds to previous findings of ZIKV antigen, RNA, and infectious particles in spermatozoa (19, 26). Spermatozoa and immature germ cells may be infected during epididymal transit (duration 1–21 days) or within the testis. To infect these cells, the virus has to cross the blood-testis barrier formed by Sertoli cell tight junctions. Direct infection of Sertoli cells is supported by our results in primary cells and those of other researchers in commercial Sertoli cells (27, 28). Sertoli cells were shown to release ZIKV particles on their adluminal side, whereas tight and adherens junction protein expression was not altered by infection (27). In agreement with these data, the ZO-1 labeling we observed in human explants suggested intact Sertoli cell barrier despite infection. In contrast, Sertoli cell exposure to inflammatory mediators produced by ZIKV-infected

blood-derived macrophages (the phenotype of which differs from that of antiinflammatory testicular macrophages) altered their barrier function (27). Thus, the alteration of the blood-testis barrier by testis-infiltrating macrophages may provide an additional way for the virus to reach seminal lumen and late germ cells. Altogether, our data show that ZIKV replicates in germ cells and suggest that the virus might be able to bypass the blood-testis barrier by infecting Sertoli cells.

In our *ex vivo* model, release of testosterone and inhibin B was not modified by infection, nor were their related gene expression. This is not a limitation of our culture system, since it is successfully used to assess the hormonal production of human testis (29). This lack of effect might be linked to low infection levels of Leydig and Sertoli cells *ex vivo*, as supported by a relatively low number of infected cells. In ZIKV-infected mouse testis, testosterone and inhibin B levels were preserved on day 7 *p.i.*, while testis integrity was maintained, whereas they decreased after orchitis (12, 13), suggesting that inflammation rather than testis infection caused altered hormone secretion. In a 4-month follow-up of a cohort of 15 ZIKV-infected men, testosterone levels were not significantly affected, whereas slightly lower inhibin B levels were reported on day 7 after onset of symptoms compared with later time points (19). Such transient imbalance of reproductive hormones can be related to fever or other systemic effects (30). However, we did not study the effect of ZIKV on LH- and FSH-stimulated hormone secretion, and a systemic impact on testicular hormones *in vivo* cannot be ruled out. Moreover, we cannot exclude effects at the single-cell level that cannot be detected when analyzing the whole tissue.

Our findings suggest that ZIKV could affect sperm production. Besides germ cell infection, somatic cell infection might disrupt the paracrine control of spermatogenesis (31, 32), and the infection of the contractile peritubular cell (33) might decrease the expulsion of spermatozoa from tubules into the epididymis. Infected men showed a decrease in sperm count and an increase in spermatozoa abnormalities during the 2 months after clinical onset (19, 34). Our results suggest that direct infection of germ and/or testicular somatic cells might be involved in such altered sperm parameters, although fever and/or immune response could be involved (35, 36).

The infection had no significant effects on testis explant morphology or viability. This is in contrast to findings in mouse models, where damaging effects of ZIKV infection on testis became evident after leukocyte infiltration (12–14). Since the *ex vivo* testis model lacks the presence of an intact immune system, we cannot rule out an effect of acquired immunity on the testis from ZIKV-infected men. However, testicular atrophy or orchitis has not been reported in clinical cases or nonhuman primate studies, and immune infiltrations were not observed in the latter (16–18), suggesting the absence of massive inflammation. The lack of cell death induction in our *ex vivo* model could be linked to culture conditions (e.g., viral strains/doses used, duration of infection) or to the limited number of infected cells. However, it might also reflect the ability of the virus to replicate in testicular cells in a noncytopathic manner. No or minimal cytopathic infection and persistence of ZIKV has been reported in different cell types, including human placental macrophages (37), brain microvascu-

lar endothelial cells (38), and, recently, male mouse germ cells (25). Absence of cytopathic effect was also reported in Sertoli cells infected with ZIKV (27, 39). In contrast, high cytotoxicity was reported in ZIKV-infected human testis organoids (40). However, in this organoid system, the architecture of the testis is not preserved and physiological cellular interactions is lost. We hypothesize that nonlytic infection, in combination with evasion from immune responses, may allow viral persistence in the human testis.

A broad range of antiviral genes was induced by ZIKV in testis explants. Several of these classically defined IFN-stimulated genes (ISGs), such as ISG15 (41), RSAD2 (42), IFITM1 (43), OAS1 (44), Mx1 (45), and IFIT gene family members (46), have an inhibiting activity on flaviviruses and/or ZIKV replication. Most interestingly, ZIKV did not increase type I, II, or III IFN secretion by testicular explants, and their transcripts consistently remained below detection at all time points. A level of IFN production below the sensitivity of our assays, or active inhibition of IFN production by ZIKV, could explain these results. Thus, ZIKV nonstructural proteins inhibit different steps of the type I IFN induction cascade (47, 48). Alternatively, the absence of IFN upregulation in testis explants might reflect a specificity of this immunosuppressed organ, in which sustained high concentrations of type I IFN trigger germ cell apoptosis and sterility (49). Detection of ISG overexpression in testis from ZIKV-infected IFNAR<sup>-/-</sup> mice further suggests IFN-independent induction of ISGs in the testis. The increased level of RIG1 mRNA in infected testis suggests it may be involved in the direct induction of ISGs, although other effectors could be in play (50). However, the broad induction of ISGs may fail to control ZIKV replication in the testis in the absence of increased type I IFN secretion to amplify and stabilize the antiviral response. In contrast to its effects on antiviral genes, ZIKV infection of the human testis did not affect any of the classic proinflammatory cytokines, except for CXCL10, secretion of which was modestly increased. Interestingly, the level of antiviral transcripts on day 9 *p.i.* positively correlated with the level of infection on days 3 and 6, suggesting that the initial level of infection influenced the intensity of the antiviral response. Accordingly, antiviral genes were not induced in explants showing lower levels of infection. Thus, host factors other than those we have studied may play a role in susceptibility to ZIKV. Altogether, our results indicate that ZIKV induces a broad induction of antiviral effectors but no IFN upregulation and minimal proinflammatory response in *ex vivo* infected human testis. We hypothesize that such innate immune response, along with a lack of cytopathic effect, might facilitate the persistence of ZIKV for extended periods in the testis and contribute to the prolonged release of ZIKV in semen.

Animal models are crucial for mechanistic studies and the *in vivo* testing of antiviral strategies. Cross-validation with human data is essential to assess their similarities and differences. Discrepant results on the interstitial (13, 22, 51) and/or intratubular localization of ZIKV (12, 14, 52, 53) were reported in mouse testis from IFN signaling-deficient mice. Our results in the IFNAR<sup>-/-</sup> mouse model reconciled these results, since ZIKV infection was exclusively located in interstitial and peritubular cells on day 5 *p.i.*, whereas by day 9 the infection had progressed to the seminiferous tubules, where it became prominent. In human testis, seminiferous tubule cell infection was consistently weaker than that in the

mouse and that in human interstitial cells. This was not modified when the infective viral dose was increased 10 times (data not shown). The difference in seminiferous tubule infection levels in mice versus humans might reflect differences in the susceptibility of mouse versus human Sertoli and germ cells to ZIKV infection and/or in their innate immune response.

We found evidence of induction of several ISG mRNAs in testis from IFNAR<sup>-/-</sup> mice, indicating type I IFN signaling-independent induction, which corroborates our results in human testis explants. Interestingly, in contrast to the human testis, the pathogen sensor RIG1 was not upregulated in the mouse testis, which may suggest a different sensing mechanism. Also unlike in human testis, type I IFNs and a number of proinflammatory genes were upregulated in mice. We previously showed that unlike their rodent counterparts, which produced large amounts of IFN, primary human Leydig cells did not produce IFN in response to paramyxovirus infection or double-stranded RNA stimulation (54, 55). This key difference between mouse and human testis in terms of IFN production may explain why in IFN signaling-competent mice, ZIKV tropism has been reported as being essentially restricted to germ cells (25), whereas a broad tropism for both somatic and germ cells is observed in human testis explants and in IFNAR<sup>-/-</sup> mice. Indeed, we previously showed in rodents that meiotic and post-meiotic male germ cells lack the functional type I IFN receptor (49) and do not express ISGs after viral or IFN stimulation, unlike testicular somatic cells (56, 57). Differences in antiviral (e.g., sensing pathways and ISG induction patterns) and proinflammatory immune responses in human versus mouse testis may explain the testis pathogenicity observed in type I IFN signaling-deficient mouse models (along with differences in infection levels). Differences in type I IFN upregulation following ZIKV infection may also explain the restricted tropism of ZIKV in testis from immunocompetent mice (25) when compared with human testis explants. Whether these differences derive from intrinsic differences between human and mouse testicular cells, differential escape mechanisms mediated by ZIKV (e.g., specific counteracting of type I IFN by ZIKV in human cells but not in immunocompetent mouse cells), or ex vivo/in vivo differences (e.g., infiltrating cell proinflammatory activity) requires further investigation.

To date, ZIKV is the only arbovirus known to be sexually transmitted within the human population. RNA from other arboviruses such as dengue, yellow fever, and chikungunya viruses were recently evidenced in semen from infected men for a prolonged period (58–60), but no cases of sexual transmission have been documented so far. Dengue virus did not productively infect/alter testicular cells in mouse models (12, 13, 25, 51) and poorly infected human Sertoli cells. Although the neurotropic West Nile virus (WNV) replicated to levels similar to ZIKV in a Sertoli cell line (27), testis from men with neuroinvasive WNV tested negative, except for one immunosuppressed patient (61). Interestingly, Japanese encephalitis virus, another mosquito-borne neurotropic flavivirus, infects boar testis and semen for a long period of time, disrupts spermatogenesis, and can be transmitted through semen (62).

Of note, other male genital organs may be involved in ZIKV shedding in human semen, as suggested by prolonged sexual

transmission of ZIKV from vasectomized men (63), and by ZIKV replication in human prostate cell lines and cell line-based organoids (64). Interestingly, we recently demonstrated in SIV-infected cynomolgus macaques that depending on the individuals, different male genital organs may be the source of the virus in semen (65). Nevertheless, the significant reduction in ZIKV titers and shorter infectivity window in semen from vasectomized mice indicate the importance of testis/epididymis contributions to infectious virus shedding (4).

In conclusion, we demonstrated that ZIKV replicates in the human testis ex vivo and infects a range of somatic cells and germ cells. Replication of ZIKV in testicular germ cells was evidenced in semen from ZIKV-infected men, along with ZIKV association with spermatozoa. ZIKV had no major deleterious effect on the morphology or hormonal production of the human testis in culture. Despite a broad induction of antiviral genes, absence of IFN upregulation and minimal proinflammatory response of the human testis ex vivo along with the lack of ZIKV cytopathic effect on testicular cells might favor the prolonged ZIKV infection observed in this organ and account for the absence of orchitis in men infected by ZIKV. Overall, our results suggest that ZIKV infection of the human testis may be involved in the persistence of the virus in semen and in altered semen parameters. These results call for further investigation of the impact of ZIKV on the reproductive health of ZIKV-infected men and warn against the potential horizontal and vertical transmission of ZIKV through the infected germ line. Finally, the ex vivo model of ZIKV infection of the human testis we developed provides a valuable tool for the testing of antiviral agents.

## Methods

**Cells lines and viruses.** Asian Zika virus strains isolated during the 2015 outbreak in the French Caribbean (MRS\_OPY\_Martinique\_PaRi-2015, passaged once in Vero cells) and during the 2013 outbreak in Polynesia (H/PF/2013, passaged 3 times in Vero cells) were obtained from the European Virus Archive (EVA) and further propagated in VeroE6 cells for 2 additional passages. VeroE6 cells (African green monkey kidney epithelial cells) were maintained in DMEM supplemented with 10% FCS, glutamine (2 mM), and 1% penicillin/streptomycin at 37°C with 5% CO<sub>2</sub> (all reagents from Gibco). To produce viral stocks, VeroE6 cells were infected at an MOI of 0.01 in serum-free medium for 2 hours, and then complete medium was added to reach a final serum concentration of 5%. When cytopathic effect was evident, supernatant was centrifuged, filtered (0.45 μm), aliquoted, and frozen at -80°C. The human testicular germ cell tumor (seminoma) cell line Tcam-2 (66) was provided by Janet Shipley (Institute of Cancer Research, London, United Kingdom).

**Organotypic culture of human testis explants and infection.** Testes were dissected into 3-mm<sup>3</sup> sections transferred onto 24-well plates (2 sections/well) containing 500 μl medium (DMEM/F12 supplemented with 1× nonessential amino acids, 1× ITS (human insulin, human transferrin, and sodium selenite), 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, all from Gibco) in the presence or absence of 10<sup>5</sup> TCID<sub>50</sub> ZIKV (corresponding to 2.2 × 10<sup>7</sup> to 2.9 × 10<sup>7</sup> vRNA copies for MRS\_OPY\_Martinique\_PaRi-2015 and 8 × 10<sup>7</sup> vRNA copies for H/PF/2013). After overnight incubation, tissue fragments were washed 3 times with PBS and transferred onto a polyethylene tere-

phthalate insert (3  $\mu\text{m}$  high-density pores) in 12-well plates containing 1 ml medium. Eight hours later, the medium was changed again to further wash away potential residual virus input (time 0 for sample collection). For each experimental condition, a minimum of 2 wells were tested. The culture was maintained up to 9 days p.i. in a humidified atmosphere containing 5%  $\text{CO}_2$  at 37°C, with medium collected and fully changed every 3 days, in order to thoroughly wash input virus and assess viral production dynamic. Media were stored frozen at -80°C for vRNA and titer measurement. Tissue fragments were either fixed in neutral buffered 4% formaldehyde or frozen and stored at -80°C.

**Isolation and infection of testicular cells.** Testis fragments were incubated in digesting medium (2 mg/ml hyaluronidase, 2 mg/ml collagenase I, 20  $\mu\text{g}/\text{ml}$  in DMEM/F12) for 60 minutes at 37°C under agitation (110 rpm) to dissociate interstitial tissue from seminiferous tubules. After centrifugation, the seminiferous tubule pellet was digested by trypsin (0.25%, 5 ml/g, 20 minutes at 37°C). Trypsin was inactivated, and cells were filtered (60  $\mu\text{m}$ ) and cultured overnight in DMEM/F12 medium supplemented with 1 $\times$  nonessential amino acids, 1 $\times$  ITS, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 10% FCS (all from Gibco). Primary testicular germ cells (TGCs) and Tcam-2 cells were incubated with ZIKV diluted in serum-free medium at an MOI of 1 (corresponding to  $1.43 \times 10^6$  TCID<sub>50</sub>/million cells) for 2 hours at 37°C, 5%  $\text{CO}_2$ . Virus was removed by washing and trypsin treatment for 5 minutes at 37°C. Primary testicular cells were cultured at a density of 0.5 million cells/ml in supplemented StemPro-34 (Invitrogen) as described elsewhere (67). T-cam2 cells were cultured at a density of 0.1 million cells/ml in RPMI1640 supplemented with P/S, glutamine (2 mM), and 10% FCS (all reagents from Gibco).

**Semen samples.** Semen was liquified at 37°C for 30 minutes and 10  $\mu\text{l}$  spread on a glass slide and dried at room temperature. Smears were fixed in 4% formaldehyde and stored at -20°C. Viral loads for ZIKV in seminal plasma and seminal cells were 7.25 log copies/ml and 6.7 log copies/ $\mu\text{g}$  total RNA, respectively, for the donor on day 7, and 7.8 log copies/ml and 7.8 log copies/ $2 \times 10^6$  cells, respectively, for the donor on day 11. Patients' serology for dengue was negative, and semen samples tested negative for dengue by RT-PCR.

**Mice.** Mice lacking the type I IFN receptor (68) were backcrossed more than 10 times onto the C57BL/6 background (referred to as IFNAR<sup>-/-</sup> mice). Seven-week-old male IFNAR<sup>-/-</sup> mice were infected through the i.p. route with  $10^4$  TCID<sub>50</sub>/100  $\mu\text{l}$  of ZIKV (H/PF/2013) or with PBS. Five and 9 days after infection, mice were sacrificed with carbon dioxide, and collected tissue was either frozen at -80°C or fixed in PFA 4%.

**RT-qPCR.** Total RNA was extracted using QIAamp vRNA (for supernatants) or RNeasy isolation kit (for tissue/cells) and treated with DNase (all from QIAGEN). Extracted RNA from explant supernatants was subjected to RT-qPCR using the GoTaq Probe 1-Step RT-qPCR System (Promega). Primers and probes for ZIKV described previously (69) were adapted as follows: ZIKV primer forward CCGCTGCCAACACAAG, ZIKV primer reverse CCACTAACGTTCTTTTGCAGACAT, ZIKV probe AGCCTACCTTGACAAGCAATCAGACACTCAA. A standard curve with serial dilution of a known number of copies of vRNA was systematically run. The relative quantification of (a) steroidogenesis enzyme mRNA (STAR; CYP11A1, cytochrome P450 family 11 subfamily A member 1; HSD3B2, hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 2; HSD17B3, hydroxysteroid 17-beta dehydrogenase 3; CYP17A1, cytochrome P450

family 17 subfamily A member 1) and (b) testicular cell marker mRNA (inhibin B; Acta2, actin alpha 2 smooth muscle aorta; PGK2, phosphoglycerate kinase 2; PRM2, protamine) was performed as previously described (29).

Primers for innate immune response effector genes (Supplemental Table 1) were designed using the Primer-BLAST tool (70). Total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). RT-qPCR reactions were performed on a Bio-Rad CFX384 instrument using iTaq SYBR green mix (Bio-Rad) and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by melt-curve analysis. Gene expression fold changes were calculated with the  $2^{-\Delta\Delta Ct}$  method normalized to  $\beta$ -actin and mock-infected sample expression levels.

**Determination of viral titer.** VeroE6 cells seeded in opaque-walled 96-well plates at a final concentration of  $1.5 \times 10^5$  cells/ml in DMEM with 5% FCS were put in contact the next day with serial dilutions of supernatant. TCID<sub>50</sub>/ml was measured on day 5 after infection using the Viral ToxGlo Assay (Promega).

**Histology, RNAscope ISH, and IHC.** Tissues or cell pellets were fixed in 4% formaldehyde and embedded in paraffin. RNA ISH was performed using RNAscope 2.5 (Advanced Cell Diagnostics) according to the manufacturer's instructions, as previously described (71, 72). RNAscope ISH is a highly specific and sensitive technique, with the ability to detect single molecules (72). Formaldehyde-fixed, paraffin-embedded tissue sections or cell pellets were deparaffinized in xylene and dehydrated in ethanol for 10 minutes at room temperature. Slides deparaffinized and  $\text{H}_2\text{O}_2$  quenched for endogenous peroxidases were boiled in RNAscope Target Retrieval Reagents (citrate buffer 10 mM, pH 6, 15 minutes) and incubated in RNAscope Protease Plus (40°C, 20 minutes), prior to probe hybridization. Sections were incubated with target probes (2 hours, 40°C), washed in buffer, and incubated with amplification reagents. Chromogenic detection was performed using Fast Red as substrate for alkaline phosphatase to generate red signal. Slides were counterstained with hematoxylin and mounted in Eukitt (O. Kindler) before observation using bright-field microscopy. The "double Z" probes targeting ZIKV RNA (consensus sequence, target region 219-5,443, catalog 467771) and positive (targeting the 2514-3,433 region of human *POLR2A* gene, catalog 310451) and negative (targeting the 414-862 region of bacterial *dapB* gene, catalog 310043) control probes were all obtained from Advanced Cell Diagnostics. Staining specificity was verified as shown in Supplemental Figure 3. Sections of ZIKV-infected Vero cell pellets and mock-infected testis tissues were systematically used as positive and negative controls.

Dual fluorescence ISH-IHC experiments were performed essentially as we previously described (71). Briefly, tissue sections were first submitted to ISH, then blocked in PBS/BSA 2% and incubated overnight at 4°C with primary antibody. Sections were washed, incubated with either anti-mouse or anti-rabbit Alexa Fluor 488 fluorescent secondary antibodies diluted 1:500 (chicken anti-mouse Alex Fluor 488, product A-21200, and chicken anti-rabbit Alexa Fluor 488 product A-21441, Life Technologies), and counterstained with ProLong medium (Thermo Fisher Scientific) containing DAPI before observation with a Zeiss Axio Imager M1 fluorescence microscope connected to a digital camera using Zen software. Fluorescent Fast Red signal was read at 550 nm.

Single IHC was performed as described previously (73). For immunofluorescence experiments, Alexa Fluor 488- or 594-coupled

secondary antibodies diluted 1:500 (goat anti-mouse Alexa Fluor 594, product A-11032; chicken anti-rabbit Alexa Fluor 594, product A21442; donkey anti-rat Alexa Fluor 488, product A21208; all from Life Technologies) were used and sections mounted with ProLong DAPI to stain the nuclei. Cell staining was never observed for isotypic controls or mock-infected samples. Primary antibodies used were mouse anti-NS1 (BioFront Technologies, clone 0102136, 4 µg/ml), anti-CD68 (Dako, clone KP1, 1.85 µg/ml), anti-CD163 (Leica Novocastra, clone 10D6, 1:100), anti- $\alpha$ -SMA (Dako, clone 1A4, 1.4 µg/ml), anti-DDX4 (GeneTex, clone 2F9H5, 1:200), anti-ZO-1 (Thermo Fisher Scientific, clone ZO1-1A12, 10 µg/ml); rabbit anti-Cyp11A1 (Sigma-Aldrich, 1:250), anti-cleaved caspase-3 (Cell Signaling Technology, Asp175, 1:50), anti-DDX4 (Abcam, 2 µg/ml), anti-STAR (Cell Signaling Technology, 1:200), anti-CD3 (Dako, A0452, 10 µg/ml), rat anti-mouse F4/80 (Abcam, clone BM8, 1:20).

The number of ZIKV RNA<sup>+</sup> CD68/CD163<sup>+</sup> macrophages, CYP11A<sup>+</sup> Leydig cells,  $\alpha$ -SMA<sup>+</sup> peritubular cells, germ cells, and Sertoli cells (identified on morphological criteria in light microscopy) in testis explants was assessed in 4 donors and at least 3 whole testis tissue sections/donor (corresponding to about 12 mm<sup>2</sup>/testis donor). Quantification of CD3<sup>+</sup> cells in mouse testis was performed in 3 mock-infected animals, 4 animals on day 5 p.i., and 4 animals on day 9 p.i., in at least 5 mm<sup>2</sup>/testis. Slides were scanned with a NanoZoomer slide scanner (Hamamatsu Photonics, at Plateforme H2P2, Biosit). Immunostained positive cells were counted with NIH ImageJ software.

**Immunocytofluorescence.** Semen smears from donors and cell pellets from testicular cell cultures put onto polylysine-coated glass coverslips were fixed in 4% paraformaldehyde for 20 minutes at room temperature. After permeabilization (0.2% Triton X-100, 10 minutes), the slides were incubated in blocking buffer (0.2% Triton X-100, 1% goat serum, 2 hours) and stained with antibodies against ZIKV NS1 (1:1,000, BioFront Technologies) or flavivirus envelope Ab 4G2 (1:1,000, Millipore). NS1 antibody was either directly coupled to Alexa Fluor 647 (Zenon labeling kit, Molecular Probes) or revealed using Alexa Fluor 555 goat anti-mouse (Life Technologies). Infected cell characterization was performed using rabbit anti-DDX4 (5 µg/ml, Abcam, ab13840), anti-STRA8 (9.6 µg/ml, Thermo Fisher Scientific PA5-35047), and anti-FSHR (10 µg/ml, Origene, TA313897), detected using Alexa Fluor 488 (Invitrogen) or Alexa Fluor 647 goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc.); mouse anti-MAGEA-4 (clone 57B, 4 µg/ml, provided by Giulio Spagnoli, Ludwig Institute for Cancer Research, Basel, Switzerland) coupled to Alexa Fluor 647 (Zenon labeling kit, Molecular Probes) and mouse anti- $\alpha$ -SMA (clone 1A4, 0.5 µg/ml, Dako, M0851) detected using Alexa Fluor 555 goat-anti mouse antibody. Isotype control antibodies or noninfected cells were used as negative controls. Slides were counterstained with ProLong medium containing DAPI. Images were acquired with the SP8 confocal system (Leica) connected to LAS software or with a DMRXA wide-field microscope (Applied Precision) and analyzed using Fiji software.

**Testosterone and inhibin B immunoassays.** Testosterone was assayed using a specific radioimmunoassay (Immunotech, Beckman Coulter). Inhibin B was assayed using a commercial ELISA kit (DSL-10-84100 Active, Beckman Coulter).

**Viability assay.** Cell viability was assessed by measuring the release of LDH using the enzymatic fluorimetric assay CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega).

**Cytokine release measurement.** A bead-based multiplex flow cytometry LEGENDplex assay (BioLegend, Ozyme) was used. Fluorescence was read using the BD LSRII Fortessa flow cytometer.

**Statistics.** Data were analyzed with nonparametric paired Friedman-Dunn or unpaired Kruskal-Wallis-Dunn test when more than 2 sets of samples were compared, as specified in the figure legends. Nonparametric Mann-Whitney *U* test was used to analyze differences in viral load of mice testis on days 5 and 9 after infection. Correlations were calculated using Spearman's test. Values were considered significant when *P* was less than 0.05. Statistical analyses were performed using commercially available software (GraphPad Prism 6, GraphPad Software).

**Study approval.** Normal testes were obtained either after orchidectomy from prostate cancer patients who had not received any hormone therapy or at autopsy, and processed within 2 hours of surgery. The procedure was approved by Ethics Committee Ouest V, Rennes, France (authorization DC-2016-2783) and the French National Agency for Biomedical Research (authorization PF S09-015). Semen samples produced by masturbation were obtained from 2 ZIKV-infected donors living in the French Caribbean at 7 and 11 days after onset of symptoms, respectively, after written informed consent was obtained, in the French Cohort of Patients Infected by an Arbovirus (CARBO; ClinicalTrials.gov identifier NCT01099852). Mice were housed at the Institut Pasteur Animal Facility, accredited by the French Ministry of Agriculture for performing experiments on live rodents. Work on animals was performed in compliance with French and European regulations on the care and protection of laboratory animals (EC Directive 2010/63, French Law 2013-118, February 6, 2013). All experiments with IFNAR<sup>-/-</sup> mice were approved by the Ethics Committee #89 and registered under reference 2016-0018.

## Author contributions

GM performed experiments, analyzed data, cowrote the manuscript, and contributed to data interpretation and study design. LH, APS, DM, FA, and ALT performed experiments, analyzed data, and contributed to writing the manuscript. LH designed primers and interpreted PCR array results. JF and SB performed experiments. GA interpreted data. KB and SL contributed testis tissues. GJ, LB, and AC contributed semen samples. TC and ML performed IFNAR mouse infection and tissue collection. NDR designed experiments, interpreted the data, and wrote the manuscript. All authors read, edited, and approved the manuscript.

## Acknowledgments

This project received funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 733176, ZikaPLAN (grant agreement no. 734584), and ZIKAlliance (grant agreement 734548) and was funded in part by LabEx IBEID, Institut Pasteur, and Inserm. GM received support from REACTing. GA and NDR received funding from CAPES-COFECUB. GJ and LB received funding from Agence de la biomédecine and Agence régionale de santé Guadeloupe for the collection of semen from infected men. AC received funding from the French Ministry of Health (Soutien exceptionnel à la recherche et à l'innovation) and support from REACTing. Experiments were conducted in part on L3, MRic, and H2P2 platforms at Biosit federative structure (Université de Rennes, CNRS, Inserm, Biosit [Biologie, Santé, Innovation Technologique de Rennes] — UMS 3480, US\_S 018). This publication was supported by the European Virus Archive Goes Global (EVAg) project, which has

received funding from the European Union's Horizon 2020 research and innovation program under grant agreement 653316. We thank Xavier Montagutelli for help with animal experiments and Laurianne Lesné and Christèle Desdoits for assistance in hormonal assays.

Address correspondence to: Nathalie Dejuçq-Rainsford, IRSET-Inserm U1085, 9 avenue du Pr Léon Bernard, F-35000 Rennes, France. Phone: 33.2.2323.5069; Email: nathalie.dejuçq-rainsford@univ-rennes1.fr.

- Coelho FC, et al. Higher incidence of Zika in adult women than adult men in Rio de Janeiro suggests a significant contribution of sexual transmission from men to women. *Int J Infect Dis.* 2016;51:128–132.
- Yakob L, Kucharski A, Hue S, Edmunds WJ. Low risk of a sexually-transmitted Zika virus outbreak. *Lancet Infect Dis.* 2016;16(10):1100–1102.
- Moreira J, Peixoto TM, Siqueira AM, Lamas CC. Sexually acquired Zika virus: a systematic review. *Clin Microbiol Infect.* 2017;23(5):296–305.
- Duggal NK, et al. Frequent Zika virus sexual transmission and prolonged viral RNA shedding in an immunodeficient mouse model. *Cell Rep.* 2017;18(7):1751–1760.
- Haddow AD, et al. High Infection rates for adult macaques after intravaginal or intrarectal inoculation with Zika virus. *Emerging Infect Dis.* 2017;23(8):1274–1281.
- Carroll T, et al. Zika virus preferentially replicates in the female reproductive tract after vaginal inoculation of rhesus macaques. *PLoS Pathog.* 2017;13(7):e1006537.
- Tang WW, Young MP, Mamidi A, Regla-Nava JA, Kim K, Shrestha S. A mouse model of Zika virus sexual transmission and vaginal viral replication. *Cell Rep.* 2016;17(12):3091–3098.
- Duggal NK, McDonald EM, Ritter JM, Brault AC. Sexual transmission of Zika virus enhances in utero transmission in a mouse model. *Sci Rep.* 2018;8(1):4510.
- Epelboin S, Duloust E, Epelboin L, Benachi A, Merlet F, Patrat C. Zika virus and reproduction: facts, questions and current management. *Hum Reprod Update.* 2017;23(6):629–645.
- Barzon L, et al. Virus and antibody dynamics in travelers with acute Zika virus infection. *Clin Infect Dis.* 2018;66(8):1173–1180.
- Mead PS, et al. Zika virus shedding in semen of symptomatic infected men. *N Engl J Med.* 2018;378(15):1377–1385.
- Govero J, et al. Zika virus infection damages the testes in mice. *Nature.* 2016;540(7633):438–442.
- Ma W, et al. Zika virus causes testis damage and leads to male infertility in mice. *Cell.* 2016;167(6):1511–1524.e10.
- Griffin BD, et al. DNA vaccination protects mice against Zika virus-induced damage to the testes. *Nat Commun.* 2017;8:15743.
- Shan C, et al. A single-dose live-attenuated vaccine prevents Zika virus pregnancy transmission and testis damage. *Nat Commun.* 2017;8(1):676.
- Osuna CE, et al. Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nat Med.* 2016;22(12):1448–1455.
- Hirsch AJ, et al. Zika virus infection of rhesus macaques leads to viral persistence in multiple tissues. *PLoS Pathog.* 2017;13(3):e1006219.
- Koide F, et al. Development of a Zika virus infection model in cynomolgus macaques. *Front Microbiol.* 2016;7:2028.
- Joguet G, et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect Dis.* 2017;17(11):1200–1208.
- Roulet V, et al. Human testis in organotypic culture: application for basic or clinical research. *Hum Reprod.* 2006;21(6):1564–1575.
- Young JC, et al. TCam-2 seminoma cell line exhibits characteristic foetal germ cell responses to TGF- $\beta$  ligands and retinoic acid. *Int J Androl.* 2011;34(4 pt 2):e204–e217.
- Uraki R, et al. Zika virus causes testicular atrophy. *Sci Adv.* 2017;3(2):e1602899.
- Xia Y, Zhu WJ, Hao SF, Liang WB, Li J. Stereological analysis of age-related changes of testicular peritubular cells in men. *Arch Gerontol Geriatr.* 2012;55(1):116–119.
- Nieschlag E, Behre HM, eds. *Andrology.* Berlin, Germany: Springer; 2010.
- Robinson CL, et al. Male germ cells support long-term propagation of Zika virus. *Nat Commun.* 2018;9(1):2090.
- Mansuy JM, et al. Zika virus in semen and spermatozoa. *Lancet Infect Dis.* 2016;16(10):1106–1107.
- Siemann DN, Strange DP, Maharaj PN, Shi PY, Verma S. Zika virus infects human sertoli cells and modulates the integrity of the in vitro blood-testis barrier model. *J Virol.* 2017;91(22):e00623-17.
- Kumar A, et al. Human Sertoli cells support high levels of Zika virus replication and persistence. *Sci Rep.* 2018;8(1):5477.
- Kristensen DM, et al. Ibuprofen alters human testicular physiology to produce a state of compensated hypogonadism. *Proc Natl Acad Sci U S A.* 2018;115(4):E715–E724.
- Adamopoulos DA, Lawrence DM, Vassilopoulos P, Contoyiannis PA, Swyer GI. Pituitary-testicular interrelationships in mumps orchitis and other viral infections. *Br Med J.* 1978;1(6121):1177–1180.
- Potter SJ, DeFalco T. Role of the testis interstitial compartment in spermatogonial stem cell function. *Reproduction.* 2017;153(4):R151–R162.
- Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the micro-environment of the seminiferous epithelium: new insights and advances. *Mol Cell Endocrinol.* 2010;315(1–2):49–56.
- Mayerhofer A. Human testicular peritubular cells: more than meets the eye. *Reproduction.* 2013;145(5):R107–R116.
- Huits R, De Smet B, Ariën KK, Van Esbroeck M, Bottieau E, Cnops L. Zika virus in semen: a prospective cohort study of symptomatic travellers returning to Belgium. *Bull World Health Organ.* 2017;95(12):802–809.
- Sergerie M, Mieuxset R, Croute F, Daudin M, Bujan L. High risk of temporary alteration of semen parameters after recent acute febrile illness. *Fertil Steril.* 2007;88(4):970.e1–970.e7.
- Evenson DP, Jost LK, Corzett M, Balhorn R. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J Androl.* 2000;21(5):739–746.
- Quicke KM, et al. Zika virus infects human placental macrophages. *Cell Host Microbe.* 2016;20(1):83–90.
- Mladinich MC, Schwedes J, Mackow ER. Zika virus persistently infects and is basolaterally released from primary human brain microvascular endothelial cells. *MBio.* 2017;8(4):e00952-17.
- Strange DP, Green R, Siemann DN, Gale M, Verma S. Immunoprofiles of human Sertoli cells infected with Zika virus reveals unique insights into host-pathogen crosstalk. *Sci Rep.* 2018;8(1):8702.
- Strange DP, et al. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. *Emerg Microbes Infect.* 2018;7(1):82.
- Singh PK, et al. Zika virus infects cells lining the blood-retinal barrier and causes chorioretinal atrophy in mouse eyes. *JCI Insight.* 2017;2(4):e92340.
- Van der Hoek KH, et al. Viperin is an important host restriction factor in control of Zika virus infection. *Sci Rep.* 2017;7(1):4475.
- Savidis G, et al. The IFITMs inhibit Zika virus replication. *Cell Rep.* 2016;15(11):2323–2330.
- Perelygin AA, Scherik SV, Zhulin IB, Stockman BM, Li Y, Brinton MA. Positional cloning of the murine flavivirus resistance gene. *Proc Natl Acad Sci U S A.* 2002;99(14):9322–9327.
- Chen J, et al. Outcomes of congenital Zika disease depend on timing of infection and maternal-fetal interferon action. *Cell Rep.* 2017;21(6):1588–1599.
- Schoggins JW, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature.* 2011;472(7344):481–485.
- Ngono AE, Shrestha S. Immune response to dengue and Zika. *Annu Rev Immunol.* 2018;36:279–308.
- Xia H, et al. An evolutionary NS1 mutation enhances Zika virus evasion of host interferon induction. *Nat Commun.* 2018;9(1):414.
- Satie AP, et al. Excess type I interferon signaling in the mouse seminiferous tubules leads to germ cell loss and sterility. *J Biol Chem.* 2011;286(26):23280–23295.
- Hasan M, et al. Trex1 regulates lysosomal biogenesis and interferon-independent activation of antiviral genes. *Nat Immunol.* 2013;14(1):61–71.
- Sheng ZY, et al. Sertoli cells are susceptible to ZIKV infection in mouse testis. *Front Cell Infect Microbiol.* 2017;7:272.
- Chan JFW, et al. Zika virus infection in dexamethasone-immunosuppressed mice demonstrating disseminated infection with multi-organ involvement including orchitis effectively treated by recombinant type I interferons. *EBioMedicine.* 2016;14:112–122.
- Winkler CW, et al. Adaptive immune responses to Zika virus are important for controlling virus infection and preventing infection in brain and testes. *J Immunol.* 2017;198(9):3526–3535.
- Dejuçq N, Lienard MO, Guillaume E, Dorval I, Jégou B. Expression of interferons- $\alpha$  and - $\gamma$  in testicular interstitial tissue and spermatogonia of

- the rat. *Endocrinology*. 1998;139(7):3081-3087.
55. Le Tortorec A, et al. Antiviral responses of human Leydig cells to mumps virus infection or poly I:C stimulation. *Hum Reprod*. 2008;23(9):2095-2103.
  56. Dejuqcq N, Liénard MO, Jégou B. Interferons and interferon-induced antiviral proteins in the testis. *J Reprod Immunol*. 1998;41(1-2):291-300.
  57. Dejuqcq N, Chousterman S, Jégou B. The testicular antiviral defense system: localization, expression, and regulation of 2'5' oligoadenylate synthetase, double-stranded RNA-activated protein kinase, and Mx proteins in the rat seminiferous tubule. *J Cell Biol*. 1997;139(4):865-873.
  58. Lalle E, et al. Prolonged detection of dengue virus RNA in the semen of a man returning from Thailand to Italy, January 2018. *Euro Surveill*. 2018;23(18):18-00197.
  59. Barbosa CM, et al. Yellow fever virus RNA in urine and semen of convalescent patient, Brazil. *Emerging Infect Dis*. 2018;24(1):176-178.
  60. Bandeira AC, et al. Prolonged shedding of Chikungunya virus in semen and urine: a new perspective for diagnosis and implications for transmission. *IDCases*. 2016;6:100-103.
  61. Armah HB, et al. Systemic distribution of West Nile virus infection: postmortem immunohistochemical study of six cases. *Brain Pathol*. 2007;17(4):354-362.
  62. Guérin B, Pozzi N. Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. *Theriogenology*. 2005;63(2):556-572.
  63. Stassen L, Armitage CW, van der Heide DJ, Beagley KW, Frentiu FD. Zika virus in the male reproductive tract. *Viruses*. 2018;10(4):E198.
  64. Spencer JL, et al. Replication of Zika virus in human prostate cells: a potential source of sexually transmitted virus. *J Infect Dis*. 2018;217(4):538-547.
  65. Houzet L, et al. Seminal simian immunodeficiency virus in chronically infected cynomolgus macaques is dominated by virus originating from multiple genital organs. *J Virol*. 2018;92(14):e00133-18.
  66. Mizuno Y, Gotoh A, Kamidono S, Kitazawa S. [Establishment and characterization of a new human testicular germ cell tumor cell line (TCam-2)]. *Nihon Hinyokika Gakkai Zasshi*. 1993;84(7):1211-1218.
  67. Sadri-Ardekani H, et al. Propagation of human spermatogonial stem cells in vitro. *JAMA*. 2009;302(19):2127-2134.
  68. Müller U, et al. Functional role of type I and type II interferons in antiviral defense. *Science*. 1994;264(5167):1918-1921.
  69. Lanciotti RS, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerging Infect Dis*. 2008;14(8):1232-1239.
  70. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 2012;13:134.
  71. Matusali G, et al. Detection of simian immunodeficiency virus in semen, urethra, and male reproductive organs during efficient highly active antiretroviral therapy. *J Virol*. 2015;89(11):5772-5787.
  72. Deleage C, Chan CN, Busman-Sahay K, Estes JD. Next-generation in situ hybridization approaches to define and quantify HIV and SIV reservoirs in tissue microenvironments. *Retrovirology*. 2018;15(1):4.
  73. Roulet V, et al. Susceptibility of human testis to human immunodeficiency virus-1 infection in situ and in vitro. *Am J Pathol*. 2006;169(6):2094-2103.

# References

1. Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis.* 1995;1(1):7-15. doi:10.3201/EID0101.950102
2. Heymann DL. Emerging and other infectious diseases : epidemiology and control. *World Health Stat Q 1997 5034 158-160.* Published online 1997. Accessed May 4, 2022. <https://apps.who.int/iris/handle/10665/55165>
3. Woolhouse MEJ, Gowtage-Sequeria S. Host range and emerging and reemerging pathogens. *Emerg Infect Dis.* 2005;11(12):1842-1847. doi:10.3201/EID1112.050997
4. WHO. A brief guide to emerging infectious diseases and zoonoses. *Online.* Published online 2014.
5. Jones KE, Patel NG, Levy MA, et al. Global trends in emerging infectious diseases. *Nature.* 2008;451(7181):990-993. doi:10.1038/NATURE06536
6. Woolhouse MEJ, Adair K, Brierley L. RNA viruses: a case study of the biology of emerging infectious diseases. *Microbiol Spectr.* 2013;1(1):10.1128/microbiolspec.OH-0001-2012. doi:10.1128/microbiolspec.OH-0001-2012
7. Mehand MS, Al-Shorbaji F, Millett P, Murgue B. The WHO R&D Blueprint: 2018 review of emerging infectious diseases requiring urgent research and development efforts. *Antiviral Res.* 2018;159:63-67. doi:10.1016/j.antiviral.2018.09.009
8. Meganck RM, Baric RS. Developing therapeutic approaches for twenty-first-century emerging infectious viral diseases. *Nat Med.* 2021;27(3):401-410. doi:10.1038/S41591-021-01282-0
9. Baker RE, Mahmud AS, Miller IF, et al. Infectious disease in an era of global change. *Nat Rev Microbiol.* 2022;20(4):193-205. doi:10.1038/S41579-021-00639-Z
10. Domingo E. Mechanisms of viral emergence. *Vet Res.* 2010;41(6). doi:10.1051/VETRES/2010010
11. Antonovics J, Wilson AJ, Forbes MR, et al. The evolution of transmission mode. *Philos Trans R Soc Lond B Biol Sci.* 2017;372(1719). doi:10.1098/RSTB.2016.0083
12. Arora N, Sadovsky Y, Dermody TS, Coyne CB. Microbial Vertical Transmission during Human Pregnancy. *Cell Host Microbe.* 2017;21(5):561-567. doi:10.1016/J.CHOM.2017.04.007
13. Prober CG, Arvin AM. Perinatal viral infections. *Eur J Clin Microbiol.* 1987;6(3):245-261. doi:10.1007/BF02017608
14. CDC. Ebola Virus Disease | Breastfeeding. *Online.* Published online 2021.



15. Lawrence RM. Transmission of Infectious Diseases Through Breast Milk and Breastfeeding. *Breastfeeding*. Published online 2011:406. doi:10.1016/B978-1-4377-0788-5.10013-6
16. Le Tortorec A, Matusali G, Mahé D, et al. From Ancient to Emerging Infections: The Odyssey of Viruses in the Male Genital Tract. *Physiol Rev*. 2020;100(3):1349-1414. doi:10.1152/PHYSREV.00021.2019
17. Saini R, Saini S, Sharma S. Oral Sex, Oral Health and Orogenital Infections. *J Glob Infect Dis*. 2010;2(1):57. doi:10.4103/0974-777X.59252
18. Wang CC, Prather KA, Sznitman J, et al. Airborne transmission of respiratory viruses. *Science*. 2021;373(6558). doi:10.1126/SCIENCE.ABD9149
19. Pandey A, Atkins KE, Medlock J, et al. Strategies for containing Ebola in West Africa. *Science*. 2014;346(6212):991. doi:10.1126/SCIENCE.1260612
20. Yeroushalmi S, Shirazi JY, Friedman A. New Developments in Bacterial, Viral, and Fungal Cutaneous Infections. *Curr Dermatol Rep*. 2020;9(2):152. doi:10.1007/S13671-020-00295-1
21. Hu Q, He L, Zhang Y. Community Transmission via Indirect Media-To-Person Route: A Missing Link in the Rapid Spread of COVID-19. *Front Public Health*. 2021;9. doi:10.3389/FPUBH.2021.687937
22. Luby SP, Rahman M, Hossain MJ, et al. Foodborne transmission of Nipah virus, Bangladesh. *Emerg Infect Dis*. 2006;12(12):1888-1894. doi:10.3201/EID1212.060732
23. Nikolay B, Salje H, Hossain MJ, et al. Transmission of Nipah Virus - 14 Years of Investigations in Bangladesh. *N Engl J Med*. 2019;380(19):1804-1814. doi:10.1056/NEJMOA1805376
24. Petrović T, D'Agostino M. Viral Contamination of Food. *Antimicrob Food Packag*. Published online 2016:65. doi:10.1016/B978-0-12-800723-5.00005-X
25. Gerba CP. Environmentally Transmitted Pathogens. *Environ Microbiol*. Published online 2009:445-484. doi:10.1016/B978-0-12-370519-8.00022-5
26. Leung NHL. Transmissibility and transmission of respiratory viruses. *Nat Rev Microbiol*. 2021;19(8):528. doi:10.1038/S41579-021-00535-6
27. Rewar S, Mirdha D. Transmission of ebola virus disease: an overview. *Ann Glob Health*. 2014;80(6):444-451. doi:10.1016/J.AOGH.2015.02.005
28. Simonsen L, Kane A, Lloyd J, Zaffran M, Kane M. Unsafe injections in the developing world and transmission of bloodborne pathogens: a review. *Bull World Health Organ*. 1999;77(10):789.
29. Zule WA, Bobashev G. High Dead-Space Syringes and the Risk of HIV and HCV Infection among Injecting Drug Users. *Drug Alcohol Depend*. 2009;100(3):204. doi:10.1016/J.DRUGALCDEP.2008.08.017
30. Weaver SC, Reisen WK. Present and future arboviral threats. *Antiviral Res*. 2010;85(2):328-345. doi:10.1016/J.ANTIVIRAL.2009.10.008

31. Halpin K, Rota P. A Review of Hendra Virus and Nipah Virus Infections in Man and Other Animals. *Zoonoses - Infect Affect Hum Anim*. Published online 2015:997. doi:10.1007/978-94-017-9457-2\_40
32. Jonsson CB, Figueiredo LTM, Vapalahti O. A Global Perspective on Hantavirus Ecology, Epidemiology, and Disease. *Clin Microbiol Rev*. 2010;23(2):412. doi:10.1128/CMR.00062-09
33. Carrasco-Hernandez R, Jácome R, Vidal YL, de León SP. Are RNA Viruses Candidate Agents for the Next Global Pandemic? A Review. *ILAR J*. 2017;58(3):343-358. doi:10.1093/ILAR/ILX026
34. Woolhouse MEJ, Brierley L. Epidemiological characteristics of human-infective RNA viruses. *Sci Data*. 2018;5:180017. doi:10.1038/sdata.2018.17
35. Giefing-Kröll C, Berger P, Lepperdinger G, Grubeck-Loebenstein B. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging Cell*. 2015;14(3):309-321. doi:10.1111/ACEL.12326
36. Plowright RK, Parrish CR, McCallum H, et al. Pathways to zoonotic spillover. *Nat Rev Microbiol*. 2017;15(8):502-510. doi:10.1038/NRMICRO.2017.45
37. Kamal M, Kenawy MA, Rady MH, Khaled AS, Samy AM. Mapping the global potential distributions of two arboviral vectors *Aedes aegypti* and *Ae. albopictus* under changing climate. *PloS One*. 2018;13(12). doi:10.1371/JOURNAL.PONE.0210122
38. Klempa B. Hantaviruses and climate change. *Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis*. 2009;15(6):518-523. doi:10.1111/J.1469-0691.2009.02848.X
39. Carlson CJ, Albery GF, Merow C, et al. Climate change increases cross-species viral transmission risk. *Nature*. Published online April 28, 2022:1-1. doi:10.1038/s41586-022-04788-w
40. Les Echos. Le réchauffement climatique va multiplier les pandémies. Les Echos. Published May 2, 2022. Accessed May 8, 2022. <https://www.lesechos.fr/monde/enjeux-internationaux/avec-le-rechauffement-climatique-le-monde-face-a-lere-des-pandemies-1404206>
41. Herrero L. for The Conversation L. Is a hybrid COVID strain behind Vietnam's latest wave? Not exactly. The Conversation. Accessed May 4, 2022. <http://theconversation.com/is-a-hybrid-covid-strain-behind-vietnams-latest-wave-not-exactly-161879>
42. Lam TTY, Hon CC, Tang JW. Use of phylogenetics in the molecular epidemiology and evolutionary studies of viral infections. *Crit Rev Clin Lab Sci*. 2010;47(1):5-49. doi:10.3109/10408361003633318
43. Webby R, Hoffmann E, Webster R. Molecular constraints to interspecies transmission of viral pathogens. *Nat Med*. 2004;10(12 Suppl):S77-S81. doi:10.1038/NM1151
44. Sanjuán R, Domingo-Calap P. Mechanisms of viral mutation. *Cell Mol Life Sci CMLS*. 2016;73(23):4433-4448. doi:10.1007/S00018-016-2299-6

45. Smith GJD, Vijaykrishna D, Bahl J, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*. 2009;459(7250):1122-1125. doi:10.1038/NATURE08182
46. Tharmalingam MD, Jorgensen A, Mitchell RT. Experimental models of testicular development and function using human tissue and cells. *Mol Cell Endocrinol*. 2018;468:95-110. doi:10.1016/J.MCE.2017.12.011
47. Clement P, Giuliano F. Anatomy and physiology of genital organs – men. 2015;130:19-37. doi:10.1016/B978-0-444-63247-0.00003-1
48. Mattoon JS, Davidson A. Prostate and testes. *Small Anim Diagn Ultrasound*. Published online 2021:635-664. doi:10.1016/B978-0-323-53337-9.00026-5
49. Mäkelä JA, Toppari J. Spermatogenesis. Published online 2017:417-455. doi:10.1007/978-3-319-44441-3\_13
50. Loveland KL, Klein B, Poeschl D, et al. Cytokines in Male Fertility and Reproductive Pathologies: Immunoregulation and Beyond. *Front Endocrinol*. 2017;8. doi:10.3389/fendo.2017.00307
51. Shalet SM. Normal testicular function and spermatogenesis. *Pediatr Blood Cancer*. 2009;53(2):285-288. doi:10.1002/PBC.22000
52. De Kretser DM, Loveland KL, Meehan T, O'Bryan MK, Phillips DJ, Wreford NG. Inhibins, activins and follistatin: actions on the testis. *Mol Cell Endocrinol*. 2001;180(1-2):87-92. doi:10.1016/S0303-7207(01)00502-0
53. Hedger MP, Winnall WR. Regulation of activin and inhibin in the adult testis and the evidence for functional roles in spermatogenesis and immunoregulation. *Mol Cell Endocrinol*. 2012;359(1-2):30-42. doi:10.1016/J.MCE.2011.09.031
54. Austin Community College. Hormonal Regulation of Spermiogenesis. *Online*.
55. Heinrich A, DeFalco T. Essential roles of interstitial cells in testicular development and function. *Andrology*. 2020;8(4):903-914. doi:10.1111/ANDR.12703
56. Chakraborty S, Pramanik J, Mahata B. Revisiting steroidogenesis and its role in immune regulation with the advanced tools and technologies. *Genes Immun*. 2021;22(3):125-140. doi:10.1038/s41435-021-00139-3
57. Zirkin BR, Papadopoulos V. Leydig cells: formation, function, and regulation. *Biol Reprod*. 2018;99(1):101-111. doi:10.1093/BIOLRE/IOY059
58. Zhou R, Wu J, Liu B, et al. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. *Cell Mol Life Sci CMLS*. 2019;76(14):2681-2695. doi:10.1007/S00018-019-03101-9
59. Sierens JE, Sneddon SF, Collins F, Millar MR, Saunders PTK. Estrogens in testis biology. *Ann N Y Acad Sci*. 2005;1061:65-76. doi:10.1196/ANNALS.1336.008

60. Shang Y, Wang H, Jia P, et al. Autophagy regulates spermatid differentiation via degradation of PDLIM1. *Autophagy*. 2016;12(9):1575-1592. doi:10.1080/15548627.2016.1192750
61. Meinhardt A, Dejuqc-Rainsford N, Bhushan S. Testicular macrophages: development and function in health and disease. *Trends Immunol*. 2022;43(1):51-62. doi:10.1016/J.IT.2021.11.003
62. Bhushan S, Theas MS, Guazzone VA, et al. Immune Cell Subtypes and Their Function in the Testis. *Front Immunol*. 2020;11. doi:10.3389/FIMMU.2020.583304
63. Mossadegh-Keller N, Sieweke MH. Testicular macrophages: Guardians of fertility. *Cell Immunol*. 2018;330:120-125. doi:10.1016/J.CELLIMM.2018.03.009
64. Mossadegh-Keller N, Gentek R, Gimenez G, Bigot S, Mailfert S, Sieweke MH. Developmental origin and maintenance of distinct testicular macrophage populations. *J Exp Med*. 2017;214(10):2829-2841. doi:10.1084/jem.20170829
65. Lokka E, Lintukorpi L, Cisneros-Montalvo S, et al. Generation, localization and functions of macrophages during the development of testis. *Nat Commun*. 2020;11(1):4375. doi:10.1038/s41467-020-18206-0
66. Wang M, Yang Y, Cansever D, et al. Two populations of self-maintaining monocyte-independent macrophages exist in adult epididymis and testis. *Proc Natl Acad Sci U S A*. 2021;118(1):e2013686117. doi:10.1073/pnas.2013686117
67. Gong J, Zeng Q, Yu D, Duan YG. T Lymphocytes and Testicular Immunity: A New Insight into Immune Regulation in Testes. *Int J Mol Sci*. 2020;22(1):E57. doi:10.3390/ijms22010057
68. Liu Y, Liang Y, Wang S, et al. Identification and characterization of telocytes in rat testis. *Aging*. 2019;11(15):5757-5768. doi:10.18632/aging.102158
69. Marini M, Rosa I, Guasti D, et al. Reappraising the microscopic anatomy of human testis: identification of telocyte networks in the peritubular and intertubular stromal space. *Sci Rep*. 2018;8(1):14780. doi:10.1038/s41598-018-33126-2
70. Rosa I, Marini M, Sgambati E, Ibba-Manneschi L, Manetti M. Telocytes and lymphatic endothelial cells: Two immunophenotypically distinct and spatially close cell entities. *Acta Histochem*. 2020;122(3). doi:10.1016/J.ACTHIS.2020.151530
71. Maekawa M, Kamimura K, Nagano T. Peritubular myoid cells in the testis: their structure and function. *Arch Histol Cytol*. 1996;59(1):1-13. doi:10.1679/aohc.59.1
72. Mayerhofer A. Peritubular cells of the human testis: prostaglandin E 2 and more. *Andrology*. 2020;8(4):898-902. doi:10.1111/ANDR.12669
73. Sawaied A, Arazi E, AbuElhija A, Lunenfeld E, Huleihel M. The Presence of Colony-Stimulating Factor-1 and Its Receptor in Different Cells of the Testis; It Involved in the Development of Spermatogenesis In Vitro. *Int J Mol Sci*. 2021;22(5):2325. doi:10.3390/ijms22052325

74. Griswold MD. 50 years of spermatogenesis: Sertoli cells and their interactions with germ cells. *Biol Reprod.* 2018;99(1):87-100. doi:10.1093/BIOLRE/IOY027
75. Kerr JB. Functional cytology of the human testis. *Baillieres Clin Endocrinol Metab.* 1992;6(2):235-250. doi:10.1016/S0950-351X(05)80149-1
76. Encyclopædia Britannica. Spermatogenesis. *Online.* Published online 2013.
77. Sharma R, Agarwal A. Spermatogenesis: An Overview. In: Zini A, Agarwal A, eds. *Sperm Chromatin.* Springer New York; 2011:19-44. doi:10.1007/978-1-4419-6857-9\_2
78. Austin Community College. Spermiogenesis. *Online.*
79. França LR, Hess RA, Dufour JM, Hofmann MC, Griswold MD. The Sertoli cell: one hundred fifty years of beauty and plasticity. *Andrology.* 2016;4(2):189-212. doi:10.1111/ANDR.12165
80. Mital P, Hinton BT, Dufour JM. The blood-testis and blood-epididymis barriers are more than just their tight junctions. *Biol Reprod.* 2011;84(5):851-858. doi:10.1095/biolreprod.110.087452
81. Mruk DD, Cheng CY. The Mammalian Blood-Testis Barrier: Its Biology and Regulation. *Endocr Rev.* 2015;36(5):564-591. doi:10.1210/ER.2014-1101
82. Su L, Mruk DD, Cheng CY. Drug transporters, the blood-testis barrier, and spermatogenesis. *J Endocrinol.* 2011;208(3):207-223. doi:10.1677/JOE-10-0363
83. Mikuz G, Damjanov I. Inflammation of the testis, epididymis, peritesticular membranes, and scrotum. *Pathol Annu.* 1982;17 (Pt 1):101-128.
84. Dadoune, J and Demoulin, A. Structure et fonction du testicule. In: *Le Reproduction Chez Les Mammifères et l'homme.* ; 2001:256-289.
85. Bart J, Hollema H, Groen HJM, et al. The distribution of drug-efflux pumps, P-gp, BCRP, MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours. *Eur J Cancer Oxf Engl 1990.* 2004;40(14):2064-2070. doi:10.1016/J.EJCA.2004.05.010
86. Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem Off J Histochem Soc.* 1990;38(9):1277-1287. doi:10.1177/38.9.1974900
87. Melaine N, Liénard MO, Dorval I, Le Goascogne C, Lejeune H, Jégou B. Multidrug resistance genes and p-glycoprotein in the testis of the rat, mouse, Guinea pig, and human. *Biol Reprod.* 2002;67(6):1699-1707. doi:10.1095/BIOLREPROD.102.003558
88. Wijnholds J, Scheffer GL, Van Der Valk M, et al. Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. *J Exp Med.* 1998;188(5):797-808. doi:10.1084/JEM.188.5.797

89. Stewart PA, Béliveau R, Rogers KA. Cellular localization of P-glycoprotein in brain versus gonadal capillaries. *J Histochem Cytochem Off J Histochem Soc.* 1996;44(7):679-685. doi:10.1177/44.7.8675989
90. Chan DJ, Ray JE. Quantification of antiretroviral drugs for HIV-1 in the male genital tract: current data, limitations and implications for laboratory analysis. *J Pharm Pharmacol.* 2007;59(11):1451-1462. doi:10.1211/JPP.59.11.0001
91. Rodríguez-Martínez H, Kvist U, Ernerudh J, Sanz L, Calvete JJ. Seminal plasma proteins: what role do they play? *Am J Reprod Immunol N Y N* 1989. 2011;66 Suppl 1(SUPPL. 1):11-22. doi:10.1111/J.1600-0897.2011.01033.X
92. Fedder J. Nonsperm cells in human semen: with special reference to seminal leukocytes and their possible influence on fertility. *Arch Androl.* 1996;36(1):41-65. doi:10.3109/01485019608987883
93. Smith DC, Barratt CLR, Williams MA. The Characterisation of Non-Sperm Cells in the Ejaculates of Fertile Men Using Transmission Electron Microscopy/Charakterisierung von nicht-Spermatozoen-Zellen in den Ejakulaten von fruchtbaren Männern unter Verwendung der Transmissions-Elektronen-Mikroskopie. *Andrologia.* 1989;21(4):319-333. doi:10.1111/J.1439-0272.1989.TB02417.X
94. Johansson E, Campana A, Luthi R, De Agostini A. Evaluation of "round cells" in semen analysis: a comparative study. *Hum Reprod Update.* 2000;6(4):404-412. doi:10.1093/HUMUPD/6.4.404
95. Wolff H. The biologic significance of white blood cells in semen. *Fertil Steril.* 1995;63(6):1143-1157. doi:10.1016/s0015-0282(16)57588-8
96. Long S, Kenworthy S. Round Cells in Diagnostic Semen Analysis: A Guide for Laboratories and Clinicians. *Br J Biomed Sci.* 2022;0. doi:10.3389/bjbs.2021.10129
97. Politch JA, Tucker L, Bowman FP, Anderson DJ. Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men. *Hum Reprod Oxf Engl.* 2007;22(11):2928-2935. doi:10.1093/humrep/dem281
98. Lackner JE, Agarwal A, Mahfouz R, du Plessis SS, Schatzl G. The association between leukocytes and sperm quality is concentration dependent. *Reprod Biol Endocrinol RBE.* 2010;8:12. doi:10.1186/1477-7827-8-12
99. Paranjpe S, Craig J, Patterson B, et al. Subcompartmentalization of HIV-1 quasispecies between seminal cells and seminal plasma indicates their origin in distinct genital tissues. *AIDS Res Hum Retroviruses.* 2002;18(17):1271-1280. doi:10.1089/088922202320886316
100. Anderson DJ, Le Grand R. Cell-Associated HIV Mucosal Transmission: The Neglected Pathway. *J Infect Dis.* 2014;210(Suppl 3):S606-S608. doi:10.1093/infdis/jiu538
101. Houzet L, Matusali G, Dejuq-Rainsford N. Origins of HIV-infected leukocytes and virions in semen. *J Infect Dis.* 2014;210 Suppl 3:S622-630. doi:10.1093/infdis/jiu328

102. Houzet L, Pérez-Losada M, Matusali G, et al. Seminal Simian Immunodeficiency Virus in Chronically Infected Cynomolgus Macaques Is Dominated by Virus Originating from Multiple Genital Organs. *J Virol*. 2018;92(14):e00133-18. doi:10.1128/JVI.00133-18
103. Rolland AD, Lavigne R, Daully C, et al. Identification of genital tract markers in the human seminal plasma using an integrative genomics approach. *Hum Reprod Oxf Engl*. 2013;28(1):199-209. doi:10.1093/HUMREP/DES360
104. Poiani A. Complexity of seminal fluid: a review. *Behav Ecol Sociobiol 2006 603*. 2006;60(3):289-310. doi:10.1007/S00265-006-0178-0
105. WHO. Sexually transmitted infections (STIs). *Online*. Published online 2021.
106. WHO. Global progress report on HIV, viral hepatitis and sexually transmitted infections, 2021. *Online*. Published online 2021:1-108.
107. Brookings C, Goldmeier D, Sadeghi-Nejad H. Sexually transmitted infections and sexual function in relation to male fertility. *Korean J Urol*. 2013;54(3):149-156. doi:10.4111/KJU.2013.54.3.149
108. Kuenzli AB, Marschall J, Schefold JC, et al. Hantavirus Cardiopulmonary Syndrome Due to Imported Andes Hantavirus Infection in Switzerland: A Multidisciplinary Challenge, Two Cases and a Literature Review. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2018;67(11):1788-1795. doi:10.1093/cid/ciy443
109. Haneche F, Leparç-Goffart I, Simon F, et al. Rift Valley fever in kidney transplant recipient returning from Mali with viral RNA detected in semen up to four months from symptom onset, France, autumn 2015. *Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull*. 2016;21(18). doi:10.2807/1560-7917.ES.2016.21.18.30222
110. Grobusch MP, van der Fluit KS, Stijnis C, et al. Can dengue virus be sexually transmitted? *Travel Med Infect Dis*. 2020;38. doi:10.1016/J.TMAID.2020.101753
111. Teixeira T, Oliveira Y, Bernardes F, et al. Viral infections and implications for male reproductive health. *Asian J Androl*. 2021;23(4):335-347. doi:10.4103/AJA.AJA\_82\_20
112. Gerendai I, Tóth IE, Kocsis K, Boldogkői Z, Rusvai M, Halász B. Identification of CNS neurons involved in the innervation of the epididymis: a viral transneuronal tracing study. *Auton Neurosci*. 2001;92(1):1-10. doi:10.1016/S1566-0702(01)00292-2
113. Le Goffic R, Mouchel T, Ruffault A, Patard JJ, Jégou B, Samson M. Mumps virus decreases testosterone production and gamma interferon-induced protein 10 secretion by human leydig cells. *J Virol*. 2003;77(5):3297-3300. doi:10.1128/jvi.77.5.3297-3300.2003
114. Ma W, Li S, Ma S, et al. Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice. *Cell*. 2016;167(6):1511-1524.e10. doi:10.1016/j.cell.2016.11.016
115. Li H, Xiao X, Zhang J, et al. Impaired spermatogenesis in COVID-19 patients. *EClinicalMedicine*. 2020;28:100604. doi:10.1016/j.eclinm.2020.100604

116. Enikeev D, Taratkin M, Morozov A, et al. Prospective two-arm study of the testicular function in patients with COVID-19. *Andrology*. Published online February 6, 2022. doi:10.1111/andr.13159
117. Schroeder M, Schaumburg B, Mueller Z, et al. High estradiol and low testosterone levels are associated with critical illness in male but not in female COVID-19 patients: a retrospective cohort study. *Emerg Microbes Infect.* 2021;10(1):1807-1818. doi:10.1080/22221751.2021.1969869
118. Mieusset R, Bujan L. Testicular heating and its possible contributions to male infertility: a review. *Int J Androl.* 1995;18(4):169-184. doi:10.1111/j.1365-2605.1995.tb00408.x
119. Sergerie M, Mieusset R, Croute F, Daudin M, Bujan L. High risk of temporary alteration of semen parameters after recent acute febrile illness. *Fertil Steril.* 2007;88(4):970.e1-7. doi:10.1016/j.fertnstert.2006.12.045
120. Abdelhamid MHM, Fella AA, Elmarghani A, Al Msellati IA. An Assessment of Men Semen Alterations in SARS-CoV-2: Is Fever the Principal Concern? *Reprod Sci Thousand Oaks Calif.* Published online February 22, 2022. doi:10.1007/s43032-022-00889-z
121. Schaefer S, Boegershausen N, Meyer S, Ivan D, Schepelmann K, Kann PH. Hypothalamic-pituitary insufficiency following infectious diseases of the central nervous system. *Eur J Endocrinol.* 2008;158(1):3-9. doi:10.1530/EJE-07-0484
122. Mäkelä S, Jaatinen P, Miettinen M, et al. Hormonal deficiencies during and after Puumala hantavirus infection. *Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol.* 2010;29(6):705-713. doi:10.1007/s10096-010-0918-y
123. Apaydin T, Sahin B, Dashdamirova S, et al. The association of free testosterone levels with coronavirus disease 2019. *Andrology*. Published online January 6, 2022. doi:10.1111/andr.13152
124. Dhindsa S, Zhang N, McPhaul MJ, et al. Association of Circulating Sex Hormones With Inflammation and Disease Severity in Patients With COVID-19. *JAMA Netw Open.* 2021;4(5):e2111398. doi:10.1001/jamanetworkopen.2021.11398
125. Moreno-Perez O, Merino E, Alfayate R, et al. Male pituitary-gonadal axis dysfunction in post-acute COVID-19 syndrome-Prevalence and associated factors: A Mediterranean case series. *Clin Endocrinol (Oxf).* 2022;96(3):353-362. doi:10.1111/cen.14537
126. Moustafa MH, Sharma RK, Thornton J, et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod.* 2004;19(1):129-138. doi:10.1093/humrep/deh024
127. Wang Z, Liu W, Zhang M, Wang M, Wu H, Lu M. Effect of Hepatitis B Virus Infection on Sperm Quality and Outcomes of Assisted Reproductive Techniques in Infertile Males. *Front Med.* 2021;8. Accessed May 9, 2022. <https://www.frontiersin.org/article/10.3389/fmed.2021.744350>



128. De Almeida Di Maio Ferreira FCP, Da Silva ASV, Recht J, et al. Vertical transmission of chikungunya virus: A systematic review. *PloS One*. 2021;16(4). doi:10.1371/JOURNAL.PONE.0249166
129. Duggal NK, McDonald EM, Ritter JM, Brault AC. Sexual transmission of Zika virus enhances in utero transmission in a mouse model. *Sci Rep*. 2018;8(1). doi:10.1038/S41598-018-22840-6
130. Mahé D, Matusali G, Deleage C, et al. Potential for Virus Endogenization in Humans through Testicular Germ Cell Infection: the Case of HIV. *J Virol*. 2020;94(24). doi:10.1128/JVI.01145-20
131. Martins EB, Silva MFB, Tassinari WS, et al. Detection of Chikungunya virus in bodily fluids: The INOVACHIK cohort study. *PLoS Negl Trop Dis*. 2022;16(3):e0010242. doi:10.1371/JOURNAL.PNTD.0010242
132. Liew CH. The first case of sexual transmission of dengue in Spain. *J Travel Med*. 2020;27(1):taz087. doi:10.1093/jtm/taz087
133. Kelley RE, Berger JR, Kelley BP. WEST NILE VIRUS MENINGO-ENCEPHALITIS: POSSIBLE SEXUAL TRANSMISSION. *J La State Med Soc Off Organ La State Med Soc*. 2016;168(1):21-22.
134. Schindell BG, Webb AL, Kindrachuk J. Persistence and Sexual Transmission of Filoviruses. *Viruses*. 2018;10(12). doi:10.3390/V10120683
135. Keita AK, Koundouno FR, Faye M, et al. Resurgence of Ebola virus in 2021 in Guinea suggests a new paradigm for outbreaks. *Nature*. 2021;597(7877):539-543. doi:10.1038/s41586-021-03901-9
136. Martines RB, Ng DL, Greer PW, Rollin PE, Zaki SR. Tissue and cellular tropism, pathology and pathogenesis of Ebola and Marburg viruses. *J Pathol*. 2015;235(2):153-174. doi:10.1002/PATH.4456
137. Zeng X, Blancett CD, Koistinen KA, et al. Identification and pathological characterization of persistent asymptomatic Ebola virus infection in rhesus monkeys. *Nat Microbiol*. 2017;2:17113. doi:10.1038/nmicrobiol.2017.113
138. Bird BH, Spengler JR, Chakrabarti AK, et al. Humanized Mouse Model of Ebola Virus Disease Mimics the Immune Responses in Human Disease. *J Infect Dis*. 2016;213(5):703-711. doi:10.1093/infdis/jiv538
139. Webb AL, Schindell BG, Griffin BD, et al. *Persistent Ebola Virus Infection within the Male Reproductive Tract Is Related to Both Viral Replication Kinetics and Host Response at the Blood-Testis Barrier*. Social Science Research Network; 2022. doi:10.2139/ssrn.4000892
140. Loske J, Röhmel J, Lukassen S, et al. Pre-activated antiviral innate immunity in the upper airways controls early SARS-CoV-2 infection in children. *Nat Biotechnol*. 2022;40(3):319-324. doi:10.1038/s41587-021-01037-9
141. Park A, Iwasaki A. Type I and Type III Interferons - Induction, Signaling, Evasion, and Application to Combat COVID-19. *Cell Host Microbe*. 2020;27(6):870-878. doi:10.1016/j.chom.2020.05.008

142. Yoshida M, Worlock KB, Huang N, et al. Local and systemic responses to SARS-CoV-2 infection in children and adults. *Nature*. 2022;602(7896):321-327. doi:10.1038/s41586-021-04345-x
143. Matusali G, Houzet L, Satie AP, et al. Zika virus infects human testicular tissue and germ cells. *J Clin Invest*. 2018;128(10):4697-4710. doi:10.1172/JCI121735
144. Le Tortorec A, Denis H, Satie AP, et al. Antiviral responses of human Leydig cells to mumps virus infection or poly I:C stimulation. *Hum Reprod Oxf Engl*. 2008;23(9):2095-2103. doi:10.1093/humrep/den207
145. Dejuçq N, Lienard MO, Guillaume E, Dorval I, Jégou B. Expression of interferons-alpha and -gamma in testicular interstitial tissue and spermatogonia of the rat. *Endocrinology*. 1998;139(7):3081-3087. doi:10.1210/endo.139.7.6083
146. Wu H, Shi L, Wang Q, et al. Mumps virus-induced innate immune responses in mouse Sertoli and Leydig cells. *Sci Rep*. 2016;6(1):19507. doi:10.1038/srep19507
147. Satie AP, Mazaud-Guittot S, Seif I, et al. Excess type I interferon signaling in the mouse seminiferous tubules leads to germ cell loss and sterility. *J Biol Chem*. 2011;286(26):23280-23295. doi:10.1074/jbc.M111.229120
148. Gubler DJ, Vasilakis N, Musso D. History and Emergence of Zika Virus. *J Infect Dis*. 2017;216(suppl\_10):S860-S867. doi:10.1093/INFDIS/JIX451
149. Yun SI, Lee YM. Zika virus: An emerging flavivirus. *J Microbiol Seoul Korea*. 2017;55(3):204-219. doi:10.1007/S12275-017-7063-6
150. Musso D, Gubler DJ. Zika Virus. *Clin Microbiol Rev*. 2016;29(3):487-524. doi:10.1128/CMR.00072-15
151. Aubry F, Jacobs S, Darmuzey M, et al. Recent African strains of Zika virus display higher transmissibility and fetal pathogenicity than Asian strains. *Nat Commun*. 2021;12(1):916. doi:10.1038/s41467-021-21199-z
152. Faye O, Freire CCM, Iamarino A, et al. Molecular evolution of Zika virus during its emergence in the 20(th) century. *PLoS Negl Trop Dis*. 2014;8(1):36. doi:10.1371/JOURNAL.PNTD.0002636
153. Ferraris P, Yssel H, Missé D. Zika virus infection: an update. *Microbes Infect*. 2019;21(8-9):353-360. doi:10.1016/J.MICINF.2019.04.005
154. Hills SL, Fischer M, Petersen LR. Epidemiology of Zika Virus Infection. *J Infect Dis*. 2017;216(suppl\_10):S868-S874. doi:10.1093/INFDIS/JIX434
155. Biswas A, Kodan P, Gupta N, et al. Zika outbreak in India in 2018. *J Travel Med*. 2020;27(4). doi:10.1093/JTM/TAAA001
156. CDC. Zika Travel Information. *Online*. Published online 2021.
157. ECDC. Zika transmission in past nine months. *Online*. Published online 2017.

158. Coutard B, Barral K, Lichière J, et al. Zika Virus Methyltransferase: Structure and Functions for Drug Design Perspectives. *J Virol*. 2017;91(5). doi:10.1128/JVI.02202-16
159. Zhou Y, Ray D, Zhao Y, et al. Structure and function of flavivirus NS5 methyltransferase. *J Virol*. 2007;81(8):3891-3903. doi:10.1128/JVI.02704-06
160. Hulo C, De Castro E, Masson P, et al. ViralZone: a knowledge resource to understand virus diversity. *Nucleic Acids Res*. 2011;39(Database issue). doi:10.1093/NAR/GKQ901
161. Wilken L, Rimmelzwaan GF. Adaptive Immunity to Dengue Virus: Slippery Slope or Solid Ground for Rational Vaccine Design? *Pathog Basel Switz*. 2020;9(6):1-48. doi:10.3390/PATHOGENS9060470
162. Hasan SS, Sevana M, Kuhn RJ, Rossmann MG. Structural biology of Zika virus and other flaviviruses. *Nat Struct Mol Biol*. 2018;25(1):13-20. doi:10.1038/S41594-017-0010-8
163. Shang Z, Song H, Shi Y, Qi J, Gao GF. Crystal Structure of the Capsid Protein from Zika Virus. *J Mol Biol*. 2018;430(7):948-962. doi:10.1016/J.JMB.2018.02.006
164. Ci Y, Liu ZY, Zhang NN, et al. Zika NS1-induced ER remodeling is essential for viral replication. *J Cell Biol*. 2020;219(2). doi:10.1083/JCB.201903062
165. Rastogi M, Sharma N, Singh SK. Flavivirus NS1: a multifaceted enigmatic viral protein. *Viol J*. 2016;13(1). doi:10.1186/S12985-016-0590-7
166. Xia H, Luo H, Shan C, et al. An evolutionary NS1 mutation enhances Zika virus evasion of host interferon induction. *Nat Commun*. 2018;9(1). doi:10.1038/S41467-017-02816-2
167. Neufeldt CJ, Cortese M, Acosta EG, Bartenschlager R. Rewiring cellular networks by members of the Flaviviridae family. *Nat Rev Microbiol*. 2018;16(3):125-142. doi:10.1038/NRMICRO.2017.170
168. Ngan NTT, Kim SJ, Lee JY, Myoung J. Zika Virus Proteins NS2A and NS4A Are Major Antagonists that Reduce IFN- $\beta$  Promoter Activity Induced by the MDA5/RIG-I Signaling Pathway. *J Microbiol Biotechnol*. 2019;29(10):1665-1674. doi:10.4014/JMB.1909.09017
169. Ding Q, Gaska JM, Douam F, et al. Species-specific disruption of STING-dependent antiviral cellular defenses by the Zika virus NS2B3 protease. *Proc Natl Acad Sci U S A*. 2018;115(27):E6310-E6318. doi:10.1073/PNAS.1803406115
170. Xing H, Xu S, Jia F, et al. Zika NS2B is a crucial factor recruiting NS3 to the ER and activating its protease activity. *Virus Res*. 2020;275. doi:10.1016/J.VIRUSRES.2019.197793
171. Xu S, Ci Y, Wang L, et al. Zika virus NS3 is a canonical RNA helicase stimulated by NS5 RNA polymerase. *Nucleic Acids Res*. 2019;47(16):8693-8707. doi:10.1093/NAR/GKZ650
172. Kumar D, Kumar A, Bhardwaj T, Giri R. Zika virus NS4A N-Terminal region (1-48) acts as a cofactor for inducing NTPase activity of NS3 helicase but not NS3 protease. *Arch Biochem Biophys*. 2020;695. doi:10.1016/J.ABB.2020.108631

173. Lin DL, Inoue T, Chen YJ, Chang A, Tsai B, Tai AW. The ER Membrane Protein Complex Promotes Biogenesis of Dengue and Zika Virus Non-structural Multi-pass Transmembrane Proteins to Support Infection. *Cell Rep.* 2019;27(6):1666-1674.e4. doi:10.1016/J.CELREP.2019.04.051
174. Ma J, Ketkar H, Geng T, et al. Zika Virus Non-structural Protein 4A Blocks the RLR-MAVS Signaling. *Front Microbiol.* 2018;9(JUN). doi:10.3389/FMICB.2018.01350
175. Grant A, Ponia SS, Tripathi S, et al. Zika Virus Targets Human STAT2 to Inhibit Type I Interferon Signaling. *Cell Host Microbe.* 2016;19(6):882-890. doi:10.1016/J.CHOM.2016.05.009
176. Issur M, Geiss BJ, Bougie I, et al. The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. *RNA N Y N.* 2009;15(12):2340-2350. doi:10.1261/RNA.1609709
177. Lanciotti RS, Kosoy OL, Laven JJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis.* 2008;14(8):1232-1239. doi:10.3201/EID1408.080287
178. Piorkowski G, Richard P, Baronti C, et al. Complete coding sequence of Zika virus from Martinique outbreak in 2015. *New Microbes New Infect.* 2016;11:52-53. doi:10.1016/J.NMNI.2016.02.013
179. Yuan L, Huang XY, Liu ZY, et al. A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Science.* 2017;358(6365):933-936. doi:10.1126/SCIENCE.AAM7120
180. Foo SS, Chen W, Chan Y, et al. Asian Zika virus strains target CD14 + blood monocytes and induce M2-skewed immunosuppression during pregnancy. *Nat Microbiol.* 2017;2(11):1558-1570. doi:10.1038/S41564-017-0016-3
181. Österlund P, Jiang M, Westenius V, et al. Asian and African lineage Zika viruses show differential replication and innate immune responses in human dendritic cells and macrophages. *Sci Rep.* 2019;9(1). doi:10.1038/S41598-019-52307-1
182. Simonin Y, van Riel D, Van de Perre P, Rockx B, Salinas S. Differential virulence between Asian and African lineages of Zika virus. *PLoS Negl Trop Dis.* 2017;11(9). doi:10.1371/JOURNAL.PNTD.0005821
183. Agrelli A, de Moura RR, Crovella S, Brandão LAC. ZIKA virus entry mechanisms in human cells. *Infect Genet Evol.* 2019;69:22-29. doi:10.1016/J.MEEGID.2019.01.018
184. Song Y, Mugavero JA, Stauff CB, Wimmer E. Dengue and Zika Virus 5' Untranslated Regions Harbor Internal Ribosomal Entry Site Functions. *mBio.* 2019;10(2). doi:10.1128/MBIO.00459-19
185. Cortese M, Goellner S, Acosta EG, et al. Ultrastructural Characterization of Zika Virus Replication Factories. *Cell Rep.* 2017;18(9):2113-2123. doi:10.1016/J.CELREP.2017.02.014
186. Ng WC, Soto-Acosta R, Bradrick SS, Garcia-Blanco MA, Ooi EE. The 5' and 3' Untranslated Regions of the Flaviviral Genome. *Viruses.* 2017;9(6). doi:10.3390/V9060137

187. Nanaware N, Banerjee A, Bagchi SM, Bagchi P, Mukherjee A. Dengue Virus Infection: A Tale of Viral Exploitations and Host Responses. *Viruses*. 2021;13(10). doi:10.3390/V13101967
188. Gao H, Lin Y, He J, et al. Role of heparan sulfate in the Zika virus entry, replication, and cell death. *Virology*. 2019;529:91-100. doi:10.1016/J.VIROL.2019.01.019
189. Kim SY, Zhao J, Liu X, et al. Interaction of Zika Virus Envelope Protein with Glycosaminoglycans. *Biochemistry*. 2017;56(8):1151-1162. doi:10.1021/ACS.BIOCHEM.6B01056
190. Perera-Lecoin M, Meertens L, Carnec X, Amara A. Flavivirus entry receptors: an update. *Viruses*. 2013;6(1):69-88. doi:10.3390/V6010069
191. Hamel R, Dejarnac O, Wichit S, et al. Biology of Zika Virus Infection in Human Skin Cells. *J Virol*. 2015;89(17):8880-8896. doi:10.1128/JVI.00354-15
192. Routhu NK, Lehoux SD, Rouse EA, et al. Glycosylation of Zika Virus is Important in Host-Virus Interaction and Pathogenic Potential. *Int J Mol Sci*. 2019;20(20). doi:10.3390/IJMS20205206
193. Pagani I, Ghezzi S, Ulisse A, et al. Human Endometrial Stromal Cells Are Highly Permissive To Productive Infection by Zika Virus. *Sci Rep*. 2017;7. doi:10.1038/SREP44286
194. Liu S, Delalio LJ, Isakson BE, Wang TT. AXL-Mediated Productive Infection of Human Endothelial Cells by Zika Virus. *Circ Res*. 2016;119(11):1183-1189. doi:10.1161/CIRCRESAHA.116.309866
195. Richard AS, Shim BS, Kwon YC, et al. AXL-dependent infection of human fetal endothelial cells distinguishes Zika virus from other pathogenic flaviviruses. *Proc Natl Acad Sci U S A*. 2017;114(8):2024-2029. doi:10.1073/PNAS.1620558114
196. Meertens L, Labeau A, Dejarnac O, et al. Axl Mediates ZIKA Virus Entry in Human Glial Cells and Modulates Innate Immune Responses. *Cell Rep*. 2017;18(2):324-333. doi:10.1016/J.CELREP.2016.12.045
197. Retallack H, Di Lullo E, Arias C, et al. Zika virus cell tropism in the developing human brain and inhibition by azithromycin. *Proc Natl Acad Sci U S A*. 2016;113(50):14408-14413. doi:10.1073/PNAS.1618029113/-/DCSUPPLEMENTAL
198. Kumar A, Jovel J, Lopez-Orozco J, et al. Human Sertoli cells support high levels of Zika virus replication and persistence. *Sci Rep*. 2018;8(1). doi:10.1038/S41598-018-23899-X
199. Strange DP, Jiyarom B, Zarandi NP, et al. Axl Promotes Zika Virus Entry and Modulates the Antiviral State of Human Sertoli Cells. *mBio*. 2019;10(4). doi:10.1128/MBIO.01372-19
200. Amara A, Mercer J. Viral apoptotic mimicry. *Nat Rev Microbiol*. 2015;13(8):461-469. doi:10.1038/NRMICRO3469
201. Wells MF, Salick MR, Wiskow O, et al. Genetic Ablation of AXL Does Not Protect Human Neural Progenitor Cells and Cerebral Organoids from Zika Virus Infection. *Cell Stem Cell*. 2016;19(6):703-708. doi:10.1016/J.STEM.2016.11.011

202. Hastings AK, Yockey LJ, Jagger BW, et al. TAM Receptors Are Not Required for Zika Virus Infection in Mice. *Cell Rep.* 2017;19(3):558-568. doi:10.1016/J.CELREP.2017.03.058
203. Wang ZY, Wang Z, Zhen ZD, et al. Axl is not an indispensable factor for Zika virus infection in mice. *J Gen Virol.* 2017;98(8):2061-2068. doi:10.1099/jgv.0.000886
204. Govero J, Esakky P, Scheaffer SM, et al. Zika virus infection damages the testes in mice. *Nature.* 2016;540(7633):438-442. doi:10.1038/NATURE20556
205. Chen J, Yang YF, Yang Y, et al. AXL promotes Zika virus infection in astrocytes by antagonizing type I interferon signalling. *Nat Microbiol.* 2018;3(3):302-309. doi:10.1038/S41564-017-0092-4
206. Tabata T, Petitt M, Puerta-Guardo H, et al. Zika Virus Targets Different Primary Human Placental Cells, Suggesting Two Routes for Vertical Transmission. *Cell Host Microbe.* 2016;20(2):155-166. doi:10.1016/J.CHOM.2016.07.002
207. Wang S, Zhang Q, Tiwari SK, et al. Integrin  $\alpha\beta 5$  Internalizes Zika Virus during Neural Stem Cells Infection and Provides a Promising Target for Antiviral Therapy. *Cell Rep.* 2020;30(4):969. doi:10.1016/J.CELREP.2019.11.020
208. Pujhari S, Brustolin M, Macias VM, et al. Heat shock protein 70 (Hsp70) mediates Zika virus entry, replication, and egress from host cells. *Emerg Microbes Infect.* 2019;8(1):8-16. doi:10.1080/22221751.2018.1557988
209. Taguwa S, Yeh MT, Rainbolt TK, et al. Zika Virus Dependence on Host Hsp70 Provides a Protective Strategy against Infection and Disease. *Cell Rep.* 2019;26(4):906-920.e3. doi:10.1016/J.CELREP.2018.12.095
210. Srivastava M, Zhang Y, Chen J, et al. Chemical proteomics tracks virus entry and uncovers NCAM1 as Zika virus receptor. *Nat Commun.* 2020;11(1). doi:10.1038/S41467-020-17638-Y
211. Sabino C, Bender D, Herrlein ML, Hildt E. The Epidermal Growth Factor Receptor Is a Relevant Host Factor in the Early Stages of The Zika Virus Life Cycle In Vitro. *J Virol.* 2021;95(20). doi:10.1128/JVI.01195-21
212. Bardina SV, Bunduc P, Tripathi S, et al. Enhancement of Zika virus pathogenesis by preexisting antinflavivirus immunity. *Science.* 2017;356(6334):175-180. doi:10.1126/SCIENCE.AAL4365
213. Castanha PMS, Nascimento EJM, Braga C, et al. Dengue Virus-Specific Antibodies Enhance Brazilian Zika Virus Infection. *J Infect Dis.* 2017;215(5):781-785. doi:10.1093/INFDIS/JIW638
214. Dejnirattisai W, Supasa P, Wongwiwat W, et al. Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nat Immunol.* 2016;17(9):1102-1108. doi:10.1038/NI.3515
215. Baud D, Gubler DJ, Schaub B, Lanteri MC, Musso D. An update on Zika virus infection. *Lancet Lond Engl.* 2017;390(10107):2099-2109. doi:10.1016/S0140-6736(17)31450-2

216. Mann TZ, Haddad LB, Williams TR, et al. Breast milk transmission of flaviviruses in the context of Zika virus: A systematic review. *Paediatr Perinat Epidemiol*. 2018;32(4):358-368. doi:10.1111/PPE.12478
217. Blohm GM, Lednicky JA, Márquez M, et al. Evidence for Mother-to-Child Transmission of Zika Virus Through Breast Milk. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2018;66(7):1120-1121. doi:10.1093/CID/CIX968
218. Foy BD, Kobylinski KC, Foy JLC, et al. Probable non-vector-borne transmission of Zika virus, Colorado, USA. *Emerg Infect Dis*. 2011;17(5):880-882. doi:10.3201/EID1705.101939
219. Blitvich BJ, Magalhaes T, Viridiana Laredo-Tiscareño S, Foy BD. Sexual Transmission of Arboviruses: A Systematic Review. *Viruses*. 2020;12(9). doi:10.3390/V12090933
220. Moreira J, Peixoto TM, Siqueira AM, Lamas CC. Sexually acquired Zika virus: a systematic review. *Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis*. 2017;23(5):296-305. doi:10.1016/J.CMI.2016.12.027
221. Gurung S, Nadeau H, Maxted M, et al. Maternal Zika Virus (ZIKV) Infection following Vaginal Inoculation with ZIKV-Infected Semen in Timed-Pregnant Olive Baboons. *J Virol*. 2020;94(11). doi:10.1128/JVI.00058-20
222. Towers S, Brauer F, Castillo-Chavez C, Falconar AKI, Mubayi A, Romero-Vivas CME. Estimate of the reproduction number of the 2015 Zika virus outbreak in Barranquilla, Colombia, and estimation of the relative role of sexual transmission. *Epidemics*. 2016;17:50-55. doi:10.1016/J.EPIDEM.2016.10.003
223. Kurscheidt FA, Mesquita CSS, Damke GMZF, et al. Persistence and clinical relevance of Zika virus in the male genital tract. *Nat Rev Urol*. 2019;16(4):211-230. doi:10.1038/S41585-019-0149-7
224. Bujan L, Mansuy JM, Hamdi S, Pasquier C, Joguet G. 1 year after acute Zika virus infection in men. *Lancet Infect Dis*. 2020;20(1):25-26. doi:10.1016/S1473-3099(19)30678-4
225. Epelboin S, Dulioust E, Epelboin L, Benachi A, Merlet F, Patrat C. Zika virus and reproduction: facts, questions and current management. *Hum Reprod Update*. 2017;23(6):629-645. doi:10.1093/HUMUPD/DMX024
226. Barzon L, Percivalle E, Pacenti M, et al. Virus and Antibody Dynamics in Travelers With Acute Zika Virus Infection. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2018;66(8):1173-1180. doi:10.1093/CID/CIX967
227. Bonaldo MC, Ribeiro IP, Lima NS, et al. Isolation of Infective Zika Virus from Urine and Saliva of Patients in Brazil. *PLoS Negl Trop Dis*. 2016;10(6). doi:10.1371/JOURNAL.PNTD.0004816
228. Rosinger AY, Olson SM, Ellington SR, et al. Evaluating Differences in Whole Blood, Serum, and Urine Screening Tests for Zika Virus, Puerto Rico, USA, 2016. *Emerg Infect Dis*. 2021;27(5):1505-1508. doi:10.3201/EID2705.203960

229. Menezes-Neto A, Castilho M da C, Calvet GA, et al. Zika virus RNA excretion in sweat with concomitant detection in other body fluid specimens. *Mem Inst Oswaldo Cruz*. 2021;115(11):1-5. doi:10.1590/0074-02760200339
230. CDC. Zika Virus. CDC. Published November 5, 2014. Accessed May 4, 2022. <https://www.cdc.gov/zika/comm-resources/infographics.html>
231. Abushouk AI, Negida A, Ahmed H. An updated review of Zika virus. *J Clin Virol Off Publ Pan Am Soc Clin Virol*. 2016;84:53-58. doi:10.1016/J.JCV.2016.09.012
232. ECDC. Recent scientific findings based on literature reviewed after the ninth update of the ECDC Rapid Risk Assessment on Zika virus infection (19 October to 27 January 2017). *Online*. Published online 2017.
233. Joguet G, Mansuy JM, Matusali G, et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect Dis*. 2017;17(11):1200-1208. doi:10.1016/S1473-3099(17)30444-9
234. Mansuy JM, Suberbielle E, Chapuy-Regaud S, et al. Zika virus in semen and spermatozoa. *Lancet Infect Dis*. 2016;16(10):1106-1107. doi:10.1016/S1473-3099(16)30336-X
235. Robinson CL, Chong ACN, Ashbrook AW, et al. Male germ cells support long-term propagation of Zika virus. *Nat Commun*. 2018;9(1). doi:10.1038/S41467-018-04444-W
236. Osuna CE, Lim SY, Deleage C, et al. Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nat Med*. 2016;22(12):1448-1455. doi:10.1038/NM.4206
237. Mead PS, Duggal NK, Hook SA, et al. Zika Virus Shedding in Semen of Symptomatic Infected Men. *N Engl J Med*. 2018;378(15):1377-1385. doi:10.1056/NEJMOA1711038
238. Almeida R das N, Braz-de-Melo HA, Santos I de O, Corrêa R, Kobinger GP, Magalhaes KG. The Cellular Impact of the ZIKA Virus on Male Reproductive Tract Immunology and Physiology. *Cells*. 2020;9(4). doi:10.3390/CELLS9041006
239. Ball EE, Pesavento P, Rompay KKAV, et al. Zika virus persistence in the male macaque reproductive tract. Published online March 4, 2022:2022.03.03.482872. doi:10.1101/2022.03.03.482872
240. ICTV. Taxonomy. *Online*. Published online 2022.
241. Millet JK, Jaimes JA, Whittaker GR. Molecular diversity of coronavirus host cell entry receptors. *FEMS Microbiol Rev*. 2021;45(3). doi:10.1093/FEMSRE/FUAA057
242. Wu Y, Zhao S. Furin cleavage sites naturally occur in coronaviruses. *Stem Cell Res*. 2020;50. doi:10.1016/J.SCR.2020.102115
243. Holmes AC, Basore K, Fremont DH, Diamond MS. A molecular understanding of alphavirus entry. *PLoS Pathog*. 2020;16(10). doi:10.1371/JOURNAL.PPAT.1008876



244. V'kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. *Nat Rev Microbiol.* 2021;19(3):155-170. doi:10.1038/S41579-020-00468-6
245. Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. *Nat Rev Microbiol.* 2021;19(3):141-154. doi:10.1038/S41579-020-00459-7
246. PAHO. COVID-19 Daily Update: 11 March 2020. *Online.* Published online 2020.
247. WHO. WHO Coronavirus (COVID-19) Dashboard. *Online.* Published online 2022.
248. Jungreis I, Sealfon R, Kellis M. SARS-CoV-2 gene content and COVID-19 mutation impact by comparing 44 Sarbecovirus genomes. *Nat Commun.* 2021;12(1):2642. doi:10.1038/s41467-021-22905-7
249. Enzo Life Sciences. COVID-19 Therapeutic Development. *Online.*
250. Yan W, Zheng Y, Zeng X, He B, Cheng W. Structural biology of SARS-CoV-2: open the door for novel therapies. *Signal Transduct Target Ther.* 2022;7(1). doi:10.1038/S41392-022-00884-5
251. Lemmin T, Kalbermatter D, Harder D, Plattet P, Fotiadis D. Structures and dynamics of the novel S1/S2 protease cleavage site loop of the SARS-CoV-2 spike glycoprotein. *J Struct Biol X.* 2020;4. doi:10.1016/J.YJSBX.2020.100038
252. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. *Nat Rev Mol Cell Biol.* 2022;23(1):3-20. doi:10.1038/S41580-021-00418-X
253. Montagutelli X, Prot M, Levillayer L, et al. Variants with the N501Y mutation extend SARS-CoV-2 host range to mice, with contact transmission. Published online December 7, 2021:2021.03.18.436013. doi:10.1101/2021.03.18.436013
254. CDC. SARS-CoV-2 Variant Classifications and Definitions. *Online.* Published online 2022.
255. Yang Q, Jian X, Syed AAS, et al. Structural Comparison and Drug Screening of Spike Proteins of Ten SARS-CoV-2 Variants. *Research.* 2022;2022:1-20. doi:10.34133/2022/9781758
256. Böttcher-Friebertshäuser E, Garten W, Klenk HD. Activation of viruses by host proteases. *Acta Viruses Host Proteases.* Published online 2018:1-335. doi:10.1007/978-3-319-75474-1
257. Millet JK, Whittaker GR. Physiological and molecular triggers for SARS-CoV membrane fusion and entry into host cells. *Virology.* 2018;517:3-8. doi:10.1016/J.VIROL.2017.12.015
258. Murgolo N, Therien AG, Howell B, et al. SARS-CoV-2 tropism, entry, replication, and propagation: Considerations for drug discovery and development. *PLoS Pathog.* 2021;17(2). doi:10.1371/JOURNAL.PPAT.1009225
259. Yu S, Zheng X, Zhou B, et al. SARS-CoV-2 spike engagement of ACE2 primes S2' site cleavage and fusion initiation. *Proc Natl Acad Sci U S A.* 2022;119(1):e2111199119. doi:10.1073/pnas.2111199119

260. Bhat EA, Khan J, Sajjad N, et al. SARS-CoV-2: Insight in genome structure, pathogenesis and viral receptor binding analysis - An updated review. *Int Immunopharmacol.* 2021;95. doi:10.1016/J.INTIMP.2021.107493
261. Petrovszki D, Walter FR, Vigh JP, et al. Penetration of the SARS-CoV-2 Spike Protein across the Blood-Brain Barrier, as Revealed by a Combination of a Human Cell Culture Model System and Optical Biosensing. *Biomedicines.* 2022;10(1). doi:10.3390/BIOMEDICINES10010188
262. Harrison AG, Lin T, Wang P. Mechanisms of SARS-CoV-2 Transmission and Pathogenesis. *Trends Immunol.* 2020;41(12):1100-1115. doi:10.1016/J.IT.2020.10.004
263. Hoffmann M, Kleine-Weber H, Pöhlmann S. A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol Cell.* 2020;78(4):779-784.e5. doi:10.1016/J.MOLCEL.2020.04.022
264. Lan J, Ge J, Yu J, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature.* 2020;581(7807):215-220. doi:10.1038/S41586-020-2180-5
265. Bojkova D, Bechtel M, McLaughlin KM, et al. Aprotinin Inhibits SARS-CoV-2 Replication. *Cells.* 2020;9(11). doi:10.3390/CELLS9112377
266. Hoffmann M, Hofmann-Winkler H, Smith JC, et al. Camostat mesylate inhibits SARS-CoV-2 activation by TMPRSS2-related proteases and its metabolite GBPA exerts antiviral activity. *EBioMedicine.* 2021;65. doi:10.1016/J.EBIOM.2021.103255
267. Jaimes JA, André NM, Chappie JS, Millet JK, Whittaker GR. Phylogenetic Analysis and Structural Modeling of SARS-CoV-2 Spike Protein Reveals an Evolutionary Distinct and Proteolytically Sensitive Activation Loop. *J Mol Biol.* 2020;432(10):3309-3325. doi:10.1016/J.JMB.2020.04.009
268. Shang J, Wan Y, Luo C, et al. Cell entry mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A.* 2020;117(21). doi:10.1073/PNAS.2003138117
269. Shang J, Ye G, Shi K, et al. Structural basis of receptor recognition by SARS-CoV-2. *Nature.* 2020;581(7807):221-224. doi:10.1038/S41586-020-2179-Y
270. Takeda M. Proteolytic activation of SARS-CoV-2 spike protein. *Microbiol Immunol.* 2022;66(1):15-23. doi:10.1111/1348-0421.12945
271. Wrobel AG, Benton DJ, Xu P, et al. SARS-CoV-2 and bat RaTG13 spike glycoprotein structures inform on virus evolution and furin-cleavage effects. *Nat Struct Mol Biol.* 2020;27(8):763-767. doi:10.1038/S41594-020-0468-7
272. Daly JL, Simonetti B, Klein K, et al. Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science.* 2020;370(6518):861-865. doi:10.1126/SCIENCE.ABD3072
273. Li ZL, Buck M. Neuropilin-1 Assists SARS-CoV-2 Infection by Stimulating the Separation of Spike Protein Domains S1 and S2. *BioRxiv Prepr Serv Biol.* Published online 2021. doi:10.1101/2021.01.06.425627

274. Cantuti-Castelvetri L, Ojha R, Pedro LD, et al. Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity. *Science*. 2020;370(6518). doi:10.1126/SCIENCE.ABD2985
275. Wang S, Qiu Z, Hou Y, et al. AXL is a candidate receptor for SARS-CoV-2 that promotes infection of pulmonary and bronchial epithelial cells. *Cell Res* 2021 312. 2021;31(2):126-140. doi:10.1038/s41422-020-00460-y
276. Wang K, Chen W, Zhang Z, et al. CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. *Signal Transduct Target Ther*. 2020;5(1). doi:10.1038/S41392-020-00426-X
277. Kalejaiye TD, Bhattacharya R, Travieso T, et al. BSG/CD147 and ACE2 receptors facilitate SARS-CoV-2 infection of human iPS cell-derived kidney podocytes. *BioRxiv Prepr Serv Biol*. Published online 2021. doi:10.1101/2021.11.16.468893
278. Shilts J, Crozier TWM, Greenwood EJD, Lehner PJ, Wright GJ. No evidence for basigin/CD147 as a direct SARS-CoV-2 spike binding receptor. *Sci Rep* 2021 111. 2021;11(1):1-10. doi:10.1038/s41598-020-80464-1
279. Mori Y, Fink C, Ichimura T, et al. KIM-1/TIM-1 is a Receptor for SARS-CoV-2 in Lung and Kidney. *MedRxiv Prepr Serv Health Sci*. Published online January 11, 2022:2020.09.16.20190694. doi:10.1101/2020.09.16.20190694
280. Koch J, Uckelely ZM, Doldan P, Stanifer M, Boulant S, Lozach PY. TMPRSS2 expression dictates the entry route used by SARS-CoV-2 to infect host cells. *EMBO J*. 2021;40(16). doi:10.15252/EMBJ.2021107821
281. Tang T, Jaimes JA, Bidon MK, Straus MR, Daniel S, Whittaker GR. Proteolytic Activation of SARS-CoV-2 Spike at the S1/S2 Boundary: Potential Role of Proteases beyond Furin. *ACS Infect Dis*. 2021;7(2):264-272. doi:10.1021/ACSINFECDIS.0C00701
282. Coutard B, Valle C, de Lamballerie X, Canard B, Seidah NG, Decroly E. The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Res*. 2020;176. doi:10.1016/J.ANTIVIRAL.2020.104742
283. Bestle D, Heindl MR, Limburg H, et al. TMPRSS2 and furin are both essential for proteolytic activation of SARS-CoV-2 in human airway cells. *Life Sci Alliance*. 2020;3(9). doi:10.26508/LSA.202000786
284. Papa G, Mallery DL, Albecka A, et al. Furin cleavage of SARS-CoV-2 Spike promotes but is not essential for infection and cell-cell fusion. *PLoS Pathog*. 2021;17(1). doi:10.1371/JOURNAL.PPAT.1009246
285. Jaimes JA, Millet JK, Whittaker GR. Proteolytic Cleavage of the SARS-CoV-2 Spike Protein and the Role of the Novel S1/S2 Site. *iScience*. 2020;23(6). doi:10.1016/J.ISCI.2020.101212
286. Bayati A, Kumar R, Francis V, McPherson PS. SARS-CoV-2 infects cells after viral entry via clathrin-mediated endocytosis. *J Biol Chem*. 2021;296. doi:10.1016/J.JBC.2021.100306

287. Zang R, Castro MFG, McCune BT, et al. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. *Sci Immunol.* 2020;5(47). doi:10.1126/SCIIMMUNOL.ABC3582
288. Kishimoto M, Uemura K, Sanaki T, et al. TMPRSS11D and TMPRSS13 Activate the SARS-CoV-2 Spike Protein. *Viruses.* 2021;13(3). doi:10.3390/V13030384
289. Zeng C, Evans JP, King T, et al. SARS-CoV-2 Spreads through Cell-to-Cell Transmission. *BioRxiv Prepr Serv Biol.* Published online 2021. doi:10.1101/2021.06.01.446579
290. Buchrieser J, Dufloo J, Hubert M, et al. Syncytia formation by SARS-CoV-2-infected cells. *EMBO J.* 2020;39(23). doi:10.15252/EMBJ.2020106267
291. Johnson BA, Xie X, Bailey AL, et al. Loss of furin cleavage site attenuates SARS-CoV-2 pathogenesis. *Nature.* 2021;591(7849):293-299. doi:10.1038/S41586-021-03237-4
292. Zhao MM, Yang WL, Yang FY, et al. Cathepsin L plays a key role in SARS-CoV-2 infection in humans and humanized mice and is a promising target for new drug development. *Signal Transduct Target Ther.* 2021;6(1). doi:10.1038/S41392-021-00558-8
293. Gallagher TM, Buchmeier MJ, Perlman S. Cell receptor-independent infection by a neurotropic murine coronavirus. *Virology.* 1992;191(1):517-522. doi:10.1016/0042-6822(92)90223-C
294. Menachery VD, Dinnon KH, Yount BL, et al. Trypsin Treatment Unlocks Barrier for Zoonotic Bat Coronavirus Infection. *J Virol.* 2020;94(5). doi:10.1128/JVI.01774-19
295. Rahman HS, Aziz MS, Hussein RH, et al. The transmission modes and sources of COVID-19: A systematic review. *Int J Surg Open.* 2020;26:125-136. doi:10.1016/J.IJSO.2020.08.017
296. Dong L, Tian J, He S, et al. Possible Vertical Transmission of SARS-CoV-2 From an Infected Mother to Her Newborn. *JAMA.* 2020;323(18):1846-1848. doi:10.1001/jama.2020.4621
297. Alzamora MC, Paredes T, Caceres D, Webb CM, Valdez LM, La Rosa M. Severe COVID-19 during Pregnancy and Possible Vertical Transmission. *Am J Perinatol.* 2020;37(8):861-865. doi:10.1055/s-0040-1710050
298. Mokhtari T, Hassani F, Ghaffari N, Ebrahimi B, Yarahmadi A, Hassanzadeh G. COVID-19 and multiorgan failure: A narrative review on potential mechanisms. *J Mol Histol.* 2020;51(6):613-628. doi:10.1007/s10735-020-09915-3
299. Haque SM, Ashwaq O, Sarief A, Azad John Mohamed AK. A comprehensive review about SARS-CoV-2. *Future Virol.* 2020;15(9):625-648. doi:10.2217/fvl-2020-0124
300. Ozkurt Z, Çınar Tanrıverdi E. COVID-19: Gastrointestinal manifestations, liver injury and recommendations. *World J Clin Cases.* 2022;10(4):1140-1163. doi:10.12998/wjcc.v10.i4.1140
301. Merad M, Blish CA, Sallusto F, Iwasaki A. The immunology and immunopathology of COVID-19. *Science.* 2022;375(6585):1122-1127. doi:10.1126/SCIENCE.ABM8108

302. Mehandru S, Merad M. Pathological sequelae of long-haul COVID. *Nat Immunol.* 2022;23(2):194-202. doi:10.1038/S41590-021-01104-Y
303. Han Y, Yang L, Lacko LA, Chen S. Human organoid models to study SARS-CoV-2 infection. *Nat Methods.* 2022;19(4):418-428. doi:10.1038/S41592-022-01453-Y
304. Del Valle DM, Kim-Schulze S, Huang HH, et al. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med.* 2020;26(10):1636-1643. doi:10.1038/s41591-020-1051-9
305. Leisman DE, Ronner L, Pinotti R, et al. Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. *Lancet Respir Med.* 2020;8(12):1233-1244. doi:10.1016/S2213-2600(20)30404-5
306. Sposito B, Broggi A, Pandolfi L, et al. The interferon landscape along the respiratory tract impacts the severity of COVID-19. *Cell.* 2021;184(19):4953-4968.e16. doi:10.1016/j.cell.2021.08.016
307. Ferreira AC, Soares VC, de Azevedo-Quintanilha IG, et al. SARS-CoV-2 engages inflammasome and pyroptosis in human primary monocytes. *Cell Death Discov.* 2021;7(1):43. doi:10.1038/s41420-021-00428-w
308. Zhang Q, Bastard P, Liu Z, et al. Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. *Science.* 2020;370(6515):eabd4570. doi:10.1126/science.abd4570
309. Bastard P, Orlova E, Sozaeva L, et al. Preexisting autoantibodies to type I IFNs underlie critical COVID-19 pneumonia in patients with APS-1. *J Exp Med.* 2021;218(7):e20210554. doi:10.1084/jem.20210554
310. Goncalves D, Mezidi M, Bastard P, et al. Antibodies against type I interferon: detection and association with severe clinical outcome in COVID-19 patients. *Clin Transl Immunol.* 2021;10(8):e1327. doi:10.1002/cti2.1327
311. Wang EY, Mao T, Klein J, et al. Diverse functional autoantibodies in patients with COVID-19. *Nature.* 2021;595(7866):283-288. doi:10.1038/s41586-021-03631-y
312. Chen T, Wu D, Chen H, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. *BMJ.* 2020;368:m1091. doi:10.1136/bmj.m1091
313. Lakbar I, Luque-Paz D, Mege JL, Einav S, Leone M. COVID-19 gender susceptibility and outcomes: A systematic review. *PLoS ONE.* 2020;15(11):e0241827. doi:10.1371/journal.pone.0241827
314. Forrester SJ, Booz GW, Sigmund CD, et al. Angiotensin II Signal Transduction: An Update on Mechanisms of Physiology and Pathophysiology. *Physiol Rev.* 2018;98(3):1627-1738. doi:10.1152/physrev.00038.2017

315. Furuhashi M, Moniwa N, Takizawa H, Ura N, Shimamoto K. Potential differential effects of renin-angiotensin system inhibitors on SARS-CoV-2 infection and lung injury in COVID-19. *Hypertens Res Off J Jpn Soc Hypertens*. 2020;43(8):837-840. doi:10.1038/s41440-020-0478-1
316. Beyerstedt S, Casaro EB, Rangel ÉB. COVID-19: angiotensin-converting enzyme 2 (ACE2) expression and tissue susceptibility to SARS-CoV-2 infection. *Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol*. 2021;40(5):905-919. doi:10.1007/s10096-020-04138-6
317. Foresta C, Rocca MS, Di Nisio A. Gender susceptibility to COVID-19: a review of the putative role of sex hormones and X chromosome. *J Endocrinol Invest*. 2021;44(5):951-956. doi:10.1007/s40618-020-01383-6
318. Galbadage T, Peterson BM, Wang JS, et al. Molecular Mechanisms Lead to Sex-Specific COVID-19 Prognosis and Targeted Therapies. *Front Med*. 2020;7:589060. doi:10.3389/fmed.2020.589060
319. Hu Y, Liu L, Lu X. Regulation of Angiotensin-Converting Enzyme 2: A Potential Target to Prevent COVID-19? *Front Endocrinol*. 2021;12:725967. doi:10.3389/fendo.2021.725967
320. Edenfield RC, Easley CA. Implications of testicular ACE2 and the renin-angiotensin system for SARS-CoV-2 on testis function. *Nat Rev Urol*. 2022;19(2):116-127. doi:10.1038/s41585-021-00542-5
321. Deng Q, Rasool RU, Russell RM, Natesan R, Asangani IA. Targeting androgen regulation of TMPRSS2 and ACE2 as a therapeutic strategy to combat COVID-19. *iScience*. 2021;24(3):102254. doi:10.1016/j.isci.2021.102254
322. Qiao Y, Wang XM, Mannan R, et al. Targeting transcriptional regulation of SARS-CoV-2 entry factors ACE2 and TMPRSS2. *Proc Natl Acad Sci U S A*. Published online December 11, 2020:202021450. doi:10.1073/pnas.2021450118
323. Samuel RM, Majd H, Richter MN, et al. Androgen Signaling Regulates SARS-CoV-2 Receptor Levels and Is Associated with Severe COVID-19 Symptoms in Men. *Cell Stem Cell*. 2020;27(6):876-889.e12. doi:10.1016/j.stem.2020.11.009
324. Stelzig KE, Canepa-Escaro F, Schiliro M, Berdnikovs S, Prakash YS, Chiarella SE. Estrogen regulates the expression of SARS-CoV-2 receptor ACE2 in differentiated airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2020;318(6):L1280-L1281. doi:10.1152/ajplung.00153.2020
325. Chakravarty D, Nair SS, Hammouda N, et al. Sex differences in SARS-CoV-2 infection rates and the potential link to prostate cancer. *Commun Biol*. 2020;3(1):374. doi:10.1038/s42003-020-1088-9
326. Fan C, Lu W, Li K, Ding Y, Wang J. ACE2 Expression in Kidney and Testis May Cause Kidney and Testis Infection in COVID-19 Patients. *Front Med*. 2020;7:563893. doi:10.3389/fmed.2020.563893
327. Li MY, Li L, Zhang Y, Wang XS. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. *Infect Dis Poverty*. 2020;9(1):45. doi:10.1186/s40249-020-00662-x

328. Sheikhzadeh Hesari F, Hosseinzadeh SS, Asl Monadi Sardroud MA. Review of COVID-19 and male genital tract. *Andrologia*. Published online November 24, 2020:e13914. doi:10.1111/and.13914
329. Wang Z, Xu X. scRNA-seq Profiling of Human Testes Reveals the Presence of the ACE2 Receptor, A Target for SARS-CoV-2 Infection in Spermatogonia, Leydig and Sertoli Cells. *Cells*. 2020;9(4):E920. doi:10.3390/cells9040920
330. Zhou L, Niu Z, Jiang X, et al. SARS-CoV-2 Targets by the pscRNA Profiling of ACE2, TMPRSS2 and Furin Proteases. *iScience*. 2020;23(11):101744. doi:10.1016/j.isci.2020.101744
331. Shen Q, Xiao X, Aierken A, et al. The ACE2 expression in Sertoli cells and germ cells may cause male reproductive disorder after SARS-CoV-2 infection. *J Cell Mol Med*. 2020;24(16):9472-9477. doi:10.1111/jcmm.15541
332. Stanley KE, Thomas E, Leaver M, Wells D. Coronavirus disease-19 and fertility: viral host entry protein expression in male and female reproductive tissues. *Fertil Steril*. 2020;114(1):33-43. doi:10.1016/j.fertnstert.2020.05.001
333. Pan F, Xiao X, Guo J, et al. No evidence of severe acute respiratory syndrome-coronavirus 2 in semen of males recovering from coronavirus disease 2019. *Fertil Steril*. 2020;113(6):1135-1139. doi:10.1016/j.fertnstert.2020.04.024
334. Ma X, Guan C, Chen R, et al. Pathological and molecular examinations of postmortem testis biopsies reveal SARS-CoV-2 infection in the testis and spermatogenesis damage in COVID-19 patients. *Cell Mol Immunol*. 2021;18(2):487-489. doi:10.1038/s41423-020-00604-5
335. Yang M, Chen S, Huang B, et al. Pathological Findings in the Testes of COVID-19 Patients: Clinical Implications. *Eur Urol Focus*. 2020;6(5):1124-1129. doi:10.1016/j.euf.2020.05.009
336. Chen L, Huang X, Yi Z, et al. Ultrasound Imaging Findings of Acute Testicular Infection in Patients With Coronavirus Disease 2019: A Single-Center-Based Study in Wuhan, China. *J Ultrasound Med Off J Am Inst Ultrasound Med*. 2021;40(9):1787-1794. doi:10.1002/jum.15558
337. Holtmann N, Edimiris P, Andree M, et al. Assessment of SARS-CoV-2 in human semen-a cohort study. *Fertil Steril*. 2020;114(2):233-238. doi:10.1016/j.fertnstert.2020.05.028
338. La Marca A, Capuzzo M, Paglia T, Roli L, Trenti T, Nelson SM. Testing for SARS-CoV-2 (COVID-19): a systematic review and clinical guide to molecular and serological in-vitro diagnostic assays. *Reprod Biomed Online*. 2020;41(3):483-499. doi:10.1016/j.rbmo.2020.06.001
339. Alkhatatbeh H, Alzaghari D, Alkhashman A, Azab M, Edwan GMA, Abufaraj M. Does severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) cause orchitis in patients with coronavirus disease 2019 (COVID-19)? *Arab J Urol*. 2020;18(3):129-133. doi:10.1080/2090598X.2020.1798862
340. He Y, Wang J, Ren J, Zhao Y, Chen J, Chen X. Effect of COVID-19 on Male Reproductive System – A Systematic Review. *Front Endocrinol*. 2021;12. Accessed May 4, 2022. <https://www.frontiersin.org/article/10.3389/fendo.2021.677701>

341. Achua JK, Chu KY, Ibrahim E, et al. Histopathology and Ultrastructural Findings of Fatal COVID-19 Infections on Testis. *World J Mens Health*. 2021;39(1):65-74. doi:10.5534/wjmh.200170
342. Poma AM, Bonuccelli D, Giannini R, et al. COVID-19 autopsy cases: detection of virus in endocrine tissues. *J Endocrinol Invest*. 2022;45(1):209-214. doi:10.1007/s40618-021-01628-y
343. Yao XH, Luo T, Shi Y, et al. A cohort autopsy study defines COVID-19 systemic pathogenesis. *Cell Res*. 2021;31(8):836-846. doi:10.1038/s41422-021-00523-8
344. Delaroche L, Bertine M, Oger P, et al. Evaluation of SARS-CoV-2 in semen, seminal plasma, and spermatozoa pellet of COVID-19 patients in the acute stage of infection. *PLoS One*. 2021;16(12):e0260187. doi:10.1371/journal.pone.0260187
345. Gacci M, Coppi M, Baldi E, et al. Semen impairment and occurrence of SARS-CoV-2 virus in semen after recovery from COVID-19. *Hum Reprod Oxf Engl*. 2021;36(6):1520-1529. doi:10.1093/humrep/deab026
346. Li D, Jin M, Bao P, Zhao W, Zhang S. Clinical Characteristics and Results of Semen Tests Among Men With Coronavirus Disease 2019. *JAMA Netw Open*. 2020;3(5):e208292. doi:10.1001/jamanetworkopen.2020.8292
347. Purpura LJ, Alukal J, Chong AM, et al. SARS-CoV-2 RNA Shedding in Semen and Oligozoospermia of Patient with Severe Coronavirus Disease 11 Weeks after Infection. *Emerg Infect Dis*. 2022;28(1):196-200. doi:10.3201/eid2801.211521
348. Kim JM, Kim HM, Lee EJ, et al. Detection and Isolation of SARS-CoV-2 in Serum, Urine, and Stool Specimens of COVID-19 Patients from the Republic of Korea. *Osong Public Health Res Perspect*. 2020;11(3):112-117. doi:10.24171/j.phrp.2020.11.3.02
349. Ruan Y, Hu B, Liu Z, et al. No detection of SARS-CoV-2 from urine, expressed prostatic secretions, and semen in 74 recovered COVID-19 male patients: A perspective and urogenital evaluation. *Andrology*. 2021;9(1):99-106. doi:10.1111/andr.12939
350. Temiz MZ, Dincer MM, Hacibey I, et al. Investigation of SARS-CoV-2 in semen samples and the effects of COVID-19 on male sexual health by using semen analysis and serum male hormone profile: A cross-sectional, pilot study. *Andrologia*. 2021;53(2):e13912. doi:10.1111/and.13912
351. Erbay G, Sanli A, Turel H, et al. Short-term effects of COVID-19 on semen parameters: A multicenter study of 69 cases. *Andrology*. 2021;9(4):1060-1065. doi:10.1111/andr.13019
352. Pazir Y, Eroglu T, Kose A, Bulut TB, Genc C, Kadihasanoglu M. Impaired semen parameters in patients with confirmed SARS-CoV-2 infection: A prospective cohort study. *Andrologia*. Published online July 15, 2021:e14157. doi:10.1111/and.14157
353. Scropo FI, Costantini E, Zucchi A, et al. COVID-19 disease in clinical setting: impact on gonadal function, transmission risk, and sperm quality in young males. *J Basic Clin Physiol Pharmacol*. 2021;33(1):97-102. doi:10.1515/jbcpp-2021-0227



354. Evenson DP, Jost LK, Corzett M, Balhorn R. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J Androl.* 2000;21(5):739-746.
355. Ivell R. Lifestyle impact and the biology of the human scrotum. *Reprod Biol Endocrinol RBE.* 2007;5:15. doi:10.1186/1477-7827-5-15
356. Best JC, Kuchakulla M, Khodamoradi K, et al. Evaluation of SARS-CoV-2 in Human Semen and Effect on Total Sperm Number: A Prospective Observational Study. *World J Mens Health.* 2021;39(3):489-495. doi:10.5534/wjmh.200192
357. Guo J, Sheng K, Wu S, Chen H, Xu W. An Update on the Relationship of SARS-CoV-2 and Male Reproduction. *Front Endocrinol.* 2021;12:788321. doi:10.3389/fendo.2021.788321
358. Rastrelli G, Di Stasi V, Inglese F, et al. Low testosterone levels predict clinical adverse outcomes in SARS-CoV-2 pneumonia patients. *Andrology.* 2021;9(1):88-98. doi:10.1111/andr.12821
359. Ma L, Xie W, Li D, et al. Evaluation of sex-related hormones and semen characteristics in reproductive-aged male COVID-19 patients. *J Med Virol.* 2021;93(1):456-462. doi:10.1002/jmv.26259
360. Song H, Seddighzadeh B, Cooperberg MR, Huang FW. Expression of ACE2, the SARS-CoV-2 Receptor, and TMPRSS2 in Prostate Epithelial Cells. *Eur Urol.* 2020;78(2):296-298. doi:10.1016/j.eururo.2020.04.065
361. Ullah I, Prévost J, Ladinsky MS, et al. Live imaging of SARS-CoV-2 infection in mice reveals that neutralizing antibodies require Fc function for optimal efficacy. *Immunity.* 2021;54(9):2143-2158.e15. doi:10.1016/j.immuni.2021.08.015
362. Campos RK, Camargos VN, Azar SR, Haines CA, Eyzaguirre EJ, Rossi SL. SARS-CoV-2 Infects Hamster Testes. *Microorganisms.* 2021;9(6):1318. doi:10.3390/microorganisms9061318
363. Li C, Ye Z, Zhang AJX, et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections by intranasal or testicular inoculation induces testicular damage preventable by vaccination in golden Syrian hamsters. *Clin Infect Dis Off Publ Infect Dis Soc Am.* Published online February 18, 2022:ciac142. doi:10.1093/cid/ciac142
364. Madden PJ, Thomas Y, Blair RV, et al. An immunoPET probe to SARS-CoV-2 reveals early infection of the male genital tract in rhesus macaques. *BioRxiv Prepr Serv Biol.* Published online March 23, 2022:2022.02.25.481974. doi:10.1101/2022.02.25.481974
365. Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis.* 1995;1(1):7-15. doi:10.3201/EID0101.950102
366. Le Tortorec A, Matusali G, Mahé D, et al. From Ancient to Emerging Infections: The Odyssey of Viruses in the Male Genital Tract. *Physiol Rev.* 2020;100(3):1349-1414. doi:10.1152/PHYSREV.00021.2019
367. Bujan L, Mansuy JM, Hamdi S, Pasquier C, Joguet G. 1 year after acute Zika virus infection in men. *Lancet Infect Dis.* 2020;20(1):25-26. doi:10.1016/S1473-3099(19)30678-4

368. Matusali G, Houzet L, Satie AP, et al. Zika virus infects human testicular tissue and germ cells. *J Clin Invest*. 2018;128(10):4697-4710. doi:10.1172/JCI121735
369. Enikeev D, Taratkin M, Morozov A, et al. Prospective two-arm study of the testicular function in patients with COVID-19. *Andrology*. Published online February 6, 2022. doi:10.1111/andr.13159
370. Ma X, Guan C, Chen R, et al. Pathological and molecular examinations of postmortem testis biopsies reveal SARS-CoV-2 infection in the testis and spermatogenesis damage in COVID-19 patients. *Cell Mol Immunol*. 2021;18(2):487-489. doi:10.1038/s41423-020-00604-5
371. Chappell JD, Dermody TS. Biology of Viruses and Viral Diseases. *Mand Douglas Bennetts Princ Pract Infect Dis*. Published online 2015:1681-1693.e4. doi:10.1016/B978-1-4557-4801-3.00134-X
372. Bomsel M, Alfsen A. Entry of viruses through the epithelial barrier: pathogenic trickery. *Nat Rev Mol Cell Biol*. 2003;4(1):57-68. doi:10.1038/nrm1005
373. Kaur G, Dufour JM. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis*. 2012;2(1):1-5. doi:10.4161/spmg.19885
374. Le Goffic R, Mouchel T, Ruffault A, Patard JJ, Jégou B, Samson M. Mumps virus decreases testosterone production and gamma interferon-induced protein 10 secretion by human leydig cells. *J Virol*. 2003;77(5):3297-3300. doi:10.1128/jvi.77.5.3297-3300.2003
375. Lejeune H, Sanchez P, Saez JM. Enhancement of long-term testosterone secretion and steroidogenic enzyme expression in human Leydig cells by co-culture with human Sertoli cell-enriched preparations. *Int J Androl*. 1998;21(3):129-140. doi:10.1046/j.1365-2605.1998.00105.x
376. Sadri-Ardekani H, Mizrak SC, van Daalen SKM, et al. Propagation of human spermatogonial stem cells in vitro. *JAMA*. 2009;302(19):2127-2134. doi:10.1001/jama.2009.1689
377. Leal MC, Pinheiro SVB, Ferreira AJ, et al. The role of angiotensin-(1-7) receptor Mas in spermatogenesis in mice and rats. *J Anat*. 2009;214(5):736-743. doi:10.1111/j.1469-7580.2009.01058.x
378. Reis AB, Araújo FC, Pereira VM, Dos Reis AM, Santos RA, Reis FM. Angiotensin (1-7) and its receptor Mas are expressed in the human testis: implications for male infertility. *J Mol Histol*. 2010;41(1):75-80. doi:10.1007/s10735-010-9264-8
379. Pan PP, Zhan QT, Le F, Zheng YM, Jin F. Angiotensin-Converting Enzymes Play a Dominant Role in Fertility. *Int J Mol Sci*. 2013;14(10):21071-21086. doi:10.3390/ijms141021071
380. Xu P, Santos RAS, Bader M, Alenina N. Alterations in gene expression in the testis of angiotensin-(1-7)-receptor Mas-deficient mice. *Regul Pept*. 2007;138(2):51-55. doi:10.1016/j.regpep.2006.11.017

381. Hu Y, Liu L, Lu X. Regulation of Angiotensin-Converting Enzyme 2: A Potential Target to Prevent COVID-19? *Front Endocrinol*. 2021;12:725967. doi:10.3389/fendo.2021.725967
382. Zhang L, Zhou L, Bao L, et al. SARS-CoV-2 crosses the blood-brain barrier accompanied with basement membrane disruption without tight junctions alteration. *Signal Transduct Target Ther*. 2021;6(1):337. doi:10.1038/s41392-021-00719-9
383. Siemann DN, Strange DP, Maharaj PN, Shi PY, Verma S. Zika Virus Infects Human Sertoli Cells and Modulates the Integrity of the In Vitro Blood-Testis Barrier Model. *J Virol*. 2017;91(22):e00623-17. doi:10.1128/JVI.00623-17
384. Alon R, Sportiello M, Kozlovski S, et al. Leukocyte trafficking to the lungs and beyond: lessons from influenza for COVID-19. *Nat Rev Immunol*. 2021;21(1):49-64. doi:10.1038/s41577-020-00470-2
385. Wennerberg E, Kremer V, Childs R, Lundqvist A. CXCL10-induced migration of adoptively transferred human natural killer cells toward solid tumors causes regression of tumor growth in vivo. *Cancer Immunol Immunother Cll*. 2015;64(2):225-235. doi:10.1007/s00262-014-1629-5
386. Roulet V, Denis H, Staub C, et al. Human testis in organotypic culture: application for basic or clinical research. *Hum Reprod Oxf Engl*. 2006;21(6):1564-1575. doi:10.1093/humrep/del018
387. Komeya M, Kimura H, Nakamura H, et al. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Sci Rep*. 2016;6:21472. doi:10.1038/srep21472
388. Gu H, Chen Q, Yang G, et al. Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine efficacy. *Science*. 2020;369(6511):1603-1607. doi:10.1126/science.abc4730
389. Shou S, Liu M, Yang Y, et al. Animal Models for COVID-19: Hamsters, Mouse, Ferret, Mink, Tree Shrew, and Non-human Primates. *Front Microbiol*. 2021;12:626553. doi:10.3389/fmicb.2021.626553
390. Dong W, Mead H, Tian L, et al. The K18-Human ACE2 Transgenic Mouse Model Recapitulates Non-severe and Severe COVID-19 in Response to an Infectious Dose of the SARS-CoV-2 Virus. *J Virol*. 2022;96(1):e0096421. doi:10.1128/JVI.00964-21
391. Kumar A, Hou S, Airo AM, et al. Zika virus inhibits type-I interferon production and downstream signaling. *EMBO Rep*. 2016;17(12):1766-1775. doi:10.15252/embr.201642627
392. Edenfield RC, Easley CA. Implications of testicular ACE2 and the renin-angiotensin system for SARS-CoV-2 on testis function. *Nat Rev Urol*. 2022;19(2):116-127. doi:10.1038/s41585-021-00542-5
393. Osuna CE, Lim SY, Deleage C, et al. Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nat Med*. 2016;22(12):1448-1455. doi:10.1038/NM.4206

394. Carossino M, Kenney D, O'Connell AK, et al. Fatal Neurodissemination and SARS-CoV-2 Tropism in K18-hACE2 Mice Is Only Partially Dependent on hACE2 Expression. *Viruses*. 2022;14(3):535. doi:10.3390/v14030535
395. Ball EE, Pesavento P, Rompay KKAV, et al. Zika virus persistence in the male macaque reproductive tract. Published online March 4, 2022:2022.03.03.482872. doi:10.1101/2022.03.03.482872
396. Mahé D, Bourgeau S, Frouard J, et al. Long-term Zika virus infection of non-sperm cells in semen. *Lancet Infect Dis*. 2020;20(12):1371. doi:10.1016/S1473-3099(20)30834-3
397. Houzet L, Pérez-Losada M, Matusali G, et al. Seminal Simian Immunodeficiency Virus in Chronically Infected Cynomolgus Macaques Is Dominated by Virus Originating from Multiple Genital Organs. *J Virol*. 2018;92(14):e00133-18. doi:10.1128/JVI.00133-18
398. Dejuqc-Rainsford N. Is SARS-CoV-2-induced testicular damage in hamsters relevant? *Nat Rev Urol*. Published online March 31, 2022:1-2. doi:10.1038/s41585-022-00589-y
399. Li C, Ye Z, Zhang AJX, et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections by intranasal or testicular inoculation induces testicular damage preventable by vaccination in golden Syrian hamsters. *Clin Infect Dis Off Publ Infect Dis Soc Am*. Published online February 18, 2022:ciac142. doi:10.1093/cid/ciac142
400. Joguet G, Mansuy JM, Matusali G, et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. *Lancet Infect Dis*. 2017;17(11):1200-1208. doi:10.1016/S1473-3099(17)30444-9
401. Kristensen DM, Desdoits-Lethimonier C, Mackey AL, et al. Ibuprofen alters human testicular physiology to produce a state of compensated hypogonadism. *Proc Natl Acad Sci*. 2018;115(4):E715-E724. doi:10.1073/pnas.1715035115
402. Travison TG, Vesper HW, Orwoll E, et al. Harmonized Reference Ranges for Circulating Testosterone Levels in Men of Four Cohort Studies in the United States and Europe. *J Clin Endocrinol Metab*. 2017;102(4):1161-1173. doi:10.1210/jc.2016-2935
403. Delaroche L, Bertine M, Oger P, et al. Evaluation of SARS-CoV-2 in semen, seminal plasma, and spermatozoa pellet of COVID-19 patients in the acute stage of infection. *PLoS One*. 2021;16(12):e0260187. doi:10.1371/journal.pone.0260187
404. Craighead JE, Mahoney EM, Carver DH, Naficy K, Fremont-Smith P. Orchitis due to Coxsackie virus group B, type 5. Report of a case with isolation of virus from the testis. *N Engl J Med*. 1962;267:498-500. doi:10.1056/NEJM196209062671008
405. Syverton JT, Scherer WF. Studies on the propagation in vitro of poliomyelitis viruses. I. Viral multiplications in tissue cultures employing monkey and human testicular cells. *J Exp Med*. 1952;95(5):355-367. doi:10.1084/jem.96.4.355
406. Muehlenbachs A, Bhatnagar J, Zaki SR. Tissue tropism, pathology and pathogenesis of enterovirus infection. *J Pathol*. 2015;235(2):217-228. doi:10.1002/path.4438

407. Gerendai I, Tóth IE, Boldogkoi Z, Medveczky I, Halász B. Central nervous system structures labelled from the testis using the transsynaptic viral tracing technique. *J Neuroendocrinol.* 2000;12(11):1087-1095. doi:10.1046/j.1365-2826.2000.00560.x
408. Matos B, Publicover SJ, Castro LFC, Esteves PJ, Fardilha M. Brain and testis: more alike than previously thought? *Open Biol.* 2021;11(6):200322. doi:10.1098/rsob.200322
409. Marocci ME, Napoletani G, Protto V, et al. Herpes Simplex Virus-1 in the Brain: The Dark Side of a Sneaky Infection. *Trends Microbiol.* 2020;28(10):808-820. doi:10.1016/j.tim.2020.03.003
410. Cheung WY, Chan AC, Loke SL, et al. Latent sites of Epstein-Barr virus infection. *Am J Clin Pathol.* 1993;100(5):502-506. doi:10.1093/ajcp/100.5.502
411. Zhang N, Zuo Y, Jiang L, Peng Y, Huang X, Zuo L. Epstein-Barr Virus and Neurological Diseases. *Front Mol Biosci.* 2021;8:816098. doi:10.3389/fmolb.2021.816098
412. Ibrahim IM, Abdelmalek DH, Elshahat ME, Elfiky AA. COVID-19 spike-host cell receptor GRP78 binding site prediction. *J Infect.* 2020;80(5):554-562. doi:10.1016/j.jinf.2020.02.026
413. Meertens L, Labeau A, Dejarnac O, et al. Axl Mediates ZIKA Virus Entry in Human Glial Cells and Modulates Innate Immune Responses. *Cell Rep.* 2017;18(2):324-333. doi:10.1016/J.CELREP.2016.12.045
414. Shen Q, Xiao X, Aierken A, et al. The ACE2 expression in Sertoli cells and germ cells may cause male reproductive disorder after SARS-CoV-2 infection. *J Cell Mol Med.* 2020;24(16):9472-9477. doi:10.1111/jcmm.15541
415. Guo J, Grow EJ, Mlcochova H, et al. The adult human testis transcriptional cell atlas. *Cell Res.* 2018;28(12):1141-1157. doi:10.1038/s41422-018-0099-2
416. Stanley KE, Thomas E, Leaver M, Wells D. Coronavirus disease-19 and fertility: viral host entry protein expression in male and female reproductive tissues. *Fertil Steril.* 2020;114(1):33-43. doi:10.1016/j.fertnstert.2020.05.001
417. Hikmet F, Méar L, Edvinsson Å, Micke P, Uhlén M, Lindskog C. The protein expression profile of ACE2 in human tissues. *Mol Syst Biol.* 2020;16(7):e9610. doi:10.15252/msb.20209610
418. Hermann BP, Cheng K, Singh A, et al. The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. *Cell Rep.* 2018;25(6):1650-1667.e8. doi:10.1016/j.celrep.2018.10.026
419. Zhou L, Niu Z, Jiang X, et al. SARS-CoV-2 Targets by the pscRNA Profiling of ACE2, TMPRSS2 and Furin Proteases. *iScience.* 2020;23(11):101744. doi:10.1016/j.isci.2020.101744
420. Wang Z, Xu X. scRNA-seq Profiling of Human Testes Reveals the Presence of the ACE2 Receptor, A Target for SARS-CoV-2 Infection in Spermatogonia, Leydig and Sertoli Cells. *Cells.* 2020;9(4):E920. doi:10.3390/cells9040920

421. Wang M, Liu X, Chang G, et al. Single-Cell RNA Sequencing Analysis Reveals Sequential Cell Fate Transition during Human Spermatogenesis. *Cell Stem Cell*. 2018;23(4):599-614.e4. doi:10.1016/j.stem.2018.08.007
422. Darde TA, Lecluze E, Lardenois A, et al. The ReproGenomics Viewer: a multi-omics and cross-species resource compatible with single-cell studies for the reproductive science community. *Bioinforma Oxf Engl*. 2019;35(17):3133-3139. doi:10.1093/bioinformatics/btz047
423. Darde TA, Sallou O, Becker E, et al. The ReproGenomics Viewer: an integrative cross-species toolbox for the reproductive science community. *Nucleic Acids Res*. 2015;43(W1):W109-116. doi:10.1093/nar/gkv345
424. Bertram S, Glowacka I, Müller MA, et al. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *J Virol*. 2011;85(24):13363-13372. doi:10.1128/JVI.05300-11
425. Kumar A, Jovel J, Lopez-Orozco J, et al. Human Sertoli cells support high levels of Zika virus replication and persistence. *Sci Rep*. 2018;8(1). doi:10.1038/S41598-018-23899-X
426. Harrison AG, Lin T, Wang P. Mechanisms of SARS-CoV-2 Transmission and Pathogenesis. *Trends Immunol*. 2020;41(12):1100-1115. doi:10.1016/J.IT.2020.10.004
427. Ding Q, Gaska JM, Douam F, et al. Species-specific disruption of STING-dependent antiviral cellular defenses by the Zika virus NS2B3 protease. *Proc Natl Acad Sci U S A*. 2018;115(27):E6310-E6318. doi:10.1073/PNAS.1803406115

# Acknowledgments

While I am running out of time and will hopefully extend them in a final version of this manuscript,

I would like to do a first round of short acknowledgements:

To the members of my jury for accepting to evaluate my work: I would like to thank Dr Béatrice Grasland and Dr Pierre Roques who accepted to be reviewers, as well as Pr Xiaozhen Liang, Pr Jin Zhong and Pr Vincent Thibault for accepting to examine this thesis. I would like to thank you all again for your valuable reviews, great discussion and kind words.

To Dr Bernard Jégou, Dr Michel Samson and Mr Shengli Si, for welcoming me to their institutes.

To Mrs Lu Ye, Hui Wang, Yudong Tan, Mr Chenhao Xu, and Ms Qian Li for their administrative help.

To Dr Wei Yu and Dr Jacques Le Seyec, for having accepted to be part of my thesis committee and for their precious comments and advice.

To Dr Nathalie Dejuçq-Rainsford and Dr Dimitri Lavillette, for having believed in me at the beginning of this adventure and for having encouraged me until the end. Thank you for your teachings, your guiding, and for your help in the various stages of this PhD.

I would like to extend my sincere thanks to Dr Dominique Mahé, for your constant help, support, understanding and caring.

I thank all the persons who participated in the presented work, which has mainly been a group effort.

I would also like to thank all the members of the URGENT team at IRSET as well as all the Arbovirus team in IPS, for their help, teaching and support.

Finally, shortly but not to forget anyone, I would like to thank my family and friends for always having been present, supportive, understanding and caring, through all the steps of my PhD/life until now:

I talk about la smala Benassaya, les Jeanzettes (boys included), les Lardons du CRI, Léo' et les Jumeaux (sans transition), les Serpentards (aka Le Noyau Dur), les Papayes, Pasteuriens et autres Niggas in Paris, the Oda'gang, the UI-team8, Jules & JLow, the CAS Cool Guys, the Silk Road Trip Gang & other Beijingers, les Français De Passage, the Girls Kingdom & the SanHe Garden Party, le Frexit, the Shanghai Zombies & the Trippy group, la PandémIRSET & Le Pain Bordel, as well as the Avengers.

They'll know.





**Titre : Infection du testicule humain par des virus émergents : le cas des virus Zika et SARS-CoV-2**

**Mots clés :** Virus émergents, testicule, sperme, Zika, SARS-CoV-2, tropisme

**Résumé :** La transmission sexuelle des virus est une préoccupation majeure de santé publique. Plus de 30 virus contemporains et émergents ont été retrouvés dans le sperme, et une partie d'entre eux sont excrétés de façon prolongée malgré une clairance systémique. Le testicule, en tant qu'organe immuno-privilegié, est suspecté être responsable de cette excrétion prolongée. A ce jour l'origine tissulaire de la plupart des virus excrétés dans le sperme, ainsi que l'effet de ces infections sur la fertilité masculine et le système endocrinien, restent mal caractérisés. Lors d'une récente épidémie, le virus Zika (ZIKV), un arbovirus en émergence, a été retrouvé de manière inattendue dans le sperme de patients plus d'un an après l'apparition des symptômes (dps). Notre équipe a révélé que le ZIKV se réplique dans le testicule humain. Mes travaux ont contribué à la mise en évidence de cellules germinales testiculaires infectées dans le sperme de patients jusqu'à 160 dps, indiquant que le testicule est un réservoir pour ZIKV.

Le SARS-CoV-2, un nouveau beta coronavirus, est responsable d'une pandémie en cours. Son principal récepteur d'entrée, ACE2, est fortement exprimé dans le testicule et des modifications hormonales et des paramètres du sperme ont été observées chez les hommes infectés. Mes travaux indiquent que le SARS-CoV-2 se réplique dans le testicule humain *ex vivo* et qu'il cible principalement les cellules de Leydig et de Sertoli, impactant la transcription d'enzymes de la stéroïdogénèse. La détection de cellules de Sertoli infectées dans le sperme d'un patient confirme l'infection du testicule. Finalement, afin d'aider à prédire la sensibilité du testicule humain à d'autres virus émergents, j'ai établi une liste de récepteurs viraux et analysé leur expression dans les cellules testiculaires à partir de données de transcriptomique à l'échelle de la cellule unique (scRNA-seq). L'ensemble de mon travail de thèse a ainsi contribué à dévoiler les interactions entre les virus émergents et le testicule humain.

**Title : Infection of the human testis by emerging viruses : the case of Zika virus and SARS-CoV-2**

**Keywords :** Emergent viruses, testis, semen, Zika, SARS-CoV-2, tropism

**Abstract:** Sexual transmission of viruses is a major public health concern. Over 30 contemporary and emerging viruses have been reported in semen, and some are excreted in a prolonged manner despite systemic clearance. The testis, as an immuno-privileged organ, is suspected to be responsible for this prolonged shedding. To date, the tissue origin of most viruses excreted in semen, as well as the effect of these infections on male fertility and the endocrine system, remain poorly characterized. In a recent epidemic, Zika virus (ZIKV), an emerging arbovirus, was unexpectedly found excreted in semen of patients over a year post-symptoms onset (dps). Our team revealed that ZIKV replicates in the human testis. My work contributed to the identification of infected testicular germ cells in patients' semen up to 160 dps, indicating that the testis is a reservoir for ZIKV.

SARS-CoV-2, a novel betacoronavirus, is responsible for an ongoing pandemic. Its main entry receptor, ACE2, is highly expressed in the testis, and changes in hormonal production and sperm parameters have been observed in infected men. My work indicates that SARS-CoV-2 replicates in the human testis *ex vivo* and that it mainly targets Leydig and Sertoli cells, impacting the transcription of steroidogenic enzymes. The detection of infected Sertoli cells in a patient's semen confirms testicular infection. Finally, to help predict the susceptibility of the human testis to other emerging viruses, I have compiled a list of viral receptors and analyzed their expression in testicular cells using single cell transcriptomic data (scRNA-seq). Altogether, my PhD work has thus contributed to unveil the interactions between emerging viruses and the human testis.