# The effect of oleic acid supplementation on lipid droplet production, beta-oxidation and rotavirus replication

By

**Tshegofatso B. Thobane**

Submitted in fulfilment of the requirements in respect of the Magister Scientiae in Biochemistry specialization in the Department of Microbial and Biochemical in the Faculty of Natural and Agricultural Sciences at the University ofthe Free State

Supervisor: Prof. H.G. O'Neill

Co-Supervisor: Prof. C.H. Pohl-Albertyn and Mr. WJ Sander

November : 2022

DECLARATION

I, Tshegofatso Benedict Thobane, declare that the master's degree research dissertation and coursework master's degree mini dissertation that I herewith submit for the master's degree qualification at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at any other institution of higher learning.

I, Tshegofatso Benedict Thobane, hereby declare that I am aware that the copyright is vested in the University of the Free State in which the study was conducted.

I, Tshegofatso Benedict Thobane, hereby declare that all royalties regarded as intellectual property that was developed during and/or in connection with the study at the University of the Free State, will accrue to the University.

I, Tshegofatso Benedict Thobane, hereby declare that I am aware that the research may only be published with the approval of the dean of the Natural and Agricultural Science at the University of the Free State.

Signature

30/11/2022

Date

I would like to take this opportunity to appreciate and thank my supervisor **Prof. Hester O'Neill** for firstly allowing me to embark on this scientific journey with her in my post-graduate studies. I thank her for her ever-lasting support and guidance for both the completion of my degree and the personal challenges I experienced. I am grateful for her honest and truthful critiques, advice and her commitment to develop and share her vast knowledge with me as an upcoming scientist.

It would entirely be wrong of me not to acknowledge two key role players of the development of this master's thesis, being my co-supervisors **Prof. Carolina Pohl-Albertyn** and **Mr. Willem Sander**. I would like to thank **Prof. Pohl-Albertyn** for eagerly and always being readily available to support and guide me in ensuring I complete this project. I will always be grateful for her sharp advice that has shaped me to be a better person and scientist. **Mr. Willem Sander,** I have not been the best of students to work with, but I am appreciative and grateful for your guidance and a hands-on approach with my experimental procedures to complete this study and always being readily available to assist with data-analysis and troubleshoot guidance.

I am grateful for the technical and personal support provided to me by **Ms. Nkhasi Lekena** and **Mr. Ayanda Thusi** from the Molecular/Clinical Biochemistry Laboratory.

I am thankful to **Dr. Annika Bagge** and colleagues (Agilent Technologies) for their efficient assistance in the optimization of the oxygen consumption assays and analysis. I acknowledge **Prof. Opperman** for allowing us to use their spectrophotometer for the analysis of fluorescence assays in this study.

Most importantly, I would like to acknowledge and thank my **family, friends and my partner Ms. Shireen Walters** for their unfailing support and encouragement.

I would like to thank and acknowledge my sponsors the **National Research Foundation** and the **Allan Gray Orbis Foundation** for their financial contributions to my studies and well-being.

Table of Contents

Table of Figures

Table of Tables

Abbreviations

ACC1 - Acetyl-CoA carboxylase 1

ADRP - Adipose differentiation-related protein

ARF1 - ADP-ribosylation factor 1

ATGL - Adipose tissue triacylglycerol lipase

ATGL - Adipose triglyceride lipase

bp - Base pairs

$Ca^{2+}$ - Cytosolic lowered calcium

CAD - Acyl-CoA dehydrogenases

CCT - CTP:phosphocholine cytidylyltransferase

CNS - Central nervous system

COX - Cyclooxygenase

DAG - Diacylglycerides

DGAT1 - Diacylglycerol acyltransferase 1

DGAT2 - Diacylglycerol acyltransferase 2

DLP – Double layered particles

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

dsRNA - Double-stranded RNA

eLDs - expanding lipid droplets

ER – Endoplasmic reticulum

ETC - Electron transport chain

ETO - Etomoxir

FA - Fatty acids

FASN - Fatty acid synthetases

FBS - Fetal bovine serum

FFAR4 - Long-chain fatty acid receptor

FIT - Fat storage-inducing transmembrane

HCV - Hepatitis C virus

HEK 293 cell line - Human embryonic kidney 293 cell line

HEPES buffer - N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffer

HSL - Hormone-sensitive lipase

IBMX - 3-isobutyl-1-methylxanthine

iLDs – Initial lipid droplets

ISP - Isoproterenol

LCFA - Long chain fatty acid

LD - Lipid droplet

MA104 cell line- African green monkey kidney cell line.

MAG - Monoacylglycerides

MGL - Monoacylglycerol lipase

MTP - Mitochondrial trifunctional protein

$N_2$ gas – Nitrogen gas

NEAA - Nonessential amino acids

NLSE - Neutral lipid-synthesizing enzymes.

NPs - Nonstructural proteins

NS - Non-structural

OA - Oleic acid

OCR - Oxygen consumption rate

ORF1 - Open reading frame 1

p.i - post-infection

PA - Phosphatidic acid

PBS - Phosphate buffered saline

$PLA_2$ - Phospholipase $A_2$

PLIN - Perilipin proteins

Rab - Ras-related protein

ROS - Reactive oxygen species

RV - Rotavirus

SA11 - Simian rotavirus strain 11

siRNA - Small interfering RNA

SLP - Single-layered particle

ssRNA - Single-stranded RNA

TAG - Triacylglycerrides

TCA - Acetyl-CoA for the citric acid

$TCID_{50}$ - 50% Tissue culture infectious doses

TLP – Triple layered particles

TOFA - 5-(tetradecyloxy)-2-furoic acid

VLS – Viroplasm like structures

VPs - Viral proteins

## Units

% - Percentage

µg/mL - Microgram per milliliter

µL - Microliter

µM - Micromolar

µmol/mg cells - Micromole per milligram of cells

cells/mL – Cells per milliliter

$cm^2$ - Square centimeter

g - Gram

h – Hour

M - Molar

mg - Milligrams

min - Minute

mL- Milliliters

mM - Millimolar

nM/mg of cells – Nanomolar per milligram of cells

nmol/well – Nanomole per well

$^oC$ – Degree Celsius

pfu/mL – Plaque forming units per milliliter

$TCID_{50}$/mL – 50% tissue culture infectious dose per milliliter

U/mL - Units per milliliter

v/v – Volume per volume

w/v – Weight per volume

Summary

Rotavirus (RV) is the most common cause of severe acute gastroenteritis in infants and young children globally. Rotavirus infections induce cytoplasmic inclusion bodies called viroplasms serving as a site of RV genome replication and assembly. Viroplasms recruit lipid droplets (LDs), which play major roles in energy homeostasis and is a site for lipid storage. The successful formation of viroplasm-LD complexes is essential for RV propagation. The extracellular supply of oleic acid (OA, C18:1) to host cells induce LD biogenesis and modulate cellular fatty acid (FA) metabolism. Rotavirus modulates the host cell lipidome and influence cell LD biogenesis and FA metabolism by β-oxidation for successful viral propagation. Previous studies have shown that chemical fragmentation of LDs by treatment with 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) to be deleterious for RV infection. In addition, the chemical modulation of FA β-oxidation by etomoxir (ETO) treatment has been shown to affect the propagation of other viruses including hepatitis C virus (HCV). In this study, we sought to investigate **(1)** the effect of OA supplementation on LDs during RV infection, **(2)** the effect of OA supplementation on FA β-oxidation during RV infection and lastly, **(3)** the effect of LD and FA β-oxidation modulation on RV infection.

Briefly, HEK293 cells were supplemented with OA in the presence and absence of IBMX and ISP as well as ETO treatment during RV infection (MOI=5) for 6 h post infection (p.i). Control cells were not treated with OA in the presence and absence of RV infection in which they were also maintained in parallel with the test samples. At the end of each timepoint p.i, LDs where isolated for triacylglyceride (TAG) quantification (LD quantification) while the test samples treated with the same conditions were analyzed for changes in oxygen consumption rate (OCR) (FA β-oxidation quantification) in HEK293 cells. For viral replication kinetics, HEK293 cells were again supplemented with OA and treated with IBMX, ISP and ETO during RV infection as previously describe for 0 h, 2 h, 6 h, 12 h and 16 h p.i, respectively. At the end of each time point, RV was harvested and quantified using tissue culture infection doses 50 ($TCID_{50}$).

Our study shows that supplementation of HEK293 cells with OA increase the overall TAG content in isolated LDs, while the treatment of HEK293 cells with IBMX and ISP reduce TAG content of isolated LDs. We further show an increase in total TAG content of isolated LDs when HEK293 cells are treated with ETO. Rotavirus infection increased the total TAG content of isolated LDs. The supplementation of HEK293 cells with OA increased HEK293 cell OCR, while the treatment with IBMX, ISP and ETO reduced the overall host cell OCR. Treatment of OA-supplemented

HEK293 cells with IBMX, ISP and ETO slightly increased the overall OCR of the treated HEK293 cells compared to the OA unsupplemented HEK293 treated with the chemical inhibitors. It is also important to note that RV infection reduced the overall OCR of HEK293 cells. Analysis of viral kinetics showed that OA supplementation increases RV replication over time, while the treatment of HEK293 cells with IBMX, ISP and ETO in the presence or absence of OA supplementation reduce the overall replication of RV over time. The OA supplementation of RV infected HEK293 cells with IBMX and ISP rescues viral propagation over time, which was not observed when HEK293 cells were treated with ETO.

The data thus show that OA supplementation increases total TAG content in isolated LDs as well as the overall host OCR while promoting RV infection over time. Rotavirus infection promotes enrichment of LDs by increasing the overall TAG content of isolated LDs while reducing the overall OCR of host cells. It was shown that the chemical modulation of both LD biogenesis and FA β-oxidation is deleterious to RV propagation over time.

Keywords: Rotavirus (RV) • Viroplasms • Lipid droplets • β-oxidation • Energy homeostasis • Fatty acids (FA) • Triacylglycerides (TAG) • Oleic acid • 3-isobutyl-1-methylxanthine (IBMX) • Isoproterenol (ISP) • Etomoxir (ETO) • Oxygen consumption rate (OCR) • Tissue culture infection doses 50 ($TCID_{50}$)

## Conferences

1. TB Thobane, WJ Sander, CH Pohl-Albertyn, HG O'Neill. "**Investigation into the effects of oleic acid (OA) supplementation on lipid droplet formation during rotavirus infection."** South African Society for Microbiology Conference.
   Host: University of the Free State, South Africa.
   Date: 4 – 6 May 2021.

## 1.1. Introduction

Rotavirus (RV) is one of the main causes of severe dehydrating diarrhea in newborn babies and young children globally, responsible for many deaths, especially in developing countries (Estes & Greenberg, 2013; Parashar et al., 1998; Troeger et al., 2018). Rotavirus is transmissible via the faecal-oral route and infects the host's villous epithelium of the small intestine (Estes & Greenberg, 2013). It is a non-enveloped, icosahedral virus belonging to the genus Rotavirus in the *Sedoreoviridae* family and named after the Latin word "Rota", that means "wheel" as it has a viral particle that appears to have a distinctive well-defined rim and short spikes radiating from the viral particle core center (Crawford et al., 2017).

The virus is composed of a double-stranded RNA (dsRNA) genome that has eleven segments transcribed and translated to produce six structural (viral proteins (VPs)) and six non-structural proteins (NSPs) (Lever & Desselberger, 2016). During RV infection, the replication of the virus occurs in viroplasms which are cytoplasmic inclusion bodies that serve as sites for viral replication, in which they also coordinate genome packaging (Cheung et al., 2010). The initial formation of viroplasms is dependent on the co-expression of various viral NSPs and these viroplasms later recruit and associate with lipid droplets (LDs) during RV infection (Cheung et al., 2010). Lipid droplets are cellular organelles that primarily serve as intracellular storage sites for neutral lipids (Brasaemle & Wolins, 2012; Guo et al., 2009a; Kuerschner et al., 2008; Walther & Farese, 2012). Lipid droplets also play major roles in energy homeostasis and eicosanoid production (Bozza et al., 1997; Dvorak et al., 1992; Robenek et al., 2005). Different viruses including RV modulate host cell total lipid content, while also affecting the metabolism of intracellular FA as well as FA liberated from LDs by FA β-oxidation (Gaunt et al., 2013b; Heaton & Randall, 2010; Lever & Desselberger, 2016; Rasmussen et al., 2011). The process of viroplasms formation, LD formation and FA β-oxidation are crucial for successful viral replication as the inhibitory effect of these processes hinder the overall replication of a wide range of viruses (Cheung et al., 2010; Crawford & Desselberger, 2016; Rasmussen et al., 2011).

In the review, the current knowledge of the association of LD formation and FA β-oxidation during RV infection will be reviewed. The impact of exogenous FAs on both processes and RV infections will be elucidated as well as the possibility of targeting both LD formation and FA β-oxidation as therapeutic sites for the reduction of RV replication.

## 1.2. Rotavirus

### 1.2.1. Genome segments, assignments, structure and function

Rotavirus genome consists of approximately 18 500 base pairs (bp) with individual segments ranging from 670 to 3 302 bps varying amongst strains (Mlera et al., 2013). The structural proteins known as viral proteins (VPs) of RV include VP1 to VP4, VP6, and VP7 while the NSPs include NSP1 to NSP5/6 (Estes and Greenberg., 2013). Rotavirus particles consist of VPs while the NSPs are found during viral replication and within non-mature viral particles (Yeager et al., 1990). Rotaviruses are subdivided into different groups/species (A–J) (Matthijnssens et al., 2022). The specific groups are distinguished between each other based on their surface proteins (VP7 and VP4) as G types (the outer shell glycoproteins) or P types (for protease-susceptible protein) and there is at least 42 different G and 58 different P types within Group A (**Table 1.1**) (Rotavirus Classification Working Group: (RCWG). Available online: https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg (accessed on 29 November 2022)). Rotavirus present a distinctive triple-layered particle (TLP) architecture enclosing the segmented dsRNA genome (**Figure 1.1**) (Jayaram et al., 2004; McClain et al., 2010; Trask et al., 2012a; Trask et al., 2012b). The viral replication complexes (VP1 and VP3) are located within the center of the RV virions, and it is suggested that the genomic RNA segments are arranged to form a conical cylinder (Lu et al., 2008). These viral replication complexes and RNA are surrounded by VP2 60 dimers, forming a single-layered particle (SLP) also known as the core-shell (McClain et al., 2010). The double-layered particles (DLPs), although transcriptionally active, are known to be non-infectious, and form through the exclusion of viral outer layer VP4 and VP7 (Teimoori et al., 2014).

Table 1.1. Summary of rotavirus genome segments in terms of size, genotypes, encoded protein, protein location and function

| Segment | Gene Size (bp) | Protein | Molecular Weight (kDa) | Number of Genotypes | Location | Function |
|---|---|---|---|---|---|---|
| **1** | 3302 | VP1 | 125 | 28R | Vertices of viral inner capsid | RNA- dependent RNA polymerase<br>Responsible for the binding of single stranded RNA<br>Forms part of the viral transcription complex |
| **2** | 2683 | VP2 | 102 | 24C | Inner capsid of virus | RNA binding<br>Structurally forms part of the viral core shell |
| **3** | 2591 | VP3 | 98 | 24L | Vertices of the inner viral capsid | Viral mRNA capping enzyme<br>Capabilities of Guanyltransferase and methyltransferase |
| **4** | 2362<br>[VP5/VP6]* | VP4 | 87 | 58P | Outer capsid | Spike protein and responsible for cell attachment<br>Fusion with host cell membrane<br>P – Type neutralization |
| **5** | 1614 | NSP1 | 59 | 39A | Nonstructural viral protein | Antagonist of host interferon response<br>RNA Binding |
| **6** | 1356 | VP6 | 48 | 32I | Viral intermediate capsid | Required for viral RNA transcription |
| **8** | 1059 | NSP2 | 34 | 28N | Nonstructural viral protein | NTPase and RNA-Binding. Responsible for the formation of viroplasms |
| **7** | 1105 | NSP3 | 37 | 28T | Nonstructural viral protein | Facilitates the translation of viral mRNA transcript and shut-off of cellular protein synthesis |
| **9** | 1063 | VP7 | 37 | 42G | Viral outer capsid | Structural and neutralizing agent.<br>Membrane penetration |
| **10** | 751 | NSP4 | 20 | 32E | Nonstructural viral protein | Intracellular receptor for new viral particles.<br>Enterotoxin.<br>Modulates intracellular $Ca^{2+}$ |
| **11** | 667 | NSP5 | 22 | 28H | Nonstructural viral protein | RNA binding capability.<br>Binding partner of NSP2 in the formation of viroplasms during rotaviral infection |
| | | NSP6 | 11 | - | Nonstructural viral protein | Viral genome replication and packaging.<br>Interaction with host mitochondria |

(Table adapted from Burrone et al., 1999; Clark & Desselberger, 1988; Desselberger, 2014; Liu et al., 1992; Matthijnssens et al., 2022; Mlera et al., 2013; Patton et al., 1997; Patton, 2001; Prasad et al., 1996; Taraporewala et al., 2002; Rotavirus Classification Working Group: (RCWG). Available online:https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg (accessed on 29 November 2022))

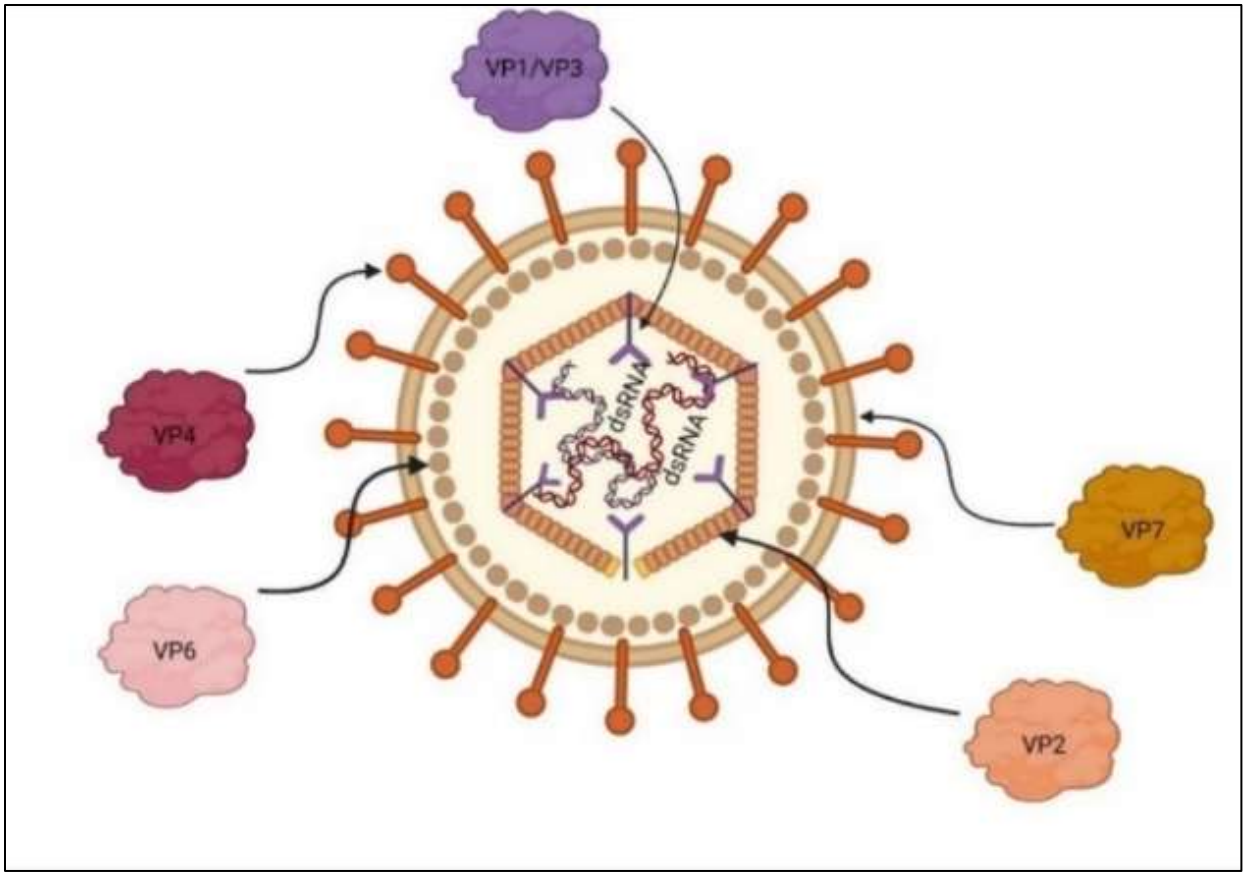


**Figure 1.1. The general structure and morphology of a rotavirus particle** (Figure adapted from Crawford et al., 2017). Designed by BioRender.

### 1.2.2. Replication overview

In general, the RV replication cycle involves RV initially attaching to the cell of the host, the formation of viroplasms, and viral particle maturation into infectious TLPs (Desselberger, 2014; McNeal & Bernstein, 2014) (**Figure 1.2**). Initially, RV attach to various host cell receptors located on the cell surface mediated by VP4 and enter the host cell by endocytosis (Dormitzer et al., 2002; Haselhorst et al., 2009; Hu et al., 2012; Huang et al., 2012; Ramani et al., 2013) (**Figure 1.2A**). This is followed by the release of viral double-layered particles (DLP) from the early endosome and the uncoating of the viral particle due to lower cytosolic calcium ($Ca^{2+}$) concentrations (Cohen et al., 1979). Once the viral DLPs that are transcriptionally active are released into the cell's cytoplasm, they release the eleven segmented dsRNAs which are transcribed by an RNA-dependent polymerase (Cohen et al., 1979; Lu et al., 2008) (**Figure 1.2B**). The transcribed mRNA act as templates for the translation of more viral proteins and the synthesis of dsRNA to be packaged into newly synthesized viral DLPs (Desselberger et al., 2013) (**Figure 1.2B**). The initial stages of viral genome production and packaging occur in viroplasms, which recruit and associate with LDs during RV replication (Cheung et al., 2010) (**Figure 1.2C**). Viroplasms are sites for viral core production and these particles normally harbor VP1-VP3 and viral RNA (Eichwald et al., 2004). Rotavirus core proteins are translocated to the cell surface of LDs where viral RNA replication complexes are recruited to the endoplasmic reticulum (ER) membrane and encapsulation occurs (Walczak et al., 2014) (**Figure 1.2D**). Viral protein 6 (induce the extracellular signal-regulated kinase (ERK) phosphorylation) resulting in an increase in calcium influx (Lobeck et al., 2016) is also found in viroplasms, leading to DLP formation which are later released into the host cell cytoplasm. The newly produced DLPs bind to NSP4 which also serves as a receptor for the ER (Walczak et al., 2014). These viral DLPs receive their outer layer through the rough ER when they are released from viroplasms, to produce infectious viral TLPs (Patton et al., 1993) (**Figure 1.2E,F**). The observed transient membrane is broken down and removed as the viral VP4 & VP7 assemble to form mature RV infectious TLP (**Figure 1.2F**) (Crawford et al., 2017; Desselberger, 2014). The mature RV TLP exit the host cell either by exocytosis or cell lysis (**Figure 1.2G**).

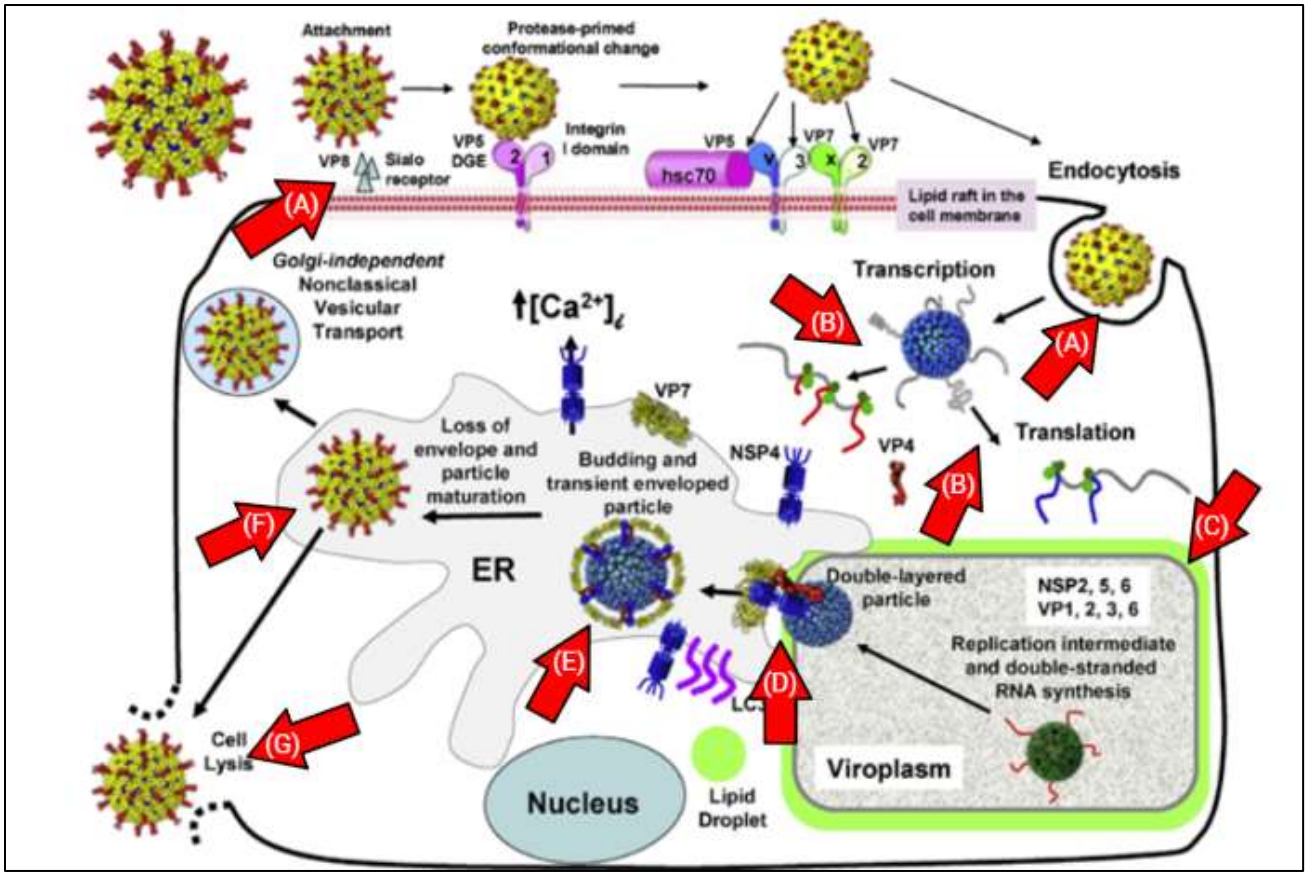


**Figure 1.2. An overview of rotavirus (RV)** (A) attachment and entry, (B) double stranded RNA (dsRNA) genome transcription and translation, (C) viroplasms formation, (D) full-length transcript encapsulation, (E) formation of inner intermediate capsid, (F) rotavirus (RV) maturation and (G) release of infectious RV triple-layered particles during viral replication (Desselberger, 2014). The figure is presented with the journal's permission (**Annexure B1**).

### 1.2.3. Viroplasms formation and structure

Viroplasms play a crucial role in ensuring that the assembly of viral capsid proteins, genome packaging, and viral replication is highly coordinated during RV infection (McDonald & Patton, 2011). Although several viral proteins (NSP6 and VP1/2/3/6) are observed in viroplasms (Altenburg et al., 1980), NSP2 and NSP5 are necessary for the initial formation of viroplasms and recruit other viral and viral dsRNA (Crawford & Desselberger, 2016). The importance of NSP2 and NSP5 in the formation of viroplasms is supported by the observation that, combined they can induce the formation of viroplasms-like structures (VLS) in non-viral infected host cells (Burrone et al., 1999; Patton et al., 2006). Modulating the function or blocking the expression of NSP2 or NSP5 in RV infected hosts is sufficient to reduce viroplasm formation and the production of

infectious viral progeny (Cheung et al., 2010). NSP5-specific small interfering RNA (siRNA) is reported to block the association of LD-associated proteins (perilipin A) with viral associated protein (NSP5) in viroplasms. (Campagna et al., 2005; Taraporewala et al., 2002; Vascotto et al., 2004). RNA interference in the expression of either NSP2 or NSP5 proteins or the use of intrabodies prevents the formation of viroplasms and subsequently effecting virion production (López et al., 2005; Silvestri et al., 2004; Taraporewala et al., 2002; Vascotto et al., 2004). The number of viroplasms-LD complexes increases during RV replication cycles and viroplasms recruit LD components during the time course of RV infection (Cheung et al., 2010). Although a model for viroplasms-LD interaction has been proposed (**Figure 1.3**), detailed mechanisms of the formation and interaction remain unknown. Both LDs and viroplasms have phosphoproteins (NSP5 and perilipin A) inserted around these structures (Eichwald et al., 2004; Marcinkiewicz et al., 2006).

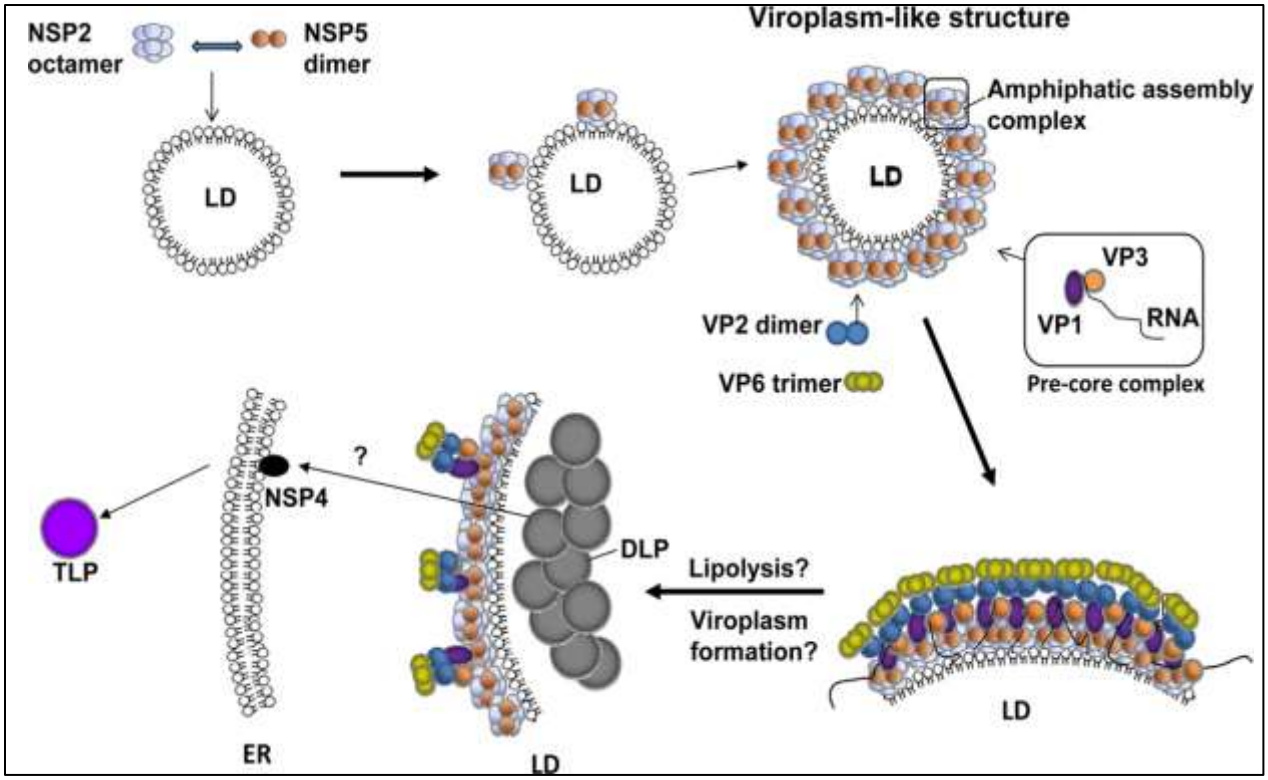


**Figure 1.3. The proposed model of early rotavirus (RV) morphogenesis.** Lipid droplets (LDs) serve as a foundation or base to which viral RV NSP 2 and NSP 5 attach and undergo condensation at low concentrations, forming protein droplets known as 'viroplasms-like structures' (Burrone et al., 1999). The formed VLS later interacts with other viral proteins (VP1, VP2, VP 3, and VP6) and RNA to form Viroplasms. This process is highly likely to be followed by lipolysis (Cheung et al., 2010). The figure is presented with the journal's permission (**Annexure B2**).

## 1.3. Lipid droplets

### 1.3.1. Structure and function

Lipid droplets (LDs) are spherical intracellular organelles that contain triacylglycerides (TAG) and sterol esters in their core and are surrounded by a phospholipid monolayer (Murphy et al., 2009). Lipid droplets are present in all eukaryotic cells and play different roles in addition to being a site for neutral lipid storage (Walther & Farese, 2009). The initial perception on LDs was that they only served as temporary storage sites for extra fat from which neutral lipids are rapidly broken down when there is a depletion of carbon sources and more energy is required by the host cell (Carmen & Víctor, 2006; Marcinkiewicz et al., 2006), Studies now show LDs as more dynamic and functional organelles that are actively involved in a wide range of cellular processes including lipid homeostasis, membrane trafficking, signal transduction, and the modulation of immune and

inflammatory responses (Ohsaki et al., 2014; Saka & Valdivia, 2012; Walther & Farese, 2009).

The different proteins and enzymes associated with LDs may also contribute to the overall function of LDs during viral replication. Proteins with predicted membrane insertion including cyclooxygenase-2 (COX-2) have also localized with LDs (Bozza et al., 1997; Dvorak et al., 1992; Robenek et al., 2005) suggesting a role of LDs in eicosanoid production. Other important metabolic proteins and enzymes that localize with LDs include lipid-synthesis enzymes (acetyl coenzyme A (CoA) carboxylase, acyl-CoA synthetase and acyl-CoA diacylglycerol acyltransferase 2 (DGAT2) (Kuerschner et al., 2008; Stone et al., 2009). Various pathogens, viruses, bacteria, and parasites specifically target host cells' LDs during their life cycle (Monson et al., 2021; Roingeard & Melo, 2017).

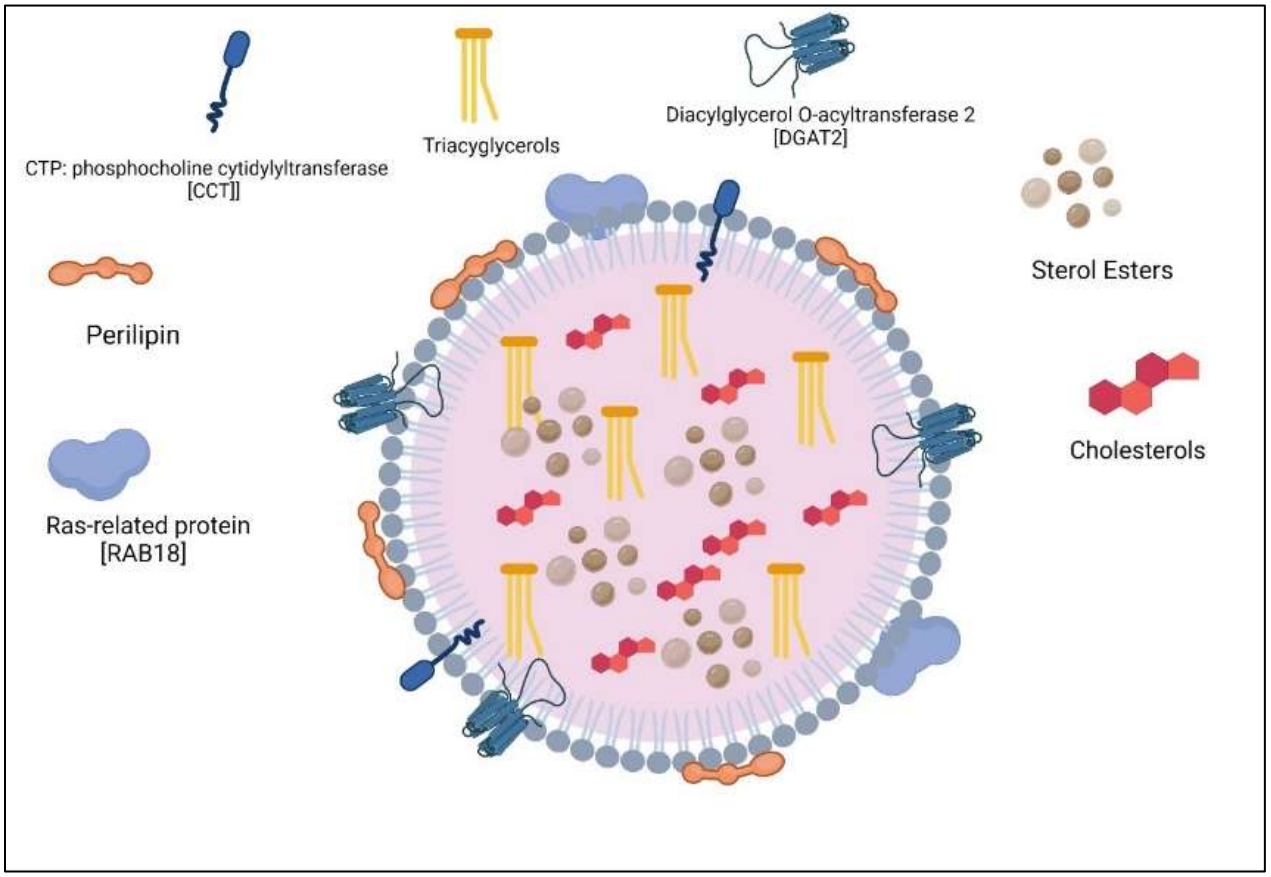


**Figure 1.4. The structure of lipid droplets with different associated lipids and proteins.** (Adapted from Bozza & Viola, 2010; Brasaemle, 2007; Krahmer et al., 2009; Murphy et al., 2009; Onal et al., 2017; Wolins et al., 2001). Designed with BioRender.

### 1.3.2. Biogenesis, enrichment and lipogenesis

There are three models that have been proposed for the formation of LDs (**Figure 1.5)**. The ER domain and budding model suggest that neutral lipids build-up between the bilayers of the ER and bud into the cytoplasm taking phospholipids from the cytosolic membrane (**Figure 1.5A**). Although the model has substantial support, the process has not been observed directly (Onal et al., 2017). The bicelle model suggests that neutral lipids build-up between the leaflets of the ER membrane and the LDs are released by the formation of a bicelle from the membrane, taking phospholipids from both the cytosolic and luminal leaflets (Ploegh, 2007) (**Figure 1.5B**). The vesicular budding model suggested that small bilayer vesicles are used as a platform for making LDs (Walther & Farese, 2009) (**Figure 1.5C**). The neutral lipids are channeled into the vesicle bilayer, eventually squeezing the vesicular lumen until it converts into small inclusion with the LDs.

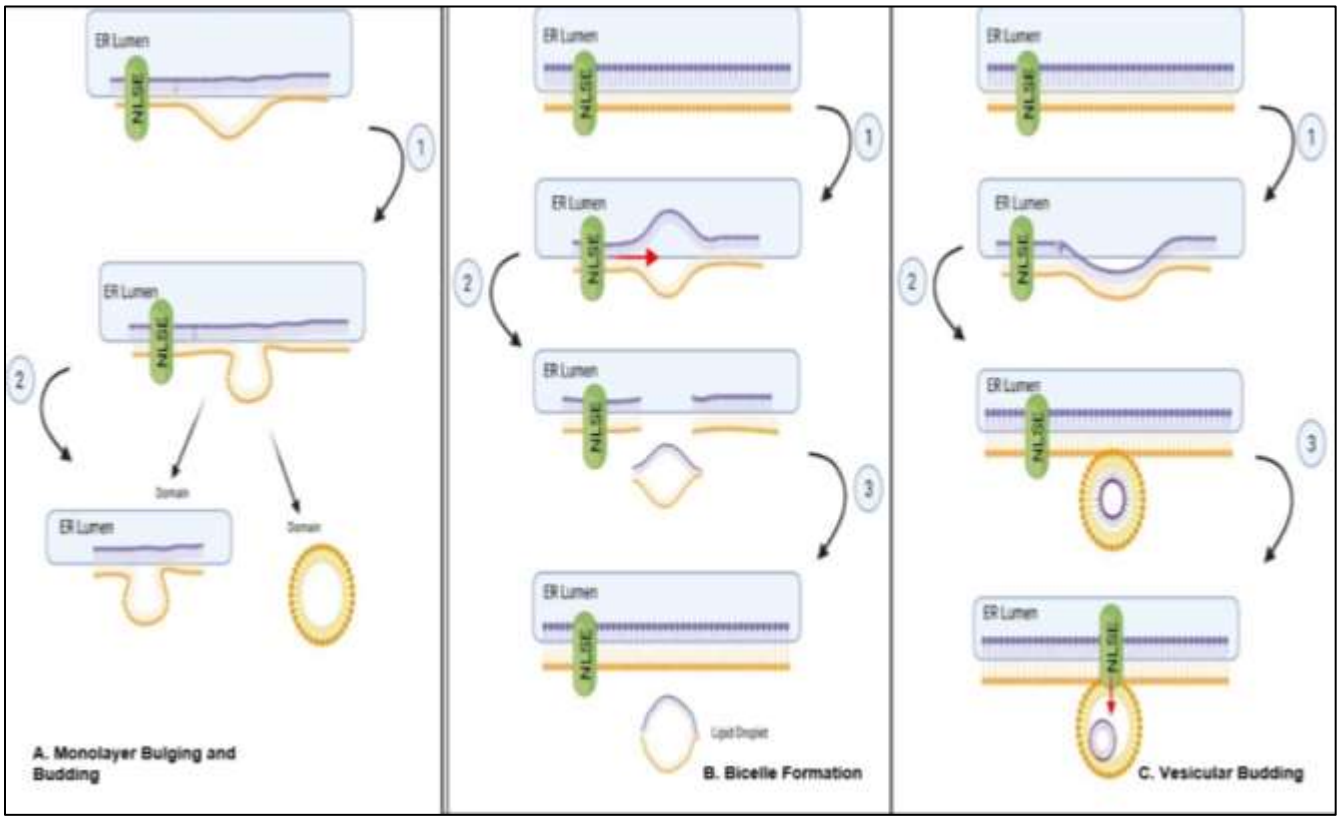


**Figure 1.5. Three models for the lipid droplets (LDs) biogenesis in cells.** The figure depicts the different models that have been proposed for the biogenesis of LDs, (A) Monolayer bulging and budding, (B) Bicelle formation, and (C) Vesicular budding. (Adapted from Choudhary et al., 2016; Guo et al., 2009b, 2009a; Ploegh, 2007). * NLSE - Neutral lipid-synthesizing enzymes. Designed with BioRender.

Lipid droplets can be divided into two types, initial LDs (iLDs) and expanding LDs (eLDs) depending on their size and lifecycle stage (Kory et al., 2016). The iLDs are formed from the endoplasmic reticulum (ER) and range from 300 to 600 nm in diameter, they bud and detach from the ER in mammals but remain attached to the bilayer in yeast cells (Kassan et al., 2013; Wilfling et al., 2013). Expanding LDs are produced from a subset of iLDs with distinct proteins (Wilfling et al., 2013). It has also been proposed that the fusion of smaller LDs results in the growth/enlargement of LDs and thus produces larger LDs in host cells (Guo et al., 2008). This proposal is also supported by the model that implicate proteins that mediate vesicle fusion (SNARE proteins) and motor proteins in LD fusion (Boström et al., 2007; Olofsson et al., 2008). It was also found that FIT proteins are essential for the promotion of proper budding of LDs from the ER (Choudhary et al., 2016).

The initial and normal formation of LDs within host cells are not dependent on externally supplied lipids, but the subsequent increase in the number, size, and quantity of LDs formed is dependent upon the uptake of externally supplied lipids (Rohwedder et al., 2014). Different types of FAs when in excess, are responsible for LD growth or increase in size with phosphatidic acid (PA) being mainly associated with the formation of 'supersized' LDs (Fei et al., 2011). Oleic acid (OA) can stimulate the formation of LDs by activating the long-chain fatty acid receptor (FFAR4) through a pertussis-toxin-sensitive G-protein signaling pathway (Rohwedder et al., 2014). Oleate generated from extracellular OA stimulates the storage of intracellular lipids into LDs, subsequently increasing total triacylglycerides (TAG) content (Rohwedder et al., 2014). It has been shown that the family of phospholipase $A_2$ ($PLA_2$) (enzymes responsible for the hydrolysis of FA present at the sn-2 position of phospholipids) are responsible for the regulation of various steps in LD biogenesis (Yu et al., 1998). During starvation, lipolysis of LDs is regulated by different types of hormones including catecholamines, that trigger perilipin A and hormone-sensitive lipase (HSL) phosphorylation resulting in LD fragmentation that liberates TAG (Gaunt et al., 2013a). Thus, it is suggested that both intracellular and extracellular FAs directly contribute to the biogenesis, enrichment and growth of LDs (**Figure 1.6**).

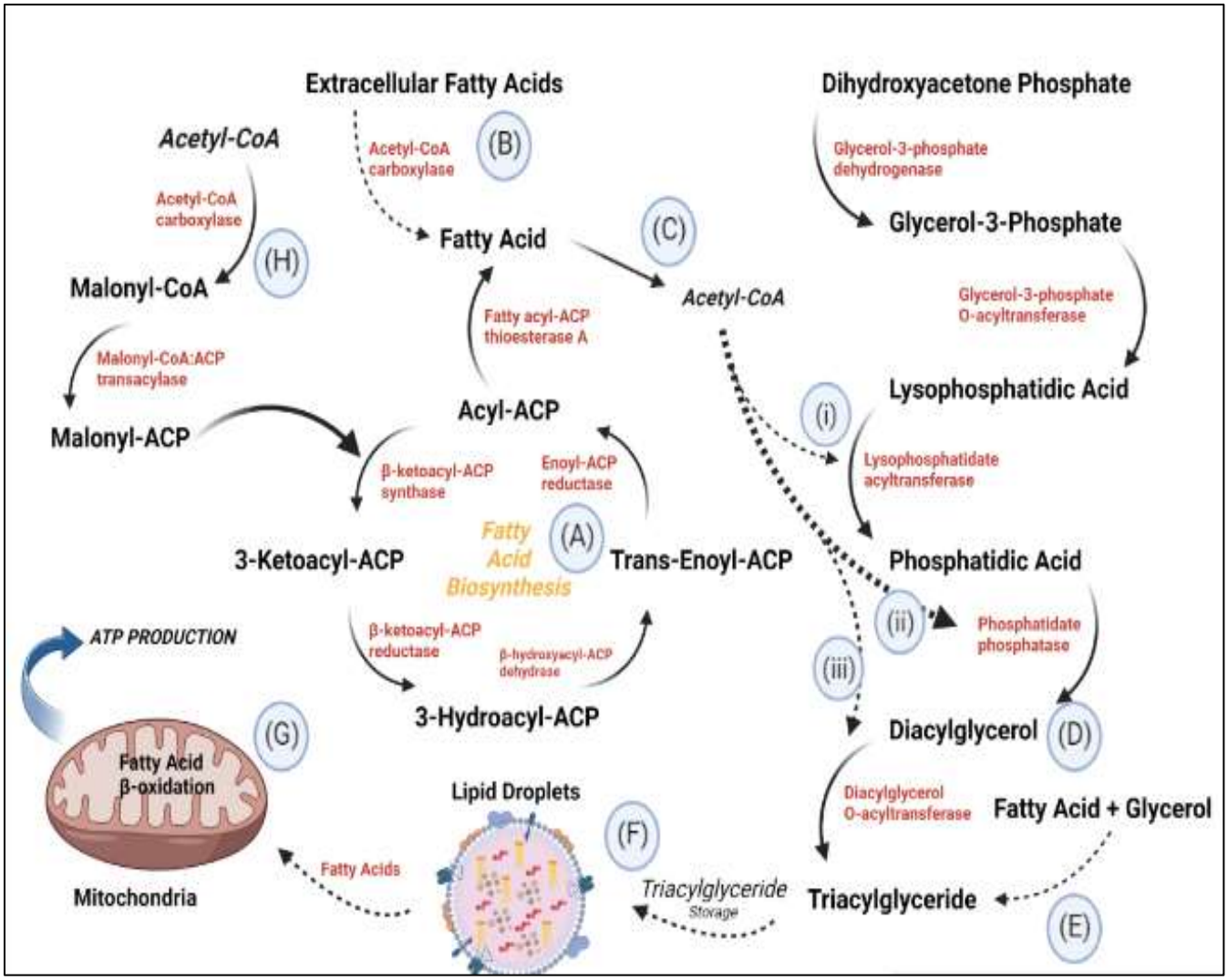


**Figure 1.6. An overview of lipid droplet (LD) biogenesis, fatty acid (FA) synthesis, storage of triacylglycerides, and the energy production by β-oxidation in the mitochondria.** (A and B) Fatty acids generated intracellularly or extracellularly supplied serve as a source of acetyl-CoA generated by both acetyl-CoA carboxylase and fatty acyl-ACP thioesterase A. (C) The generated acetyl-coA enters the triacylglycerides (TAG) biosynthetic pathway at 3 stages (i, ii and iii). (D) The generated diacylglycerols as later converted to TAG by diacylglycerol O-acyltransferase 1 (DGAT1). (E) Free FAs also react with glycerol to produce more TAG for (F) storage in LDs when FAs are in excess. During cell starvation, FAs are released from LDs (G) to enter the mitochondria for FA degradation by β-oxidation to generate ATP. (Adapted from Ahowesso et al., 2015; Ando et al., 2012; Brasaemle & Wolins, 2012; Fei et al., 2011; Gao et al., 2014; Welte, 2015). Designed with BioRender.

Triacylglycerides (TAGs) are liberated from LDs conversion to diacylglycerides (DAG) and monoacylglicerides (MAG) by adipose triglyceride lipase (ATGL) and monoacylglycerol lipase (MGL) which are finally released into the cytosol as free FAs, these free FAs are later primed and activated with acyl-CoA for degradation via FA β-oxidation and ATP production (Ando et al., 2012; Ducharme & Bickel, 2008; Gao et al., 2014).

### 1.3.3. Viral association with FAs and LDs

Lipids are involved in various steps of viral infection, including viral entry, RNA replication, viral assembly, and energy supply (**Table 1.2**). In turn, viruses are known to alter cellular lipid metabolic processes for efficient viral replication (Belov and van Kuppeveld, 2012; Miller and Krinse-Locker, 2008). Fatty acids produced from acetyl-CoA by lipogenesis may serve as precursors to produce lipid components or be broken down for ATP production via FA β-oxidation (Lam et al., 2005).

Table 1.2. The different roles of selected lipids and association with LDs during viral infection and replication (Adapted from Martín-Acebes et al., 2011).

| Virus | Lipid | Function | Effect on lipid droplets | Reference |
|---|---|---|---|---|
| **Rotavirus (RV)** | Cholesterol / lipid rafts | The transportation of RV to the cell surface in vitro and in vivo (Lipid rafts) | Total lipid content increase correlates with increase of LDs<br>Viroplasms recruit LDs. This association is critical for viral replication | (Cuadras and Greenberg, 2003; Gaunt et al., 2013b; Kim and Chang, 2011) |
| **Dengue virus (DENV)** | Bisoleoyl-lysobisphosphatidic acid (LBPA) | Cofactor for that promotes membrane fusion | Virus induces autophagy of LDs for the release of FAs, resulting in an increase of β-oxidation<br>LDs are used as energy and lipids reservoir<br>Resulting in an increase in ATP production | (Heaton and Randall 2010; Zaitseva et al., 2008) |
| **Vaccinia virus (VACV)** | Phosphatidylserine | Induces viral particle internalization | Tubular membranes localize around LDs suggesting a role of LDs in VACV assembly and viral replication | (Chung et al., 2005; Mercer et al., 2008) |
| **Human papillomavirus (HPV)** | Phosphatidylinositol 4-phosphate (PI4P) | Anchor viral replicase to replication complex | Virus found to increase LDs levels when cell density is also increased | (Guštin et al., 2017; Hsu et al., 2010) |
| **Hepatitis C virus (HCV)** | Sphingomyelin | Activation of RNA polymerase activity | Virus induces autophagy of LDs for the release of FAs, resulting in an increase of β-oxidation<br>LDs are used as energy and lipids reservoir<br>Resulting in an increase in ATP production | (Heaton and Randall, 2010; Weng et al., 2010) |
| **West Nile virus (WNV)** | Cholesterol | Innate immune evasion | Virus induces autophagy of LDs for the release of FAs, resulting in an increase of β-oxidation.<br>LDs are used as energy and lipids reservoir<br>Resulting in an increase in ATP production. LDs are used by viruses as an energy and lipids reservoir | (Mackenzie et al., 2007; Heaton and Randall, 2010) |

The replication of viruses with positive-strand RNA is highly associated with intracellular lipid membranes that are derived from different organelles including endoplasmic reticulum (ER), Golgi complex, mitochondria, peroxisomes, endosomes, or lysosomes (Monson et al., 2021; Stapleford & Miller, 2010). Studies have shown that RV infection modulates host cell FA composition (Gaunt, et al., 2013b; Sander et al., 2022). Furthermore, the interference with the intracellular biosynthetic pathways of FAs reduces the production of RV infectious particle yield and replication (Lever & Desselberger, 2016).

The close interaction and formation of complexes between viroplasms and LDs are important for RV replication and propagation in a host cell, as different compounds that interfere with LD formation or disperse LDs by enhancing the phosphorylation of perilipin A, leading to lipolysis (3-isobutyl-1-methylxanthine (IMBX) and isoproterenol (ISP)) (Marcinkiewicz et al., 2006) significantly reduce the number and size of viroplasms and subsequently decrease RV infectious progeny yield (**Figure 1.7**) (Cheung et al., 2010). Chueng and co-workers 2010 also demonstrated that RV viral proteins (VP1, VP2, VP6, NSP2 and NSP5) co-localizes with LD proteins perilipin A and ADRP in viroplasms (**Figures 1.8 and 1.9**) (Cheung et al., 2010). The controls without infected MA104 cells only depicted stained perilipin A and/ or ADRP. This showed that uninfected MA104 cells only show LDs with no NSP2 being observed (**Figures 1.8C**).

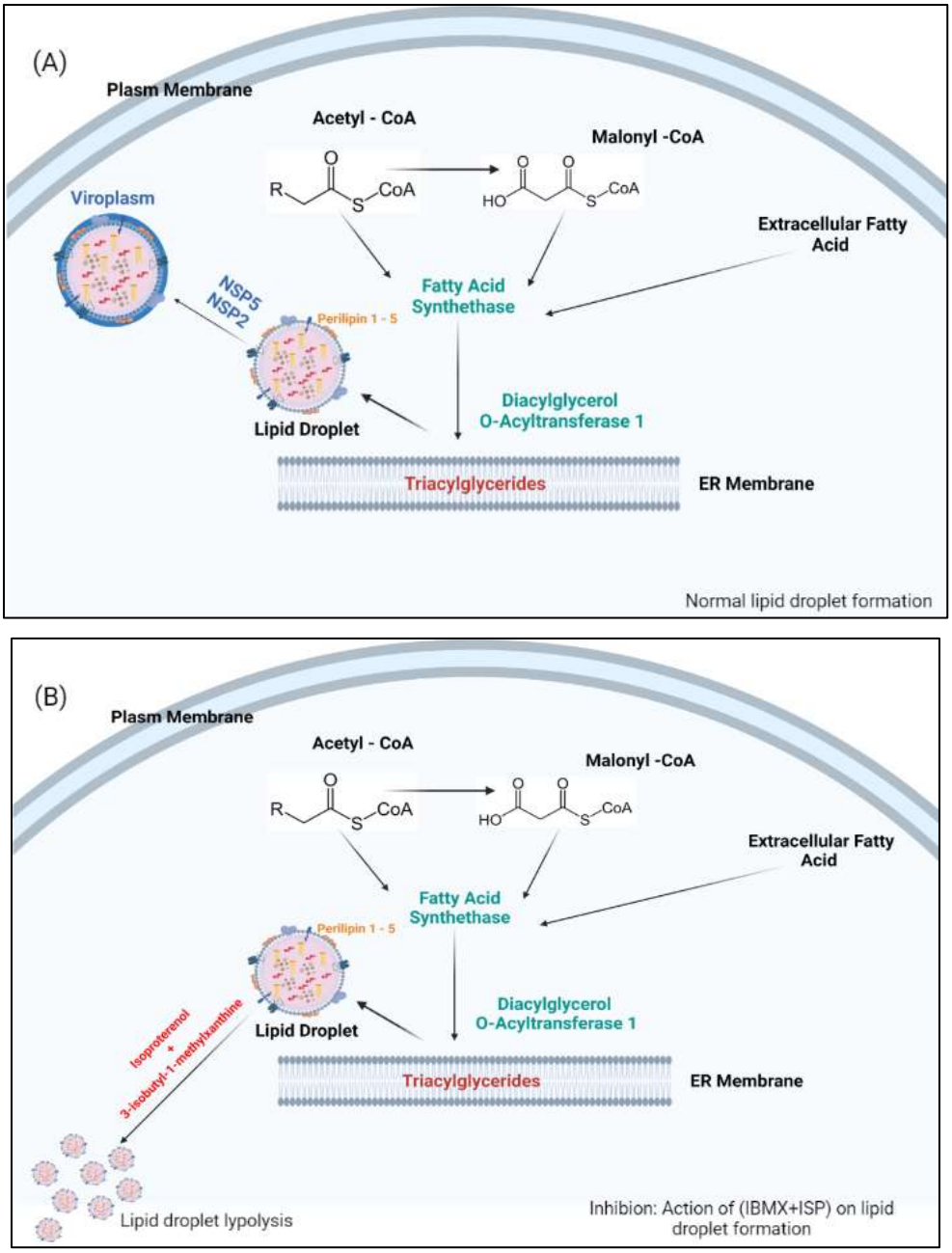


**Figure 1.7. The action and effect of compounds 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) on the normal formation of lipid droplets (LDs).** (A) Normal lipid droplet formation: Excess fatty acids (FAs) from internal or external sources are converted to triacylglycerides (TAGs) which are later channeled and stored in LDs. These LDs interact with viral NSP2 and NSP5 for the formation of the viroplasms-LD complexes. (B) The presence of IBMX and ISP results in the phosphorylation of LD perilipin proteins that induce the lipolysis of LDs into microdroplets, thus preventing the formation of viroplasms-LD complexes (Adapted from Crawford & Desselberger, 2016). Designed with BioRender.

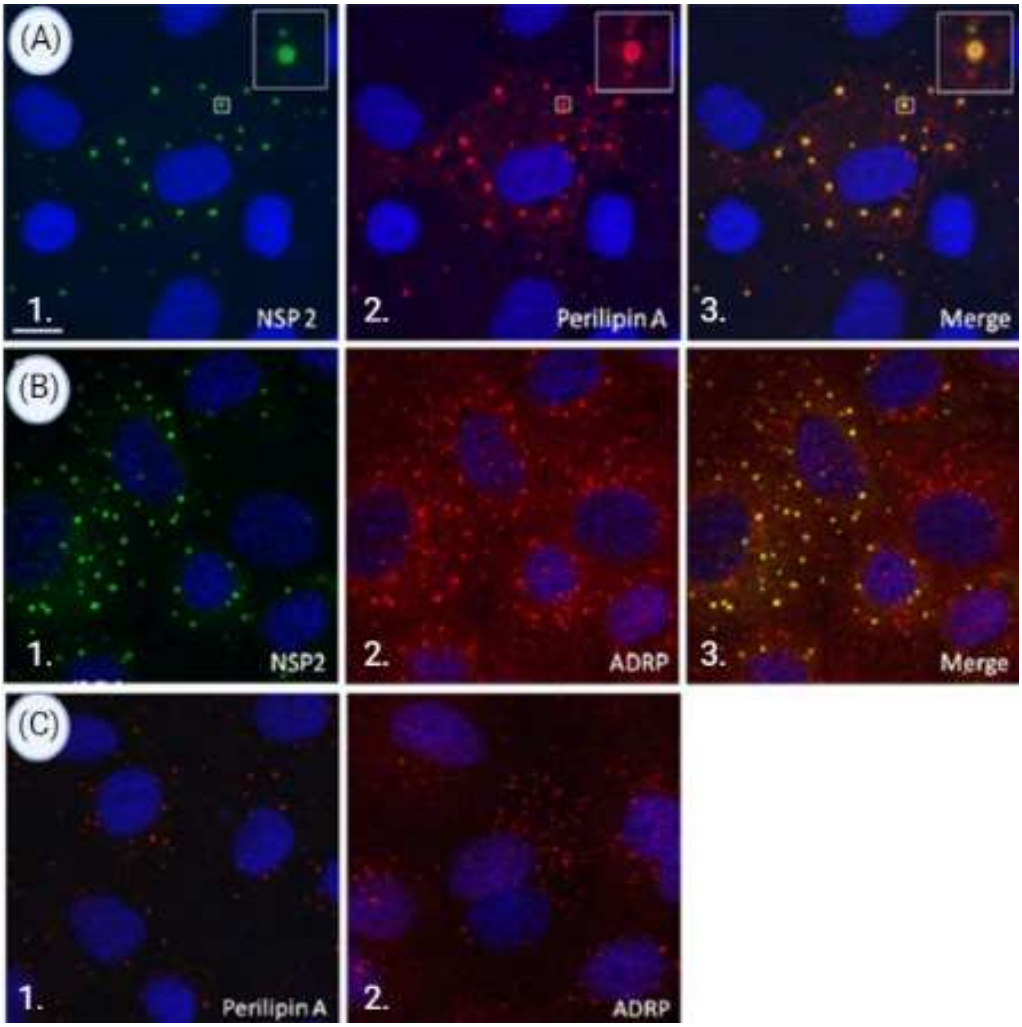


**Figure 1.8. Co-localization of rotavirus-associated proteins (NSP2) and lipid droplets-associated proteins, (perilipin A and adipose differentiation-related protein (ADRP,)) in RV infected MA104 cells, at 8 h p.i**. The confocal images show the close association and co-localization of RV (A1 and B2) NSP2 with LD associated proteins (A2) Perilipin A and (B2) ADRP. (C) Uninfected MA104 control cells only show presence of both stained (C1,2) LD proteins (perilipin A and ADRP) (Cheung et al., 2010). The figure is presented with the journal's permission (**Annexure B2**).

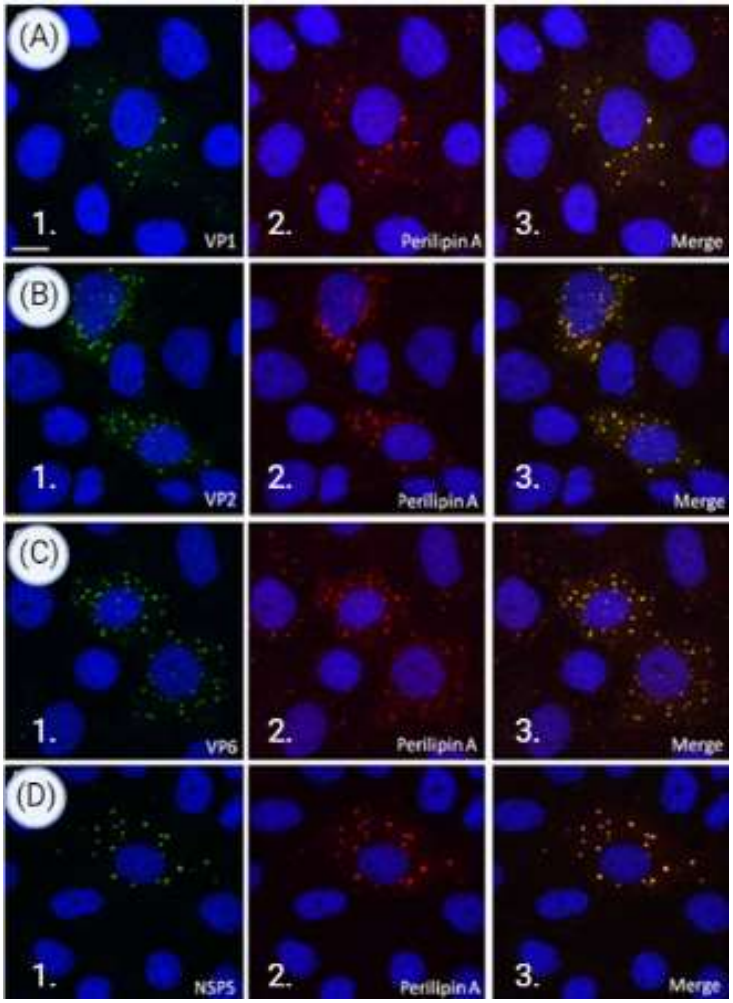


**Figure 1.9. Rotavirus viral proteins co-localizing with perilipin A, LD-associated protein. The confocal image of rotavirus infected M104 cells at 8 h p.i.** The confocal imaging show that viral proteins (A1) VP1, (B1) VP2, (C1) VP6 and (D1) NSP5 co-localize with (A2, B2, C2 and D2) LD perilipin A (A1, A2 and A3) (Cheung et al., 2010). The figure is presented with the journal's permission (**Annexure B2**).

## 1.5. Fatty acid β-oxidation

### 1.5.1. β-oxidation overview

During FA metabolism, β-oxidation is an important catabolic process for the breakdown of FAs in the mitochondria and peroxisomes of cells to generate acetyl-CoA for the citric acid cycle and the electron transport chain (Houten & Wanders, 2010). Host cell β-oxidation is facilitated by the mitochondrial trifunctional protein (MTP) (**Figure 1.10**), which is an enzyme complex associated with the inner mitochondrial membrane that catalyze the final three steps of β-oxidation (Blenda et., al 2019). Briefly, during β-oxidation a long chain FA is dehydrogenated to produce a trans bond between carbon (C1 and C2) giving rise to trans-delta 2-enoyl-CoA catalyzed by acyl-CoA dehydrogenase (**Figure 1.10A**). This is then followed by the hydration of the trans-delta 2-enoyl-CoA by the enoyl-CoA hydratase to produce a L-3-hydroxyacyl-CoA (**Figure 1.10B**). The hydrated L-3-hydroxyacyl-CoA subsequently dehydrogenated to produce a 3-ketoacyl-CoA, catalyzed by 3-hydroxyacyl-CoA dehydrogenase (**Figure 1.10C**). The final step involves the thiolysis of C1 and C2 of the produced 3-ketoacyl CoA to produce the precursor molecule acetyl-CoA (**Figure 1.10D**). The process of β-oxidation will continue until all the carbon molecules of the initial FA are converted into acetyl-CoA (Blenda et. al 2019; Houten and Wanders, 2010). Although β-oxidation occurs in both the mitochondria and peroxisomes for energy production, short chain FAs are broken-down in the mitochondria while long chain FAs are degraded in peroxisomes (Eaton, 2002; Houten & Wanders, 2010; Kerner & Hoppel, 2000). Nonstructural protein of a wide range of viruses including RV have been shown to interact directly with both the mitochondria and the MTP to modulate mitochondrial-based FA metabolism (Altenburg et al., 1980; Korenaga et al., 2005; Piccoli et al., 2009; Piccoli et al., 2007; Seo et al., 2011; Seo & Cresswell, 2013).

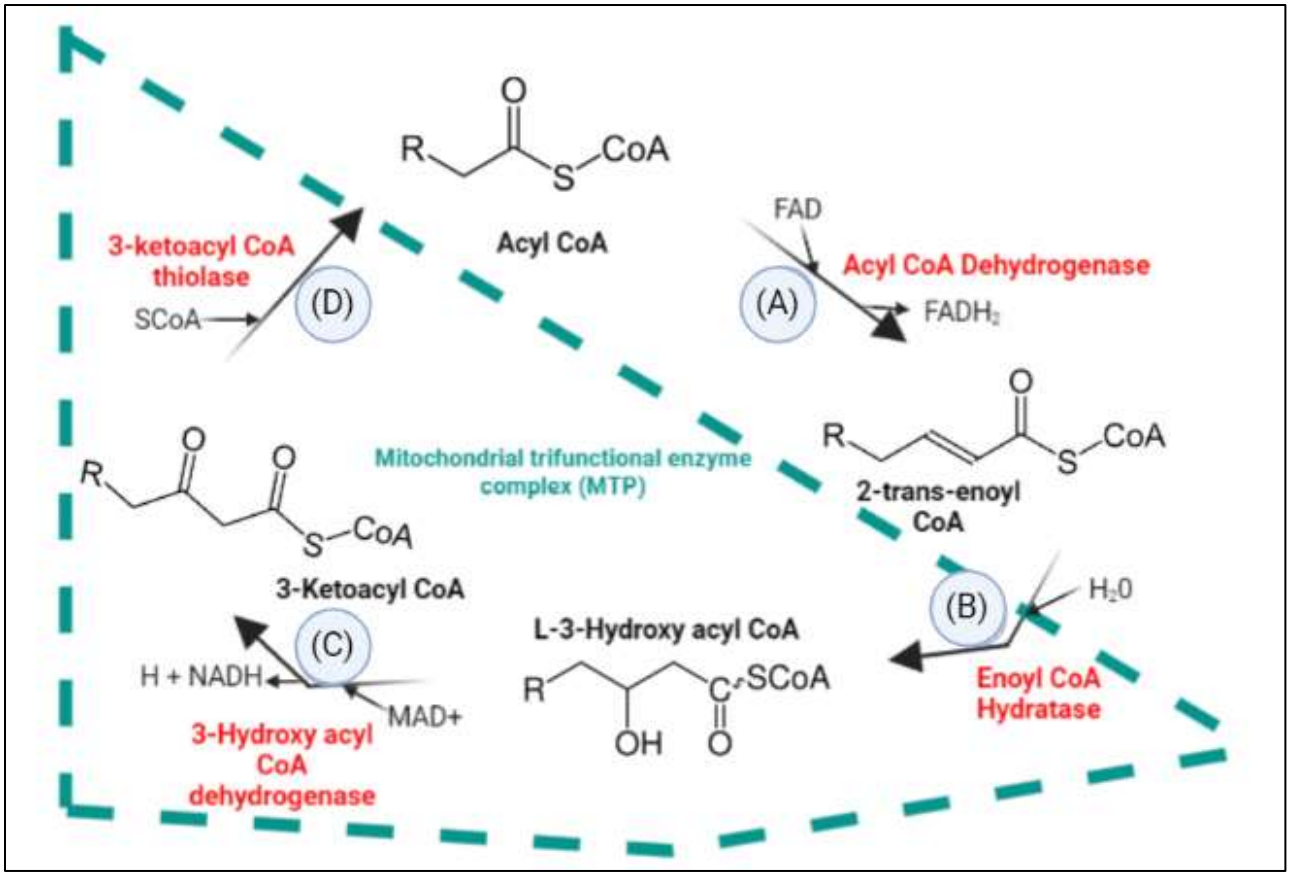


**Figure 1.10. An overview of the β-oxidation steps** (A-D) and the mitochondrial trifunctional enzyme complex (MTP) is indicated within the green outline. (Adapted from Rector et al., 2008). Designed with BioRender.

### 1.5.2. Viral association with β-oxidation

To further evaluate the importance of FA β-oxidation during viral infection, the inhibition of FA β-oxidation by inhibiting the carnitine palmitoyltransferase I (CPT1) function with etomoxir (ETO) (**Figure 1.11**) has been shown to reduce the viral load of various viruses including Dengue virus (DENV) and Hepatitis C virus (HCV) (Heaton & Randall, 2010; Rasmussen et al., 2011). However, the inhibition of FA β-oxidation does not reduce the viral load of Japanese encephalitis virus (JEV) compared with the above-mentioned viruses. This provides evidence that different viruses may modulate FA β-oxidation in different ways.

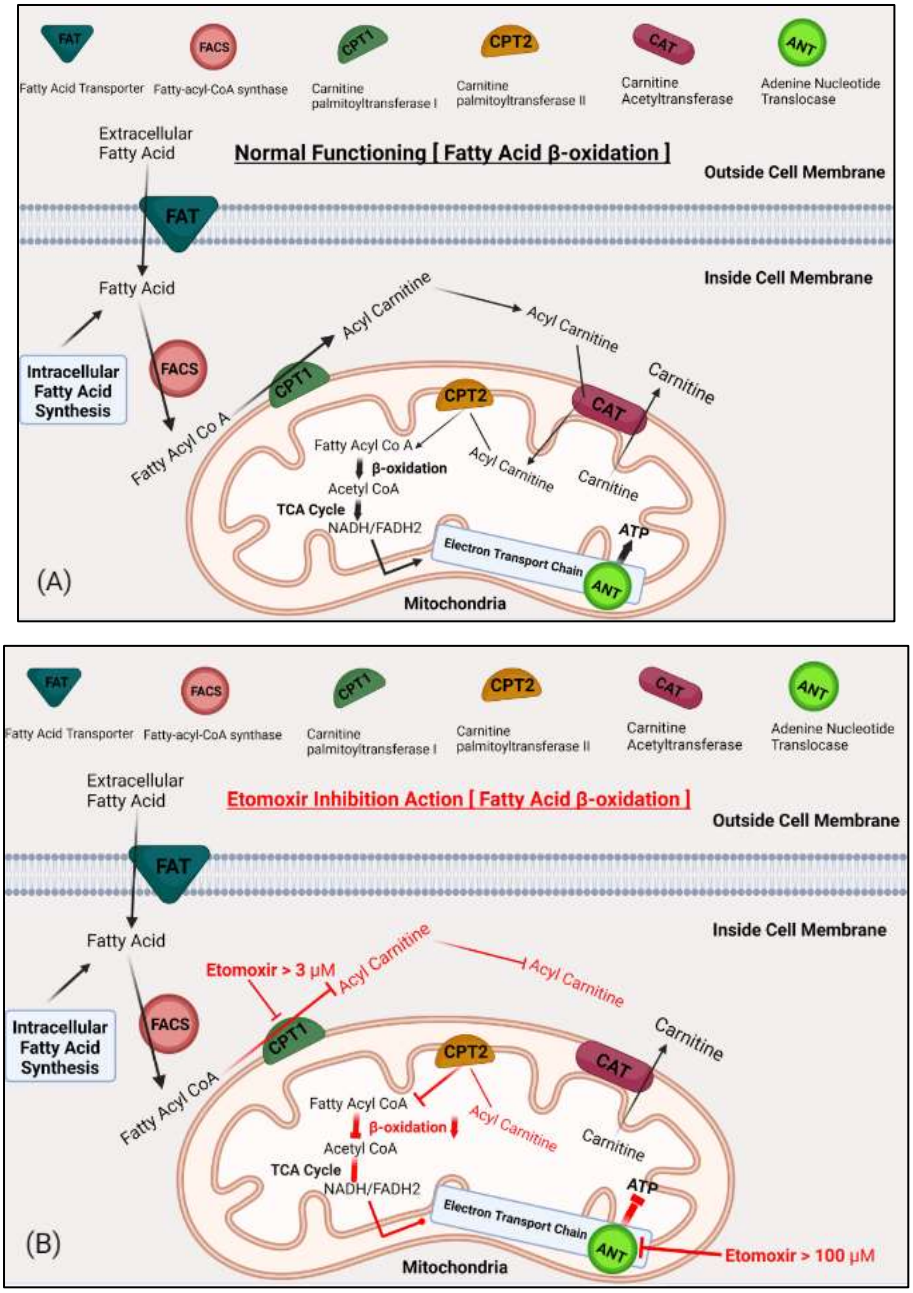


**Figure 1.11. The action of etomoxir (ETO) inhibition on fatty acid (FA) β-oxidation.** (A) Normal functioning of fatty acid (FA) β-oxidation indicated by successful synthesis of acyl carnitine from fatty acyl-coA by CPT1. Thus, making acyl carnitine available for transportation into the mitochondria to regenerate fatty acyl-CoA by CPT2 for β-oxidation and ATP production. (B) The action of > 3μM ETO irreversibly inhibits CPT1 preventing the production of acyl carnitine, thus reducing available acyl carnitine for translocation into the mitochondria and β-oxidation. The concentration of ETO > 100μM also inhibits ANT reducing the overall production of ATP by β-oxidation (Adapted from Divakaruni et al., 2018; Raud et al., 2018). Designed by BioRender.

Metabolic profiling studies show significant reduction in cellular ATP concentrations induced by HCV infections (Ando et al., 2012; Mankouri et al., 2010). Hepatitis C virus interacts with the mitochondria to induce reactive oxygen species (ROS) production, thought to lead to impairment of lipid β-oxidation (Piccoli et al., 2009). Besides RV NSP6 binding to the single-stranded RNA (ssRNA) and dsRNA independently of nucleotide sequence (Rainsford & McCrae, 2007), it was also reported that NSP6 may be present at RV viral replication sites withing the cytoplasm and localize to the mitochondria via its conserved sequences in the N-terminal a-helix (Holloway et al., 2015). This also provides a possible mechanism of how RV affects mitochondrial metabolic processes during infection (Holloway et al., 2015). The RV NSP6 also interacts with NSP5 (Mattion et al., 1991; Rainsford & McCrae, 2007; Torres-Vega et al., 2000).

In RV infected host cells, a cluster of mitochondria organelles are observed to localize at the area of viral replication and assembly (Rojo et al., 1998). Rotavirus has also been observed in the mitochondria between the cristae and these infected mitochondria have dense outer layer membranes suggesting that RV entered the mitochondria by budding into the inner and outer mitochondrial membranes (**Figure 1.12**) (Altenburg et al., 1980). The infected mitochondria containing viral particles are found in close proximity with viroplasms, further indicating the close relation of the RV, viroplasms, LDs and the mitochondria (**Figure 1.12A)** (Altenburg et al., 1980). This close association of these organelles at the site of viral assembly may suggest the mechanism in which RV infections influence mitochondrial based β-oxidation and how cellular ATP production is altered to the energy demands of viral replication. Rotavirus infections are reported to increase lactate production which correlates with a decrease in mitochondrial oxygen consumption and ATP production in Caco-2 cells (Dickman et al., 2000).

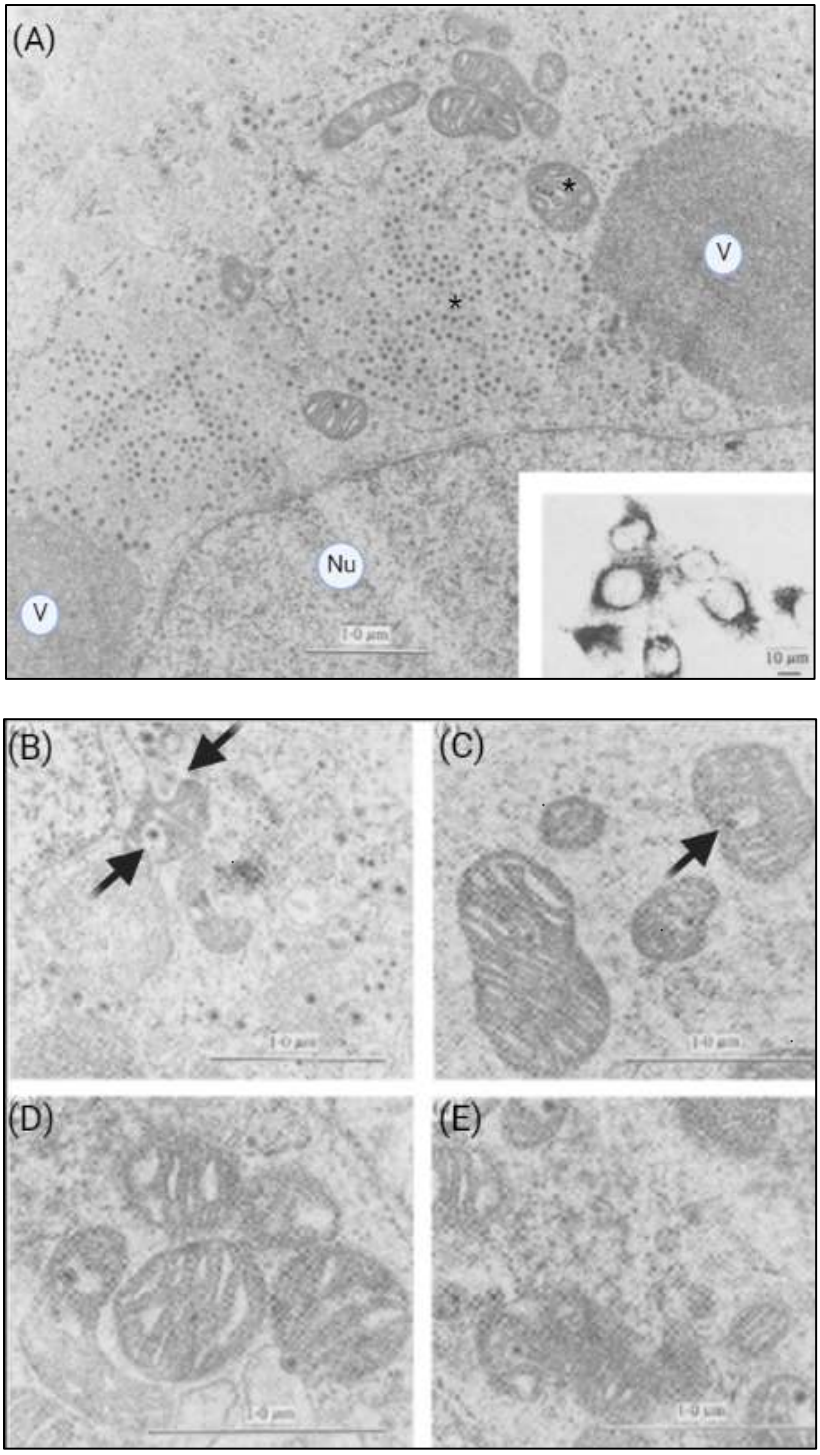


**Figure 1.12. The perinuclear region of mitochondria of MA104 cells infected with SA11 24 h p.i.** Viroplasms (V) are associated with cristae which contains multiple RV particles (*). The direct and physical interaction of RV (arrows) with the mitochondria is also shown (B-E). The cell nucleus is indicated (Nu) (Altenburg et al., 1980). Figure presented with journal's permission (**Annexure B3**).

## 1.6. Problem statement

Rotavirus (RV) is a well-known agent that induces severe and fatal dehydrating diarrhea in infants and young children globally (Estes and Greenberg., 2013; Troeger et al., 2018). Researchers have also shown that RV in its infection and replication alters the hosts FA lipidome and recruits cellular LDs for its successful replication (Cheung et al., 2010; Crawford & Desselberger, 2016; Gaunt et al., 2013b). Rotavirus is one of the viruses that alter the host cell's FA β-oxidation to either promote lipolysis and energy production or lipogenesis for viral replication (Dickman et al., 2000).The modulation of FA synthesis, LD formation and FA metabolism by β-oxidation negatively affects viral replication while OA is one of the major unsaturated FAs shown to be modulated during RV infection (Cheung et al., 2010; Gaunt et al., 2013a; Sander, 2019). Understanding the association of RV infection with these processes may provide therapeutic target insights for reduction of RV infections. To date, it remains unclear why some FAs during RV infection are modulated and the possible mechanisms may include (**1**) RV infection reducing free FAs for storage in LDs as to increase the number and size of LDs interacting with viroplasms or (**2**) RV reduce cellular FAs by channeling FA degradation by β-oxidation to create an energy rich environment promoting and driving viral replication or reduce β-oxidation to elevate FA concentration for LD formation. Studies showed previously that exogenous OA promotes the formation of LDs which also increase viral load (Rohwedder et al., 2014; Sander., 2019). However, it remains unclear how exogenous OA affects LDs and the overall FA metabolism and, therefore, β-oxidation during RV infection. The fragmentation of LDs are shown to reduce RV replication (Cheung et al., 2010), but it remains unclear how β-oxidation modulation may affect RV infection as compared to other viruses. It is also unclear how RV infection may affect LDs and FA β-oxidation in the presence or absence of LD fragmenting compounds, FA β-oxidation inhibitors and OA. The possible association between LDs and FA β-oxidation during RV infection remains unclear.

**Aim**

To investigate how oleic acid (OA) supplementation affects lipid droplets (LDs), fatty acid (FA) β-oxidation, and subsequent rotavirus (RV) replication.

**Objectives**

1. Investigate the effect of OA supplementation on LDs during RV infection.
2. Investigate the effect of OA supplementation on FA β-oxidation during RV infection.
3. Investigate the effect of LDs and FA β-oxidation modulation on RV infection.

## 2.1. Ethical clearance

The ethical clearance for the study was obtained from the Biosafety & Environmental Research Ethics Committee of the University of the Free State, with ethical clearance number, UFS-ESD2020/0081/1610 (**Annexure A**).

## 2.2. Virus, cell lines and culture conditions

Rotavirus simian agent 11 (SA11) (Mlera et al., 2013) was used to conduct all experiments. African green monkey kidney (MA104) cells (Dr. Ulrich Desselberger, University of Cambridge) were used in conducting SA11 (Mlera et al., 2013) quantification assays using 50% tissue culture infectious doses ($TCID_{50}$), as these cells are known to be susceptible to SA11 infection resulting in higher viral detection (Estes et al., 1979) for viral kinetic assays. Human embryonic kidney 293 cells, (HEK293) are a specific immortalized cell line derived from human embryonic kidney cells grown and adapted for tissue culture taken from a female fetus (Graham et al., 1977; Kavsan et al., 2011). The HEK293 cells were used to conduct all our experimental assays, as they are considered to have reliable growth and give a direct metabolic response to human cells (Abaandou et al., 2021; Swiech et al., 2012; Thomas & Smart, 2005). Both cell lines were maintained in complete growth media (Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 5% (v/v) foetal bovine serum (FBS) (Gibco, USA), 1% (v/v) antibiotic mixture ((penicillin (10 000 U/mL), streptomycin (10 000 µg/mL) and amphotericin B (0.25 µg/mL)) (Gibco, USA)), 1% (v/v) nonessential amino acids (NEAA) (Gibco, USA) and 1% (v/v) N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES buffer) (Gibco, USA)). The HEK293 cells were cultured to 70 - 80% confluency at 37°C and 5% $CO_2$ (Air liquid, SA) in a Steri-Cycle $CO_2$ incubator (Thermo Scientific, USA). During SA11 infections, serum-free or infection media consisting of DMEM (Gibco, USA) supplemented with 1% (v/v) antibiotic mixture (Gibco, USA), 1% (v/v) NEAA (Gibco, USA), 1% (v/v) HEPES buffer (Gibco, USA) and 500 µL 1 µg/mL trypsin IX (Sigma Aldrich, USA) was used.

### 2.3. HEK293 cell count and viability in serum-free media

Human embryonic kidney 293 cells were seeded at 1.2 x $10^6$ cells/mL in 25 $cm^2$ cell culture flasks (Thermo Scientific, USA) in complete growth media and incubated at 37°C, 5% $CO_2$ for 24 h. The cells were washed once with 0.01 M pH 7.4 Phosphate buffered saline (PBS) (Sigma-Aldrich, USA), followed by a 16 h incubation at 37°C, 5% $CO_2$ in serum-free media (**Figure 2.1A**). Following incubation, HEK293 cells were trypsinized with 0.05% (v/v) trypsin-EDTA (Gibco, USA) for 5 mins and were collected by centrifugation (Yingtai TD 5) 800 x g for 5 mins. The cells were resuspended in 1 mL complete growth medium and mixed in a 1:1 ratio with trypan blue (Thermo Scientific, USA) to determine the HEK293 live cell count over time using a countess automated cell counter (Invitrogen, USA) at 0 h and 16 h time points (**Figure 2.1B**). To determine cell viability over time, the tetrazolium dye XTT cell proliferation assay kit (Sigma Aldrich, USA) was used. HEK293 cells were seeded at a concentration of 4 × $10^3$ cells/well into a 96 - well culture plate (Thermo Scientific, USA) and cultured at 37ºC and 5% $CO_2$ overnight. The cells were washed once with 0.01 M pH 7.4 PBS (Sigma-Aldrich, USA), followed by the addition of 100 µL serum-free media for a 16 h period (**Figure 2.1C**). This was then followed by addition of 50 µL XTT labeling mixture ((master mix: 5 mL 1 mg/mL sodium 3´- [1- (phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate and 0.1 mL N-methyl dibenzopyrazine methyl sulfate) (Sigma Aldrich, USA) per well and incubated for 4 h at 37 °C and 5% $CO_2$ (**Figure 2.1D**). The spectrophotometric absorbance of each well was measured using Victor Nivo multimode microplate reader (Perkin Elmer, USA) at a wavelength of 480 nm (**Figure 2.1E**).

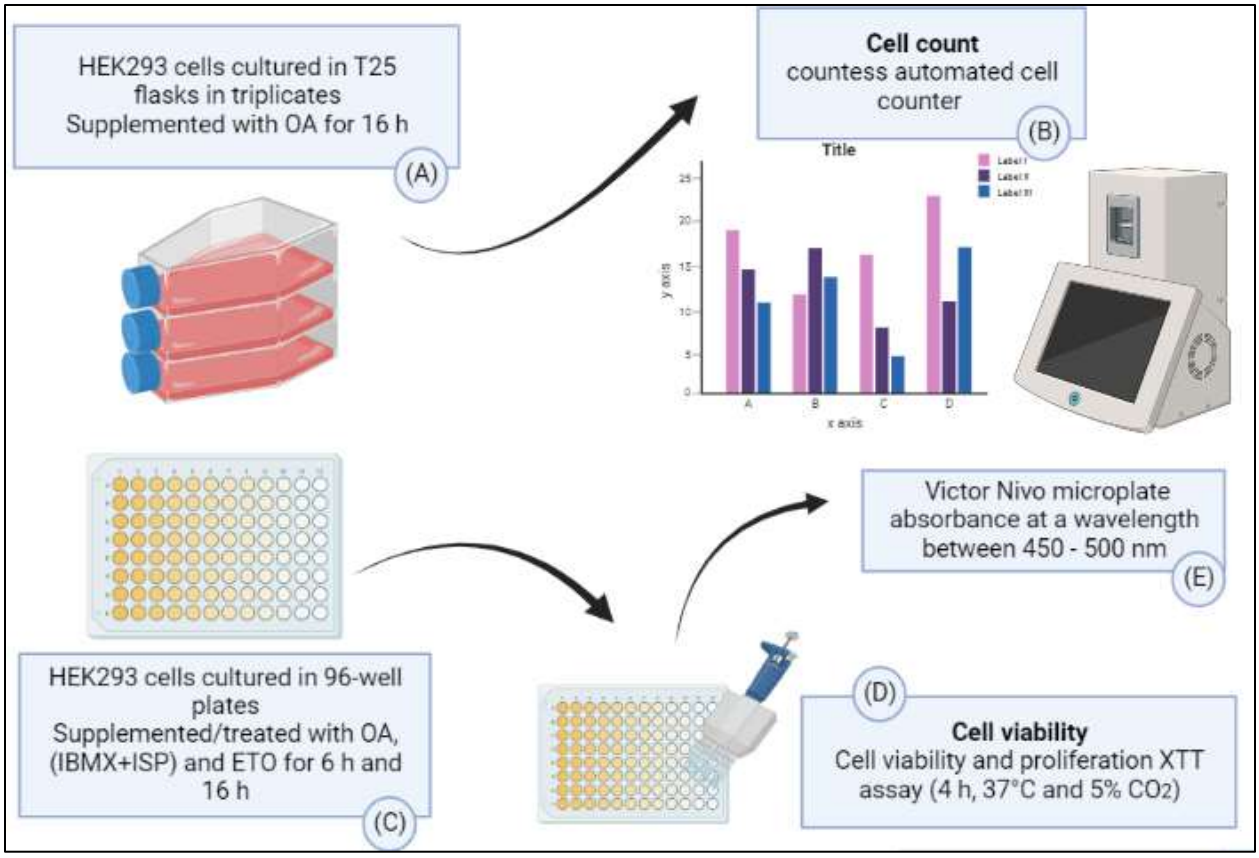


**Figure 2.1. Experimental scheme depicting the determination of** (A-B) cell number and (C-E) cell viability after the supplementation and treatment with oleic acid (OA), (3-isobutyl-1-methylxanthine (IBMX)+isoproterenol (ISP)) and etomoxir (ETO). Designed with BioRender.

## 2.4. Lipid droplets and FA β-oxidation modulation compounds

### 2.4.1. Lipid droplet fragmentation compounds

To generate the working stocks, LD fragmenting compounds, 3-isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) to generate a 240 mM stock solution while isoproterenol (ISP) (Sigma Aldrich, USA) was dissolved in $dH_2O$ to generate a 10 mM stock solution and filter sterilized before use in cell culture. HEK293 cells were incubated in complete growth media supplemented with 1 mM IBMX (Sigma Aldrich, USA) and 20 µM ISP (Sigma Aldrich, USA) as performed by Cheung and co-workers in MA104 cells (Cheung et al., 2010). The concentrations used are similar to the concentrations used by Cheung and co-workers in MA104 cells and those used by Violin and co-workers in HEK 293 cells, thus being deemed non-toxic for the study (Cheung et al., 2010; Orlicky et al., 2013; Violin

et al., 2008). These compounds are known to disperse and/or fragment LDs to produce microdroplets (Cheung et al., 2010; Marcinkiewicz et al., 2006).

#### 2.4.2. Fatty acid β-oxidation inhibitors

Etomoxir (ETO) (Sigma Aldrich, USA) was dissolved in $dH_2O$ to generate a 14 mM stock solution. A cytotoxicity assay was conducted to determine the ETO concentration that were least toxic to HEK293 cells but can still induce the inhibitory effect required for the study on fatty acid (FA) β-oxidation. The XTT cell proliferation assay kit (Sigma Aldrich, USA) was used as described above (**2.2**). Once the cells reached 70 – 80% confluency, several dilutions of ETO (Sigma Aldrich, USA) (20 µM, 40 µM, 60 µM, 80 µM, 100 µM and 120 µM) were added to the wells. The plate was incubated for 16 h at 37$^{o}$C and 5% $CO_2$. Once incubation was complete, 50 µL XTT labeling mixture (Sigma Aldrich, USA) was added to the wells. Incubation and plate analysis was carried out as described previously (**2.1**). Twenty-four hours prior to SA11 infection, the complete growth media was supplemented with ETO (Sigma Aldrich, USA) at a final concentration of 60 µM. The cells were treated again at the time of SA11 infection as described by Rasmussen and co-workers (Rasmussen et al., 2011).

### 2.5. Oleic acid supplementation of HEK293 cells

Oleic acid (OA) (18:1) (Sigma Aldrich, USA) was initially prepared and stored in absolute ethanol (Merk, USA). It was later dried under $N_2$ gas (Air liquid, SA) and dissolved in an albumin fraction VI (33.8% w/v dissolved in 100 mL $dH_2O$) (Roche, Germany) by sonication. The fatty acid (FA) was added to the complete growth media as an albumin fraction VI (Roche, Germany) complex with a molar ratio of 3:1 to the FA (Tanaka et al., 2001). The FA-albumin complex was then filter-sterilized prior to supplementation in complete growth media. HEK293 cells were seeded at 0.7 x $10^6$ cells/mL into 25 $cm^2$ culture flasks (Thermo Scientific, USA) and cultured as previously described (**2.1**), these cells were supplemented with 50 µM OA (Sigma Aldrich, USA) when reaching cell confluency of 60 - 70% 24 h prior to commencement of the assays. The cells were washed once with 0.01 M pH 7.4 PBS (Sigma Aldrich, USA) followed by the addition of 5 mL complete growth media containing 50 µM OA (Sigma Aldrich, USA) as described by Tanaka and co-workers (Tanaka et al., 2001).

### 2.6. Infection of HEK293 cells with SA11 and viral kinetics

In the study, MA104 and HEK293 cells were infected with rotavirus strain SA11 (Mlera et al., 2013). The titer and viability of the used RV stocks with unknown passage levels was determined using 50% tissue culture infectious doses ($TCID_{50}$). Large variations between the viral stocks were not observed. Therefore, 1 mL SA11 viral stock was activated at 37 °C for 30 min using 10 µg/mL

trypsin IX (Sigma Aldrich, USA) (Arnold et al., 2009). MA104 cells were seeded at 1 x $10^5$ cells/well into 96 - well culture plates (Thermo Scientific, USA) and cultured to reach 70 - 80% confluency at 37ºC and 5% $CO_2$. The cells were subsequently washed three times with 0.01 M pH 7.4 PBS (Sigma Aldrich, USA). The MA104 monolayers were infected with the SA11 lysate serial dilutions ($10^{-1}$ to $10^{-12}$). The cells were incubated for 45 min on an orbital rocker (Bench Rocker 2D) for infection. After infection, the cells were washed once with 0.01 M pH 7.4 PBS (Sigma Aldrich, USA), and 100 µL serum-free media was added to the wells. The infected MA104 monolayers were incubated at 37°C and 5% $CO_2$ for 5 days in which viral titers were read on day five (Reed & Muench, 1938).

Calculation of $TCID_{50}$/mL and final SA11 titer in plaque forming units per milliliter (pfu/mL)

$$I = \frac{(\%\text{ of wells infected by dilution above } 50\% - 50\%)}{(\%\text{ of wells infected by dilution above } 50\% - \%\text{ of wells infected by dilution below } 50\%)}$$

$$TCID_{50}\ (TCID_{50}/mL) = 10^{(\text{Log total dilution above } 50\% - (\ I \text{ x log } D)}$$

I = Interpolated value of the 50% endpoint.

D = Dilution factor.

Once the final SA11 titer was known, the multiplicity of infection (MOI) was determined to conduct SA11 infections in a known number of HEK293 cells.

Calculation of MOI:

$$MOI = \frac{\text{Viral titre (T) (pfu/mL)}}{\text{Cell number in flask (cell/mL)}}$$

HEK293 cells supplemented and non-supplemented (with OA, IBMX, ISP), and ETO were infected with activated SA11 at a MOI of 5 and incubated in serum-free media for 45 min on an orbital rocker (Bench Rocker 2D). After infection, the cells were washed once with 0.01 M pH 7.4 PBS (Sigma Aldrich, USA) and later incubated in serum-free media at 37ºC and 5% $CO_2$ until different time points (0 h, 2 h, 6 h, 12 h, and 16 h) (**Figure 2.2A**). The SA11 lysate were harvested by freeze-thawing the 25$m^2$ culture flasks (Thermo Scientific, USA) three times, after which the cell debris was removed by centrifugation (Yingtai TD5) at 4000 x g for 10 min at the above-mentioned time points post infection (p.i) (**Figure 2.2B**). Controls consisted of uninfected cells as well as un-supplemented infected cells. These cells were processed in parallel under the same conditions. Once the SA11 lysates at each time point (0 h, 2 h, 6 h, 12 h, and 16 h) had been harvested as describe above, $TCID_{50}$ assays were performed to determine the SA11 viral titer at

each time point as described above (**2.6**). The infected MA104 monolayers were incubated at 37°C and 5% $CO_2$ for 5 days and titers were read on day five (Reed & Muench, 1938) (**Figure 2.2C,D**).

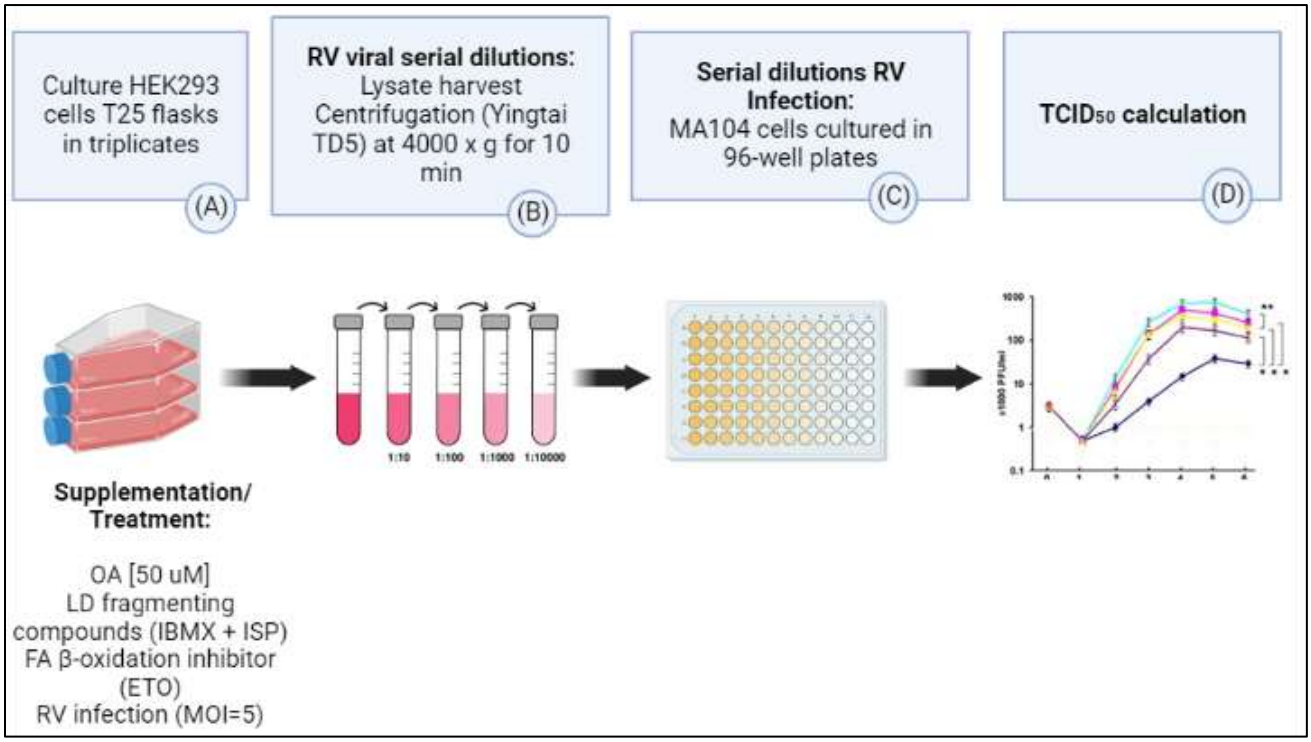


**Figure 2.2. Experimental overview of rotavirus (RV) replication kinetics** (A) HEK293 cell culture, (B-D) rotavirus (RV) dilution and tissue culture infectious dose 50 ($TCID_{50}$s) followed by (D) result analysis and representation. Designed with BioRender.

2.7. Lipid droplet isolation from HEK293 cells

Approximately 1.5 - 3 x $10^7$ HEK293 cells (roughly 50-100 mg) initially seeded in 175 $cm^2$ culture flasks (Thermo Scientific, USA) were trypsinized with 0.05% (v/v) trypsin-EDTA (Gibco, USA) and 10 mL of complete growth media was added. The cells were then collected by centrifugation at 1000 x g for 5 mins (Yingtai TD5) (**Figure 2.3A**). The complete growth media was discarded, and the mass of the cells was measured using an analytical balance (Radwag, Poland). The HEK293 cells were once more collected by centrifugation at 1000 x g for 5 mins (Yingtai TD5) followed by resuspension in 1 mL 0.01 M pH 7.4 PBS (Sigma-Aldrich, USA) and transferred to a 2 mL microcentrifuge tubes. The cells were collected at 1000 x g for 5 min. The lipid droplet isolation kit (Abcam, UK) was used to isolate LDs from HEK293 cells. Briefly, cells were resuspended in

200 µL 1X kit reagent A and incubated for 10 min on ice. The incubation period was followed by the addition of 800 µL of 1X kit reagent B and incubated on ice for 10 min. The homogenate was passed five times through a one inch 27-gauge needle connected to a 3 mL syringe (Avacare, SA). The homogenate was centrifuged at 100 x g for 5 min followed by the addition of 600 µL 1X kit reagent B in a gentle and dropwise procedure. The homogenate was centrifuged at 20 000 x g for 3 h at 4$^{o}$C to produce a gradient isolation of LDs. Once centrifugation was complete, the top five fractions were collected for triacylglycerides (TAG) quantification (**Figure 2.3B**).

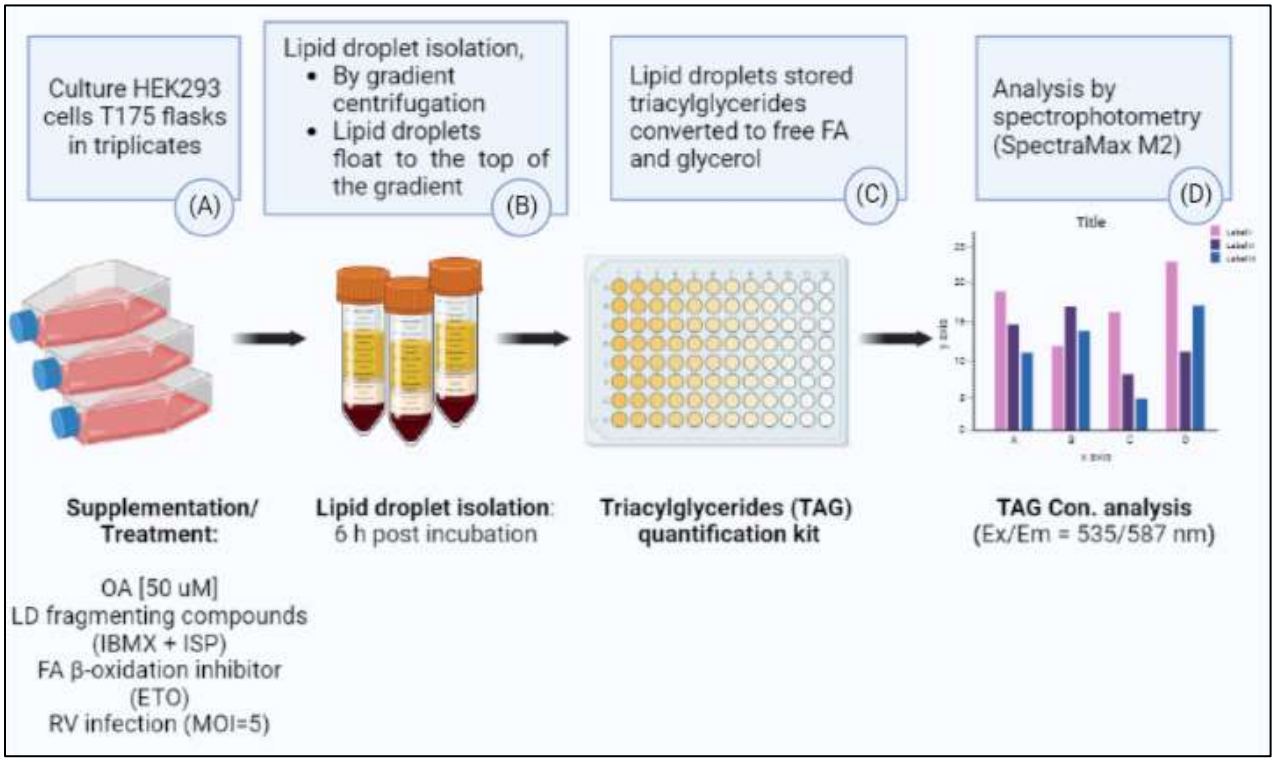


**Figure 2.3. Schematic representation of** (A-B) lipid droplet isolation and (C-D) lipid droplet triacylglycerides (TAG) quantitation. Designed with BioRender.

2.8. Quantification of triacylglyceride (TAG) concentration in LDs

The concentration of TAG in the isolated LDs was determined using a Pico-probe triglyceride quantification assay kit (Abcam, UK). A TAG standard curve was prepared with a 0.2 to 0.8 mM TAG concentration range (**Table 2.1**) and 50 µL of each standard sample was added to an appropriate well onto a 96 well-culture plate (Thermo Scientific, USA) in duplicate. This was followed by the addition of 2 µL lipase provided in the kit into standard sample wells, followed by an incubated period of 20 mins at room temperature (RT) under constant agitation to produce free FA (**Figure 2.4)**. After the time has elapsed, 50 µL of kit reaction mixture consisting of TAG assay buffer (47.6 µL), pico-probe (0.4 µL) and TAG enzyme mix (2 µL) was added followed by an incubation period of 60 mins at RT while protected from light (**Figure 2.3C**). The TAG measurement output was determined using the SpectraMax M2 plate reader (Molecular Devices, USA) at Ex/Em = 535/587 nm (**Figure 2.3D**). The TAG concentration of the isolated samples was determined by adding 50 µL of the samples into separate wells and analyzed as described for the standard curve (**2.8**). The TAG standard 6 was used as a positive control while the assay buffer was regarded as a negative control for TAG quantification.

Table 2.1. Experimental set-up for TAG standard curve determination using the Pico-probe triglyceride quantification assay kit (Abcam, UK).

| Standard | TAG standard (µL) | Assay buffer (µL) | Final concentration amount of TAG standard (nmol/well) |
|---|---|---|---|
| 1 | 0 | 150 | 0.2 |
| 2 | 30 | 120 | 0.4 |
| 3 | 60 | 90 | 0.5 |
| 4 | 90 | 60 | 0.6 |
| 5 | 120 | 30 | 0.7 |
| 6 | 150 | 0 | 0.8 |

The determined weight of the trypsinized cells was used to give the final concentration of TAG per 175 $cm^2$ culture flask as TAG concentration µmol. per cell mass (mg).

Calculation TAG concentration:

Triacylglycerol concentration (TAG Con.) = $\frac{\mathrm{B}}{\mathrm{V}} * D$ (mM)

$\mathrm{B}$ = Amount of triglyceride in the sample well calculated from the standard curve in nmol.

$V$ = Sample volume added in the sample wells (µL)

$D$ = Sample dilution factor before reaction well set up.

Normalized concentration per cell mass= $\frac{(\text{TAG Con.})}{m}$ (nM/mg of cells)

$m$ **=** Mass of cells from which lipid droplets were isolated (mg)

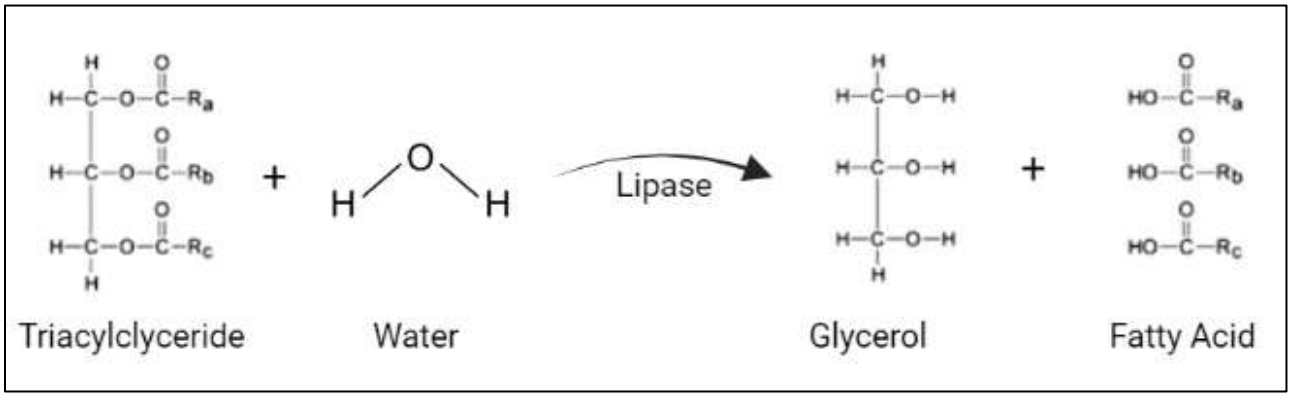


**Figure 2.4. Reaction mechanism for the conversion of triacyglyceride (TAG) to glycerol and free fatty acids (FA).** The HEK293 cell's lipid droplets (LDs) liberated TAG is converted to free FA and glycerol molecules by the lipase. The produced glycerol molecule is later oxidized to generate a colorimetric product used to quantify liberated FA. (Adapted from Díaz et al., 2014). Designed with BioRender.

2.9. Oxygen consumption rate (OCR) analysis

Assay optimization was achieved using the MitoXpress Xtra – Oxygen consumption assay (HS method) Kit, (Agilent, USA) according to the manufacturer's instructions. HEK293 cells were seeded at 8 x $10^4$ cell/well in a 96-well fluorometric black plates (Thermo scientific, USA) in complete growth media. The cells were incubated overnight at 5% $CO_2$ and 37°C. The cells were either supplemented and incubated with 50 µM OA, a combination of (1 Mm IBMX and 20µM ISP) and/or 60 µM ETO with and without SA11 infection (**2.6**) as previously described (**2.3 and 2.5**) for a period of 6 h (**Figure 2.5A**). The used growth media was removed, and the cells were washed once with 0.01 M, pH 7.4 PBS (Sigma Aldrich, USA) and replaced with 80 µL of fresh complete growth media for SA11 infections. Subsequently, 20 µL of reconstituted MitoXpress reagent (Agilent, USA) was added to each well, except for wells used as blank controls to which 20 µL of fresh complete growth media was added to make the final volume 100 µL. All

experimental wells were immediately sealed by adding two drops of prewarmed HS Mineral Oil (Agilent, USA). The plate was analyzed using a SpectraMax M2 plate reader (Molecular Devices, USA) kinetically for 120 mins every 2min at 30$^{o}$C (Ex/Em = 380 nm / 650 nm) in quadruplicates (**Figure 2.5B**). The slope of each generated kinetic curve was determined to give a direct value of HEK293 cells oxygen consumption rate (OCR) and all values were corrected by subtracting the blank signal. Glucose Oxidase (GOx) (Sigma Aldrich, USA), an enzyme that readily deplete $O_2$ in cell free wells was used as a positive control while complete growth media was used as cell free negative control for the assay (Agilent technologies, USA)

To ensure changes in host cell OCR was not due to reduction in HEK293 cell viability, the XTT cell proliferation assay kit (Sigma Aldrich, USA) was used to evaluate HEK293 cell viability post supplementation/treatment with IBMX, ISP, ETO and OA during RV infection. Briefly, HEK293 cells were seeded at a concentration of $4 \times 10^3$ cells/well into a 96 - well culture plate (Thermo Scientific, USA) and cultured at 37$^{o}$C and 5% $CO_2$ overnight. The cells were washed once with 0.01 M pH 7.4 PBS (Sigma-Aldrich, USA). The HEK293 cells were supplemented and incubated with 50 µM OA, a combination of (1 Mm IBMX and 20µM ISP) and/or 60 µM ETO with and without SA11 infection for a period of 6 has described (**2.3, 2.5 and 2.6**) (**Figure 2.1C**). This was then followed by addition of 50 µL XTT labeling mixture ((master mix: 5 mL 1 mg/mL sodium 3´- [1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate and 0.1 mL N-methyl dibenzopyrazine methyl sulfate) (Sigma Aldrich, USA) per well and incubated for 4 h at 37 °C, 5% $CO_2$ (**Figure 2.1D**). The spectrophotometric absorbance of each well was measured using a Victor Nivo multimode microplate reader (Perkin Elmer, USA) a wavelength of 480 nm (**Figure 2.1E**).

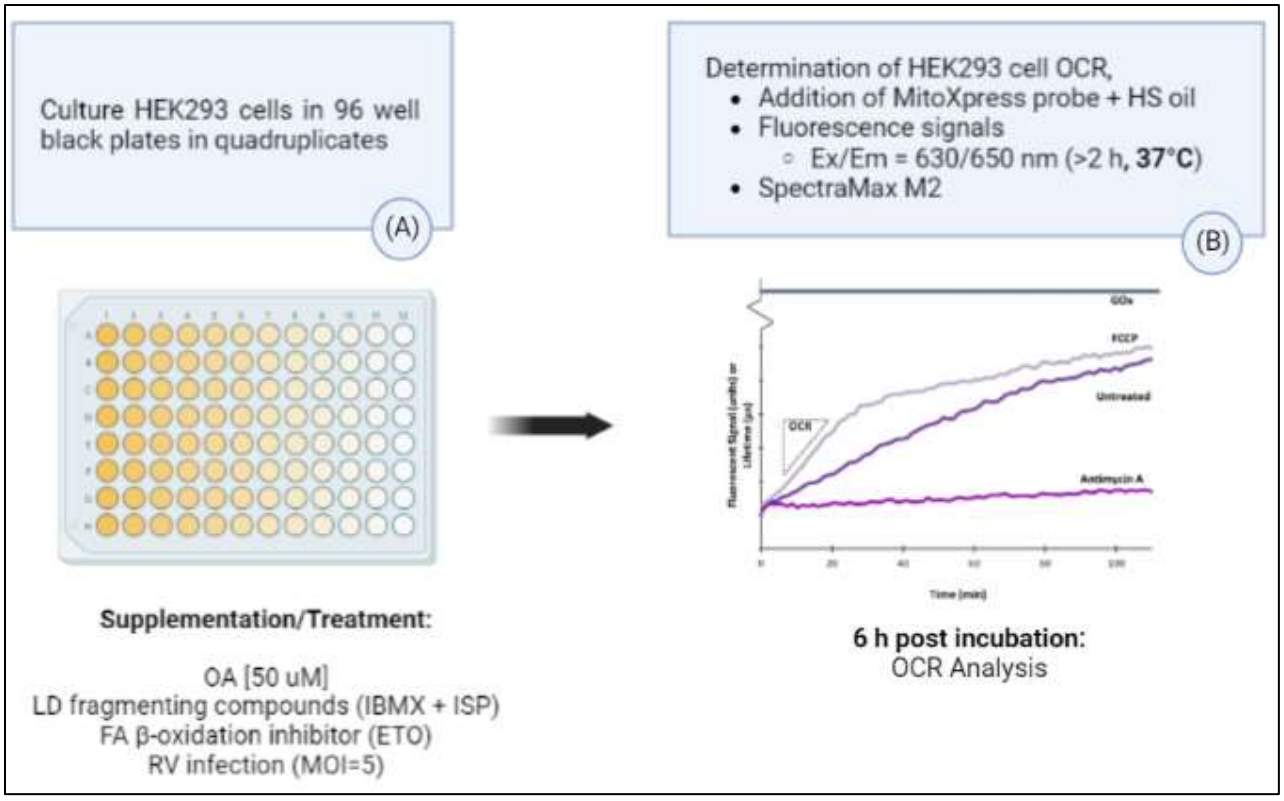


**Figure 2.5. Experimental overview of HEK293 cells oxygen consumption (OCR) quantification, depicting** (A) HEK293 cell culture in 96-well plates and (B) signal profiles used to determine profile sample slopes (OCR). Designed with BioRender.

Calculations OCR and normalizing to overall % effect:

OCR (slope) RFU = $\frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$

Where the values of $\bar{x}$ and $\bar{y}$ are the sample means (the averages) of the known x and y-values of the regression line of the linear part of the sample signal profile.

Normalization of sample slopes as % HEK293 cells where the untreated control is 100% Effect.

% effect = (x/m)*100

x = is the calculated slope for a given sample

m = is the calculated slope for the untreated HEK293 cells

2.10. Statistical analysis

Each assay was carried out at least in three biological replicates on separate days unless stated otherwise. Each biological replicate was analyzed at least in triplicate (three technical replicates) unless stated otherwise (**Figure 2.6**). Data are presented as standard error of the mean. For statistical analysis, two-way ANOVA was performed using a Tukey-Kramer test in GraphPad Prism version 9.4.1 for Windows (GraphPad Software, San Diego, Calif.). GraphPad t-test was used to determine data statistical significance. In all tests $p<0,05$ was considered significant.

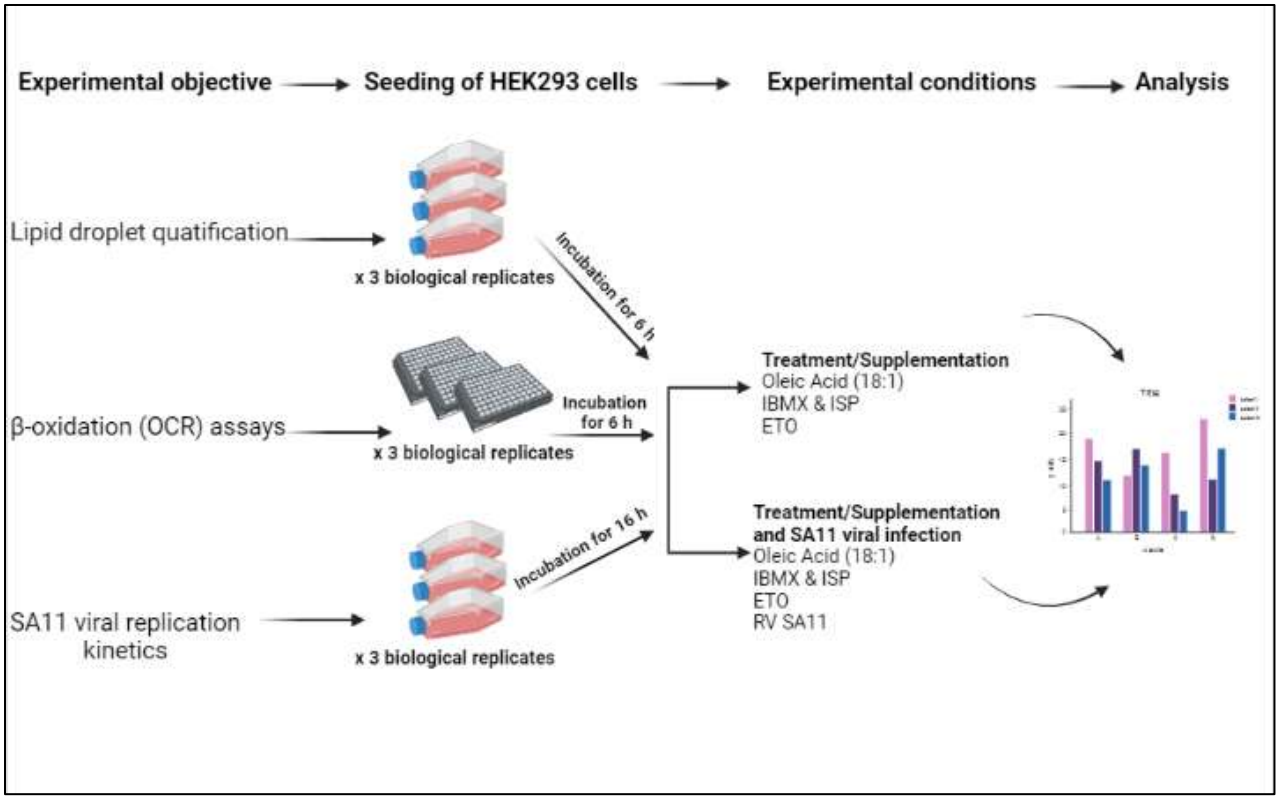


**Figure 2.6. An overview experimental design of the study including biological replicates, incubation times and experimental conditions.** Designed with BioRender.

## Chapter 3 – Results

### 3.1. HEK293 cell viability

#### 3.1.1. Effect of serum-free media on HEK293 total cell number and viability

During the study, to understand the interaction between HEK293 cells and rotavirus (RV) infection under a variety of conditions, serum-free media was used to conduct viral infections. Serum-free media is used for viral infections as it is not supplemented with foetal bovine serum (FBS) (a component of complete growth media) reported to hinder viral activation (Arnold et al., 2009). It was important to investigate the effect of serum-free media on HEK293 total cell number and cell viability over the time course that all experiments will be conducted. The data show that at time point 0 h, there is no significant change in the total cell number of HEK293 cells supplemented with serum-free media [$1.7x10^6$ ($\pm5x10^4$) cells/mL] as compared to HEK293 cells supplemented with complete growth media [$1.7x10^6$ ($\pm5x10^4$) cells/mL]. Similarly, at 16 h, there were no adverse effects of the lack of serum on HEK293 cells, with the cell number of HEK293 cells in serum-free media [$4.4x10^6$ ($\pm1x10^5$) cells/mL] comparable to the cell number in complete growth media [$3.2x10^6$($\pm5x10^5$) cells/mL] (**Table 3.1; Figure 3.1A**). The maintenance of HEK293 cells in serum-free media caused a slight, but significant, reduction in cell viability [98% ($\pm3.6x10^{-3}$) viability] compared to cells maintained in 5% serum complete growth media [100% ($\pm2.0x10^{-2}$) % viability] over a 16 h incubation period (**Table 3.2; Figure 3.1B**). The data thus show that the supplementation of HEK293 cells with serum-free media only caused slight changes in both cell number and viability **(Figure 3.1**).

Table 3.1: The total cell number of HEK293 cells maintained in serum-free media over a period of 16 h

| HEK293 cell number: | 5% Serum (Cell/mL) | 0% Serum-free (Cell/mL) | *P* – value |
|---|---|---|---|
| 0 h | $1.7x10^6$ ($\pm5x10^4$) | $1.7x10^6$ ($\pm5x10^4$) | 0.8471 |
| 16 h | $3.2x10^6$ ($\pm5x10^5$) | $4.4x10^6$ ($\pm1x10^5$)* | 0.0466 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

Table 3.2: The total cell viability of HEK293 cells maintained in serum-free media over a period of 16 h

| HEK293 cells % viability: | 5% Serum (%Viability) | 0% Serum-free (%Viability) | *P* – value |
|---|---|---|---|
| 16 h | 100% ($\pm2.0x10^{-2}$) | 100% ($\pm2.0x10^{-2}$)* | <0.0001 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

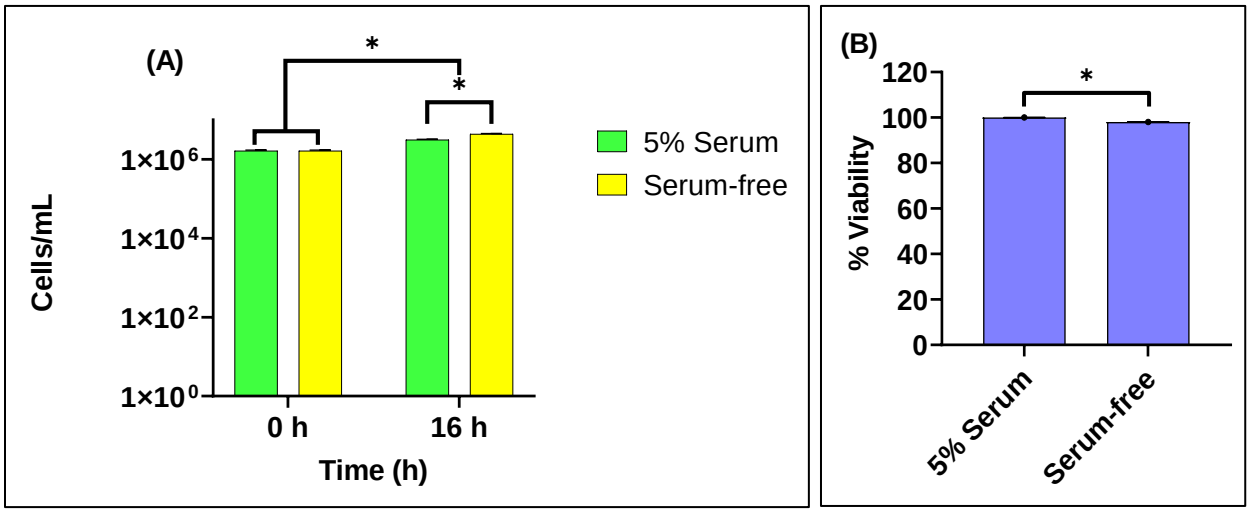


**Figure 3.1. The total cell number and total cell viability of HEK293 cells maintained in serum-free media.** (A) show the HEK293 total cell number over a period of 16 h post-serum-free media supplementation, while (B) reflects HEK293 total cell viability 16 h post-serum-free media supplementation. Values represent the mean of three biological replicates. Standard deviation is indicated by error bars. ***** Indicate significance, *P* <0.05.

### 3.1.2. Evaluation of etomoxir toxicity in HEK293 cells

The initial step in inducing β-oxidation inhibition in HEK293 cells using etomoxir (ETO), is to determine the toxicity of different ETO concentrations in host cells to determine which concentration can be utilized in the experiments without significantly reducing the overall cell viability to <80%, while still ensuring β-oxidation inhibition without off-target effects. A reduction of less than 30% of the overall cell viability is seen to be acceptable to conclude that a compound in question is non-toxic to host cells and may not affect result significantly (Rudolf, 2021). The treatment of HEK293 cells with ETO concentrations below 60 µM caused no significant reduction in total cell viability compared to the control and untreated HEK293 cells (**Table 3.3; Figure 3.2**). However, a significant reduction in cell viability at ETO concentration from 60 µM [88% ($\pm 1.4 \times 10^{-1}$) viability], 80 µM [52% ($\pm 3 \times 10^{-1}$) viability], 100 µM [51% ($\pm 8.6 \times 10^{-2}$) viability] to 120 µM [17% ($\pm 2.1 \times 10^{-1}$)viability] compared to the untreated HEK293 cells, was seen. Previous studies found that ETO concentrations > 3 µM were effective in inhibiting carnitine palmitoyltransferase I (CPT1), and thus β-oxidation (Divakaruni et al., 2018; Raud et al., 2018). However, it is also known that ETO concentrations >100 µM have an off-target effect on serine/threonine kinase, thus, although 100 µM ETO was used in literature (Divakaruni et al., 2018; Rasmussen et al.,

2011; Raud et al., 2018), 60 µM ETO was selected as the highest ETO concentration that can be used and that allowed >80% of HEK293 cells to remain viable.

Table 3.3: The total cell viability of HEK 293 cells after treatment with different concentrations of etomoxir (ETO)

| | Viability | *P* – value |
|---|---|---|
| Control | 100% ($\pm 8.8 \times 10^{-2}$) | |
| 20 | 100% ($\pm 8.0 \times 10^{-3}$) | 1 |
| 40 | 100% ($\pm 9.6 \times 10^{-2}$) | 1 |
| 60 | 88% ($\pm 1.4 \times 10^{-1}$) * | <0.0001 |
| 80 | 52% ($\pm 3 \times 10^{-1}$) * | <0.0001 |
| 100 | 51% ($\pm 8.6 \times 10^{-2}$) * | <0.0001 |
| 120 | 17% ($\pm 2.1 \times 10^{-1}$) * | <0.0001 |

*: Significantly different from rotavirus infected HEK293 controls cells (*P* < 0.05)

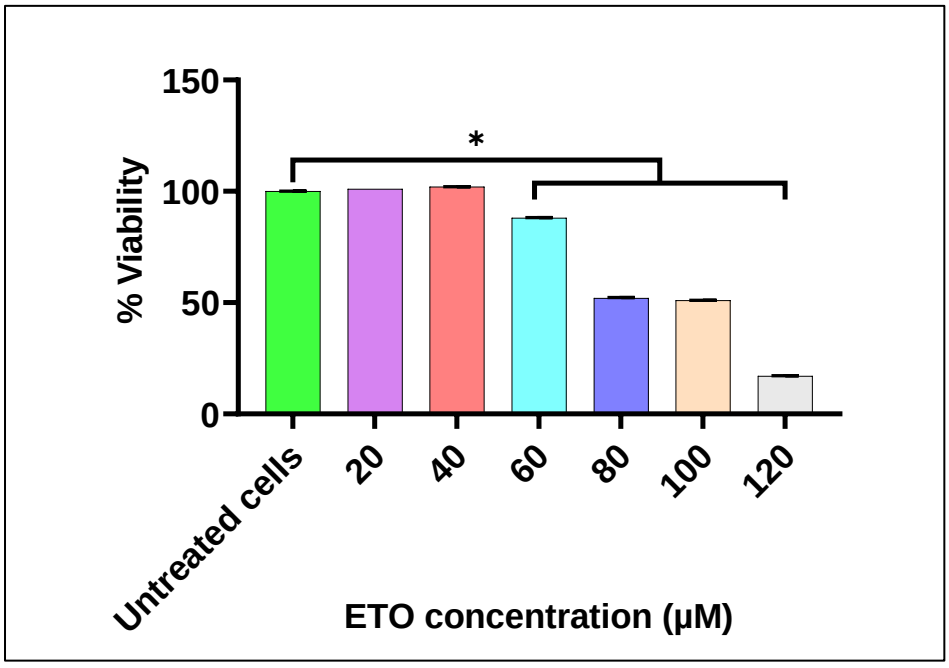


**Figure 3.2. HEK293 total cell viability after treatment with different concentrations of etomoxir (ETO).** Values depict the mean of three biological replicates. Standard deviation is indicated by error bars. * Indicate significance, *P* <0.05.

## 3.3. Investigation of lipid droplets in HEK293 cells

### 3.3.1. Effect of oleic acid supplementation, lipid droplet fragmentation, and fatty acid β-oxidation inhibition on overall triacylglyceride content in lipid droplets

Triacylglycerides (TAGs) are one of the major lipid classes stored in lipid droplets (LDs), and various studies have shown that an effective way to quantify LDs is to determine the total TAG content in isolated LDs (Cheung et al., 2010). In the study, the fragmentation of LDs by the treatment of HEK293 cells with a combination of 3-isobutyl-1-methylxanthine (IBMX) and Isoproterenol (ISP) resulted in the reduction of total TAG content of host cells [$7x10^{-4}$ ($\pm 2.1x10^{-4}$) mM/mg] compared to the untreated HEK293 cells control [$10x10^{-4}$ ($\pm 1.4x10^{-4}$) mM/mg], while the treatment with the β-oxidation inhibitor (ETO) caused a significant increase in total TAG content [$2x10^{-3}$ ($\pm 1.949x10^{-4}$) mM/mg] compared to both the untreated cells as well as cells treated with IBMX and ISP (**Table 3.4; Figure 3.3**). It is also important to note that the supplementation of HEK293 cells with oleic acid (OA) significantly increased the total TAG content of host cells [$3x10^{-3}$ ($\pm 2.2x10^{-4}$) mM/mg] compared to the unsupplemented cells (**Table 3.4; Figure 3.3**). The same increase in total TAG content was observed when HEK293 cells were supplemented with OA in combination with IBMX and ISP or ETO (**Table 3.4; Figure 3.3)**.

Table 3.4: The total triacylglyceride (TAG) content of HEK293 cells supplemented with oleic acid (OA) in the presence or absence of the combination of 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) or etomoxir (ETO) treatment

| | OA (-) (nM/mg) | OA (+) (nM/mg) | *P* – value |
|---|---|---|---|
| HEK293 | $10x10^{-4}$ ($\pm 1.4x10^{-4}$) | $3x10^{-3}$ ($\pm 2.2x10^{-4}$) ***** | 0.0252 |
| IBMX+ISP | $7x10^{-4}$ ($\pm 2.1x10^{-4}$) | $1x10^{-3}$ ($\pm 1.4x10^{-4}$) | 0.2485 |
| ETO | $2x10^{-3}$ ($\pm 1.9x10^{-4}$) | $4x10^{-3}$ ($\pm 3.0x10^{-5}$) ***** | 0.0122 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

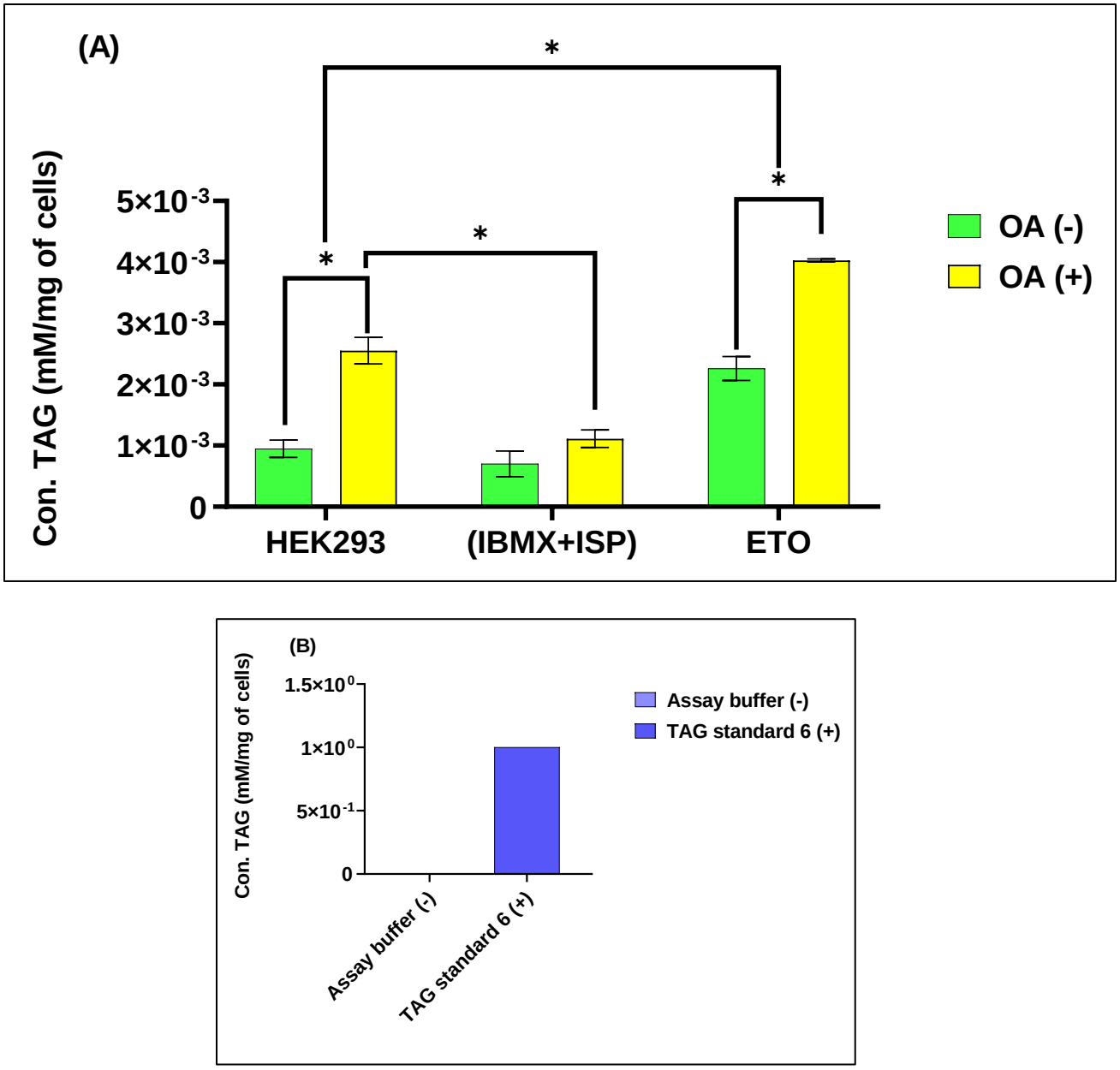


**Figure 3.3. The total triacylglyceride (TAG) content of HEK293 cells supplemented with oleic acid (OA) in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) and etomoxir (ETO) treatment.** (A) HEK293 cells were supplemented with 50 µM of OA and treated with IBMX (1 mM) and ISP (20µM) and 60 µM ETO as previously described. The total TAG content was determined after 6 h incubation period with OA and the different inhibitors. (B) Negative control: assay buffer, positive control: TAG standard 6. Values depict the mean of three biological replicates. Standard deviation is indicated by error bars. ***** Indicate significance, *P* <0.05.

### 3.3.2. Effect of oleic acid supplementation, lipid droplet fragmentation, and β-oxidation inhibition on triacylglycerol content of lipid droplets during rotavirus infection

The study also investigated the overall effect of LD fragmentation and β-oxidation inhibition on overall host cell total TAG content during RV infection. The most significant difference in total host TAGs between infected and uninfected cells was that, even in the absence of OA supplementation, RV infection caused an increase in TAG content. In general, the effect of the different inhibitors was similar to those observed for uninfected cells. (**Table 3.5; Figure 3.4).**

Table 3.5: The total triacylglyceride (TAG) content of HEK293 cells supplemented with oleic acid (OA) in the presence or absence of a combination of 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) or etomoxir (ETO) during rotavirus (RV) infection

| | OA (-) (nM/mg) | OA (+) (nM/mg) | *P* – value |
|---|---|---|---|
| HEK293 | $10x10^{-4}$ ($\pm 1.4x10^{-4}$) | $3x10^{-3}$ ($\pm 2.2x10^{-4}$) ***** | 0.0252 |
| RV | $2x10^{-5}$ ($\pm 4.0x10^{-6}$) | $2x10^{-5}$ ($\pm 3.0x10^{-6}$) | 0.9261 |
| RV+(IBMX+ISP) | $1x10^{-5}$ ($\pm 2.4x10^{-7}$) | $1x10^{-5}$ (0.0) ***** | 0.0343 |
| RV+ETO | $7.0x10^{-6}$ ($\pm 2.4x10^{-7}$) | $2x10^{-5}$ ($\pm 1.0x10^{-6}$) ***** | 0.0070 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

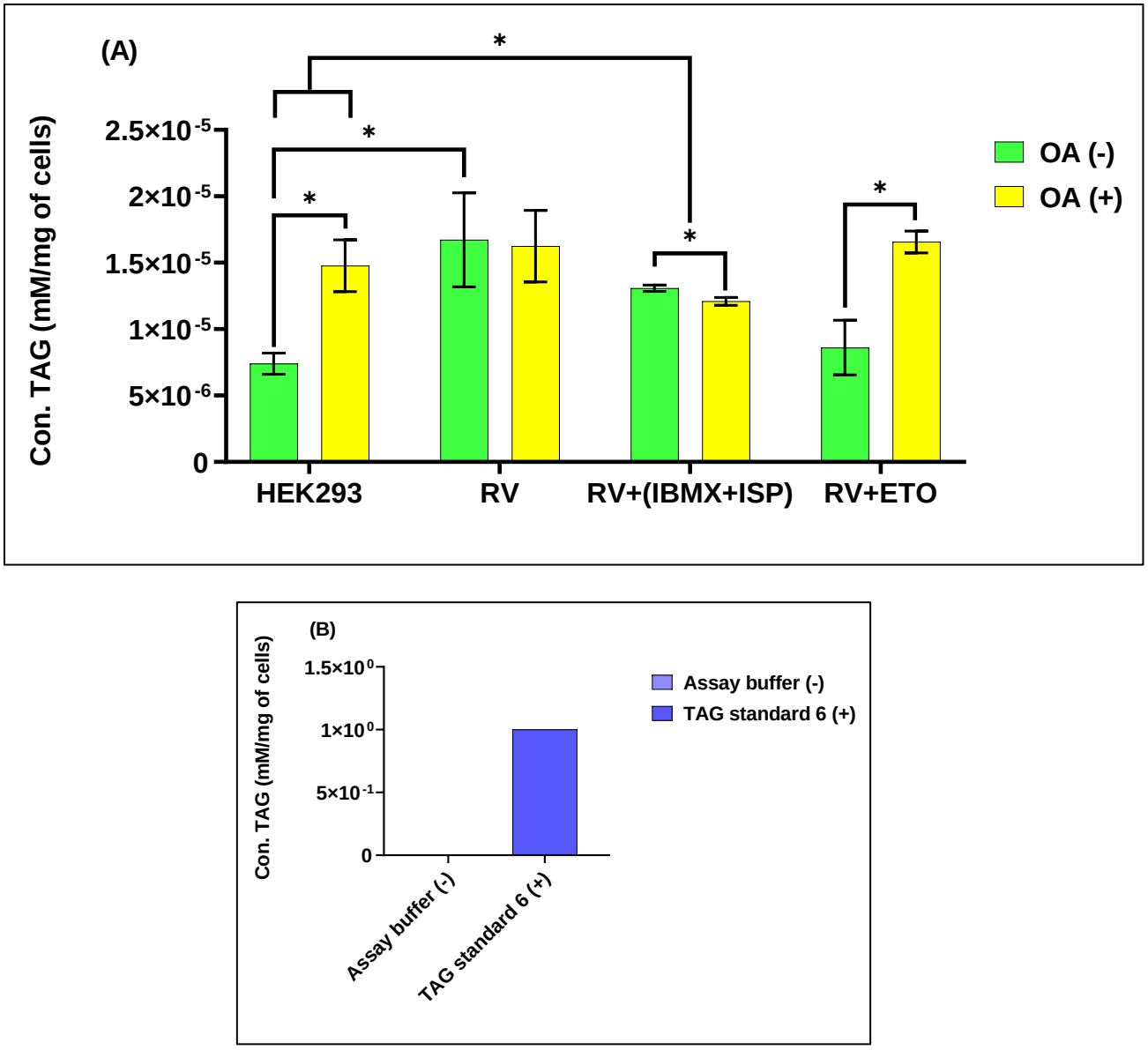


**Figure 3.4. The total triacylglyceride (TAG) content of HEK293 cells supplemented with oleic acid (OA) in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) and etomoxir (ETO) treatment during rotavirus (RV) infection.** (A) HEK293 cells were supplemented with 50 µM of OA and treated with IBMX (1 mM) and ISP (20µM) and 60 µM ETO during RV infection at MOI = 5. Total TAG content was determined 6 h p.i as previously described. (B) Negative control: assay buffer, positive control: TAG standard 6. Values depict the mean of three biological replicates. Standard deviation is indicated by error bars. ***** Indicate significance, *P* <0.05.

### 3.4. HEK293 cell oxygen consumption rate (OCR)

#### 3.4.1. Effect of oleic acid supplementation, lipid droplet fragmentation, and β-oxidation inhibition on the oxygen consumption rate in HEK293 cells

Fatty acid β-oxidation is an important pathway in cellular respiration, used for the breakdown of fatty acids for the generation of energy for the host cell. The rate of $O_2$ utilization has been reported to be an accurate measure of β-oxidation in host cells (Houten & Wanders, 2010; Ojuka et al., 2016). In our study, HEK293 cells were subjected to OA supplementation, induced LD fragmentation, β-oxidation inhibition, and RV infection while analyzing the rate of $O_2$ consumption (OCR) over time. Our study showed that the treatment of HEK293 cells with IBMX and ISP resulted in a significant reduction in host cell OCR compared to the untreated cells over time (**Table 3.6; Figure 3.5**). The treatment of HEK293 cells with ETO also resulted in a significant reduction in the overall OCR over time [from 100% to 58% (±3.1) effect] compared to the untreated HEK293 cells, although the decrease is less than that observed when HEK293 cells were treated with IBMX and ISP [from 100% to 16% ($\pm 5x10^{-1}$) effect] (**Table 3.6; Figure 3.5**). The supplementation of HEK293 cells with OA resulted in a significant increase in OCR [from 100% to 175% ($\pm 1.0x10^{1}$) effect] as compared to the OA unsupplemented HEK293 control cells over time (**Table 3.6; Figure 3.5**). Cells supplemented with OA generally exhibited the same response to all the inhibitors as unsupplemented cells, however the inhibitory effect was slightly overcome by OA supplementation (**Table 3.6; Figure 3.5**).

Table 3.6: The overall oxygen consumption rate (OCR) of HEK293 cells supplemented with oleic acid (OA) in the presence and absence of both 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) or etomoxir (ETO) treatment

| | OA (-) (% Effect) | OA (+) (% Effect) | *P* – value |
|---|---|---|---|
| HEK293 | 100 (±3.7) | 175 (±10.0) ***** | 0.0001 |
| IBMX+ISP | 16 ($\pm 1.0x10^{-1}$) | 26 ($\pm 6.0x10^{-1}$) ***** | 0.0053 |
| ETO | 58 (±3.1) | 84 (±4.0) ***** | 0.0021 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

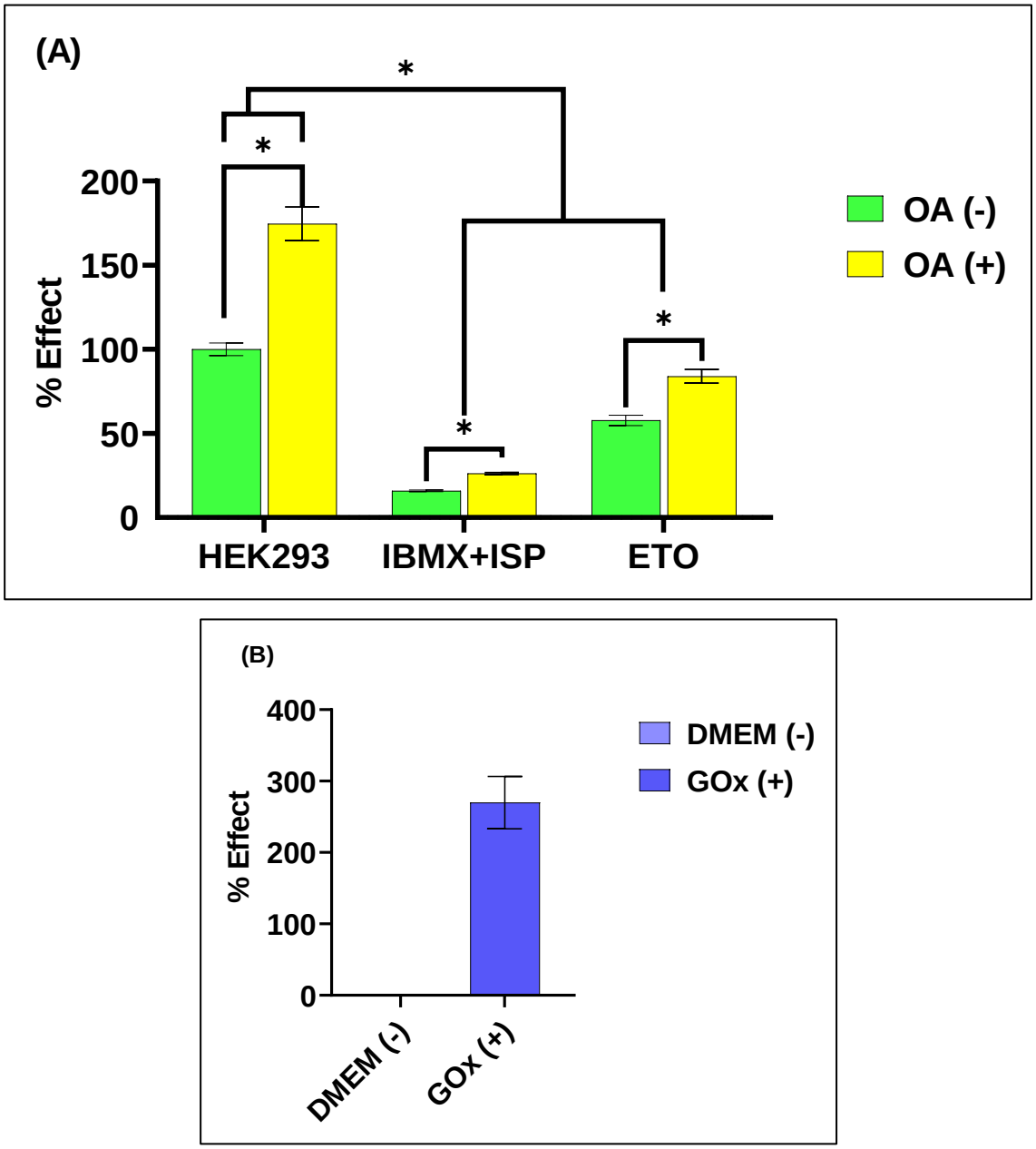


**Figure 3.5. The overall oxygen consumption rate (OCR) of HEK293 cells supplemented with oleic acid (OA) in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) and etomoxir (ETO) treatment.** (A) HEK293 cells were supplemented with 50 µM of OA and treated with (IBMX (1 mM) and ISP (20µM)) and 60 µM ETO as previously described. The overall OCR was determined after 6 h of incubation with OA and the different inhibitors. (B) Cell free negative control: complete growth media (DMEM), cell free positive control: glucose oxidase (GOx). Values depict the mean of three biological replicates. Standard deviation is indicated by error bars. ***** Indicate significance, *P* <0.05.

### 3.4.2. Effect of OA supplementation, LD fragmentation, and FA β-oxidation inhibition on HEK293 cell OCR during RV infection

An important aspect of the study was to determine the effect of RV infection on HEK293 cell OCR over time, during OA supplementation, and in the presence of LD fragmentation compounds and fatty acid β-oxidation inhibitors. In the study, it was observed that RV infection results in the reduction of the overall host cell OCR [from 100% to 75% (±11.9) effect] and the addition of inhibitors to RV-infected cells resulted in a further reduction of the overall host cell OCR over time (**Table 3.7; Figure 3.6**). The OA supplementation of HEK293 cells resulted in an increase in OCR [from 100% to 170% (±10.0) effect] compared to the unsupplemented HEK293 cells (**Table 3.7; Figure 3.6**). However, OA supplementation could not overcome the inhibitory effect of RV infection or treatment with inhibitors observed (**Table 3.7; Figure 3.6**).

Table 3.7: The overall oxygen consumption rate (OCR) of HEK293 cells supplemented with oleic acid (OA) in the presence and absence of (3-isobutyl-1-methylxanthine (IBMX) and Isoproterenol (ISP)) and etomoxir (ETO) treatment during rotavirus (RV) infection

| | OA (-) (% Effect) | OA (+) (% Effect) | *P* – value |
|---|---|---|---|
| HEK293 | 100 (±4.4) | 175 (±10.0) ***** | 0.0081 |
| RV | 75 (±12) | 82 (±13.5) | 0.6971 |
| RV+(IBMX+ISP) | 40 (±9.6) | 52 (±13.34) | 0.4896 |
| RV+ETO | 45 (±4.2) | 40 (±3.3) | 0.4221 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

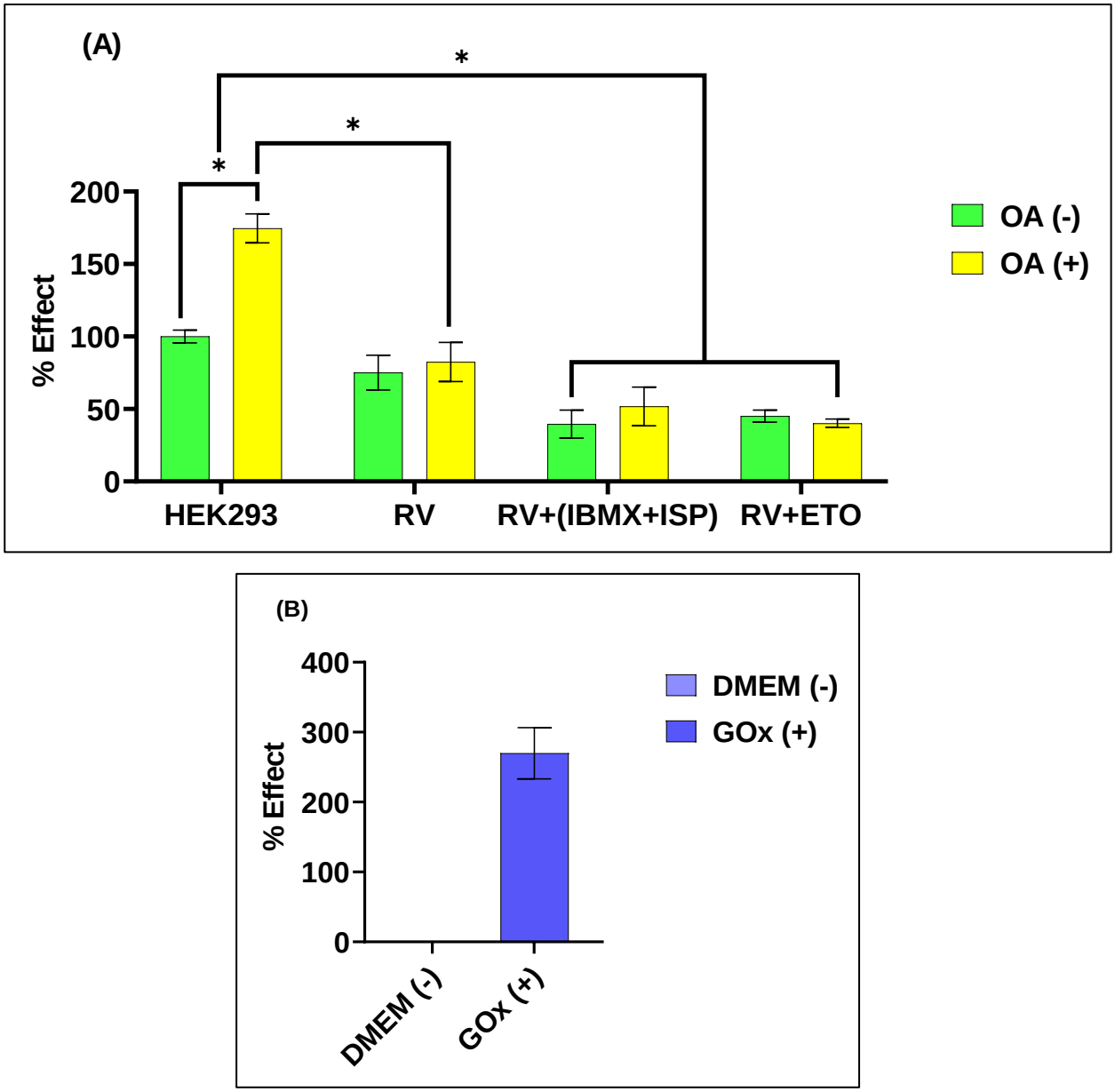


**Figure 3.6. The overall oxygen consumption rate (OCR) of HEK293 cells supplemented with oleic acid (OA) in the presence and absence of (3-isobutyl-1-methylxanthine (IBMX) and Isoproterenol (ISP)) and etomoxir (ETO) treatment during rotavirus (RV) infection.** (A) HEK293 cells were supplemented with 50 µM of OA and treated with (IBMX (1 mM) and ISP (20µM)) and ETO 60 µM as previously described. The overall OCR was determined after 6 h of incubation with OA and the different inhibitors. (B) Cell free negative control: complete growth media (DMEM), Cell free positive control: glucose oxidase (GOx). Values depict the mean of three biological replicates. Standard deviation is indicated by error bars. ***** Indicate significance, *P* <0.05.

### 3.5. Rotavirus replication kinetics

#### 3.5.1. Effect of oleic acid supplementation on rotavirus replication in HEK293 cells

As previously described, RV infections are reported to alter the lipidome of the infected host cells, thus implicating the role of fatty acids in RV infection and replication (**1.3.4**). To determine the effect of OA supplementation on RV replication, HEK293 cells were infected with RV (MOI = 5) post OA supplementation, and the viral titer was quantified using $TCID_{50}$ over 16 h post infection. In our study, it was observed that RV infection of OA-supplemented HEK293 cells results in a significant increase in RV replication over the 16 h time course, compared to the OA unsupplemented RV-infected cells **(Table 3.8; Figure 3.7)**. This suggests that OA supplementation increases viral load over time.

Table 3.8. Viral titer of rotavirus infectious progeny over a period of 16 h post infection as ($TCID_{50}$/mL) after oleic acid supplementation

| Condition / Time points | $TCID_{50}$/mL | | | | |
|---|---|---|---|---|---|
| | 0 h | 2 h | 6 h | 12 h | 16 h |
| RV | $5x10^5$ ($\pm 3.2x10^5$) | $2x10^6$ ($\pm 9.0x10^5$) | $6x10^8$ ($\pm 3.3x10^8$) | $2x10^{10}$ ($\pm 1.1x10^{10}$) | $4x10^{10}$ ($\pm 1.7x10^{10}$) |
| RV+OA | $5x10^5$ ($\pm 3.2x10^5$) | $2x10^7$ ($\pm 2.1x10^7$) | $2x10^{10}$ ($\pm 4.1x10^9$) ***** | $4x10^{11}$ ($\pm 2.0x10^{11}$) ***** | $7x10^{11}$ ($\pm 4.5x10^{11}$) ***** |
| *P* – value | 1.00 | < 0.0001 | <0.0001 | 0.0030 | 0.0388 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

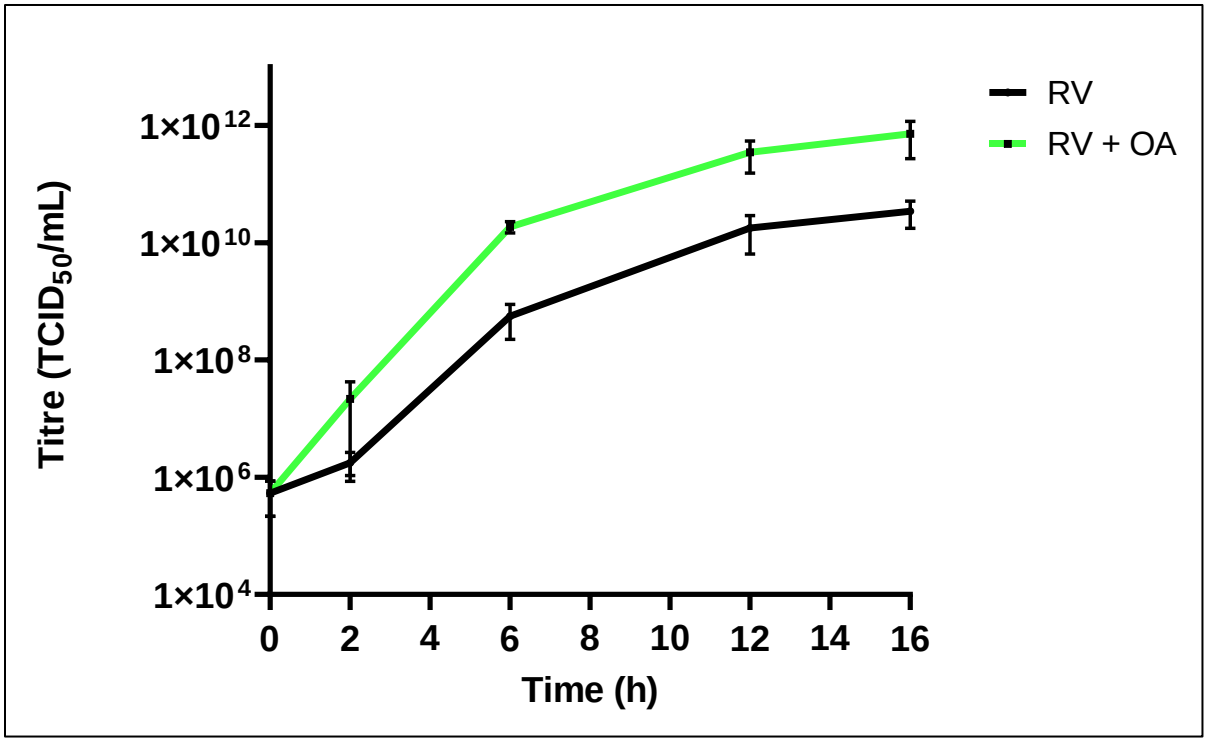


**Figure 3.7. Viral replication kinetics of rotavirus during oleic acid (OA) supplementation in HEK293 cells.** HEK293 cells were supplemented with 50 µM oleic acid (OA) and infected with RV for a period of 16 h. $TCID_{50}$ were used to determine RV titer at various time points post infection. Cells were counted before seeding and again prior to infection. Values depict the mean of three biological replicates. Standard deviation is indicated by error bars.

### 3.5.2. Effect of lipid droplet fragmentation on rotavirus replication

HEK293 cells treated with IBMX (1 mM), and ISP (20 µM) were infected with RV (MOI = 5) with and without OA supplementation to determine the effect of LD fragmentation on RV replication. From our data, it was evident that the treatment of RV infected HEK293 cells with IBMX and ISP significantly reduces the overall production of RV infectious progeny in unsupplemented cells (**Table 3.9; Figure 3.8**). This effect can be partially rescued by OA supplementation. Thus, the provided data show that treatment of HEK293 cells with IBMX and ISP in the presence or absence of OA supplementation results in the reduction of overall RV replication.

Table 3.9. Viral titer of rotavirus infectious progeny over a period of 16 h post infection as ($TCID_{50}$/mL) after IBMX and ISP treatment

| Condition / Time points | $TCID_{50}$/mL | | | | |
|---|---|---|---|---|---|
| | 0 h | 2 h | 6 h | 12 h | 16 h |
| RV | $5x10^5$ ($\pm3.2x10^5$) | $2x10^6$ ($\pm9.0x10^5$) | $6x10^8$ ($\pm3.3x10^8$) | $2x10^{10}$ ($\pm1.1x10^{10}$) | $4x10^{10}$ ($\pm1.7x10^{10}$) |
| RV+OA | $5x10^5$ ($\pm3.2x10^5$) | $2x10^7$ ($\pm2.1x10^7$) | $2x10^{10}$ ($\pm4.1x10^9$) * | $4x10^{11}$ ($\pm2.0x10^{11}$) * | $7x10^{11}$ ($\pm4.5x10^{11}$) * |
| *P* – value | 1.00 | < 0.0001 | <0.0001 | 0.0030 | 0.0388 |
| RV+(IBMX+ISP) | $5x10^5$ ($\pm3.2x10^5$) | $1x10^6$ ($\pm9.7x10^5$) | $1x10^6$ ($\pm0.00$) * | $7x10^7$ ($\pm6.1x10^7$) * | $1x10^8$ ($\pm9.5x10^7$) * |
| *P* – value | 1.00 | 0.3543 | 0.0184 | 0.0215 | 0.0775 |
| RV+(IBMX+ISP)+OA | $5x10^5$ ($\pm3.2x10^5$) | $2x10^5$ ($\pm1.2x10^5$) * | $3x10^6$ ($\pm1.5x10^6$) * | $1x10^9$ ($\pm5.6x10^8$) * | $2x10^9$ ($\pm4.2x10^8$) * |
| *P* – value | 1.00 | 0.0085 | 0.0032 | 0.0183 | 0.0474 |

*: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

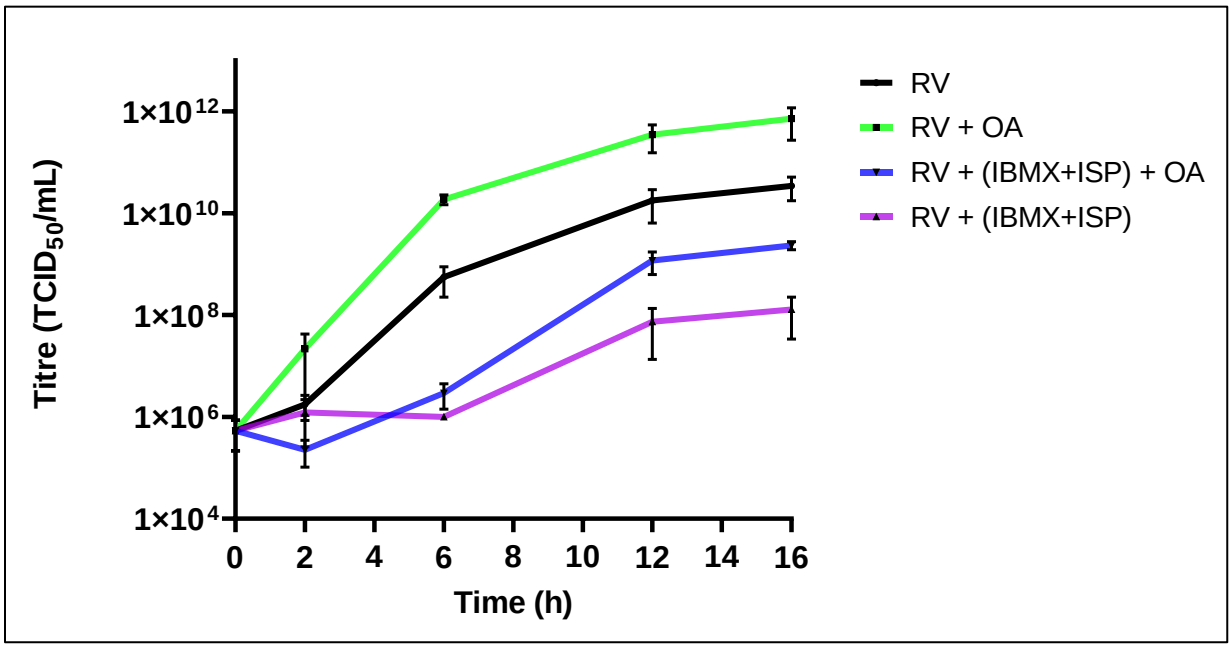


**Figure 3.8. The viral replication kinetics of rotavirus (RV) during lipid droplet (LD) fragmentation in HEK293 cells.** HEK293 cells were treated with 1 mM IBMX and 20 µM of ISP for 16 h post infection. This was done in the presence or absence of 50 µM OA supplementation. $TCID_{50}$ were used to determine RV titer at various time points post infection. Cells were counted before seeding and again prior to infection. Values depict the mean of three biological replicates. Standard deviation is indicated by error bars.

### 3.5.3. Effect of β-oxidation inhibition on rotavirus replication

To determine the effect of β-oxidation modulation on RV replication, HEK293 cells treated with ETO (60 µM) followed by RV infection (MOI = 5) in the presence and absence of OA supplementation was studied. Our results **(Table 3.10; Figure 3.9)** show that the treatment of HEK293 cells with ETO in the absence of OA supplementation, significantly reduces the overall production of RV infectious progeny compared to untreated cells over the period of 16 h post infection. Interestingly, this effect could not be rescued by OA supplementation.

Table 3.10. Viral titer of rotavirus infectious progeny over a period of 16 h post infection as (TCID50/mL) after ETO treatment

| Condition / Time points | $TCID_{50}$/mL | | | | |
|---|---|---|---|---|---|
| | 0 h | 2 h | 6 h | 12 h | 16 h |
| RV | $5x10^5$ ($\pm3.2x10^5$) | $2x10^6$ ($\pm9.0x10^5$) | $6x10^8$ ($\pm3.3x10^8$) | $2x10^{10}$ ($\pm1.1x10^{10}$) | $4x10^{10}$ ($\pm1.7x10^{10}$) |
| RV+OA | $5x10^5$ ($\pm3.2x10^5$) | $2x10^7$ ($\pm2.1x10^7$) | $2x10^{10}$ ($\pm4.1x10^9$) ***** | $4x10^{11}$ ($\pm2.0x10^{11}$) ***** | $7x10^{11}$ ($\pm4.5x10^{11}$) ***** |
| *P* – value | 1.00 | < 0.0001 | <0.0001 | 0.0030 | 0.0388 |
| RV+ETO | $5x10^5$ ($\pm3.2x10^5$) | $1x10^6$ ($\pm6.1x10^5$) | $1x10^5$ ($\pm4.4x10^4$) ***** | $5x10^6$ ($\pm2.4x10^6$) ***** | $4x10^7$ ($\pm3.5x10^7$) * |
| *P* – value | 1.00 | 0.0877 | <0.0001 | 0.0003 | 0.0019 |
| RV+ETO+OA | $5x10^5$ ($\pm3.2x10^5$) | $4x10^6$ ($\pm3.1x10^6$) | $2x10^5$ ($\pm8.9x10^4$) ***** | $8x10^6$ ($\pm3.5x10^6$) ***** | $4x10^7$ ($\pm2.8x10^7$) * |
| *P* – value | 1.00 | 0.0220 | 0.0003 | 0.0062 | 0.0006 |

*****: Significantly different from rotavirus infected HEK293 controls cells ($P < 0.05$)

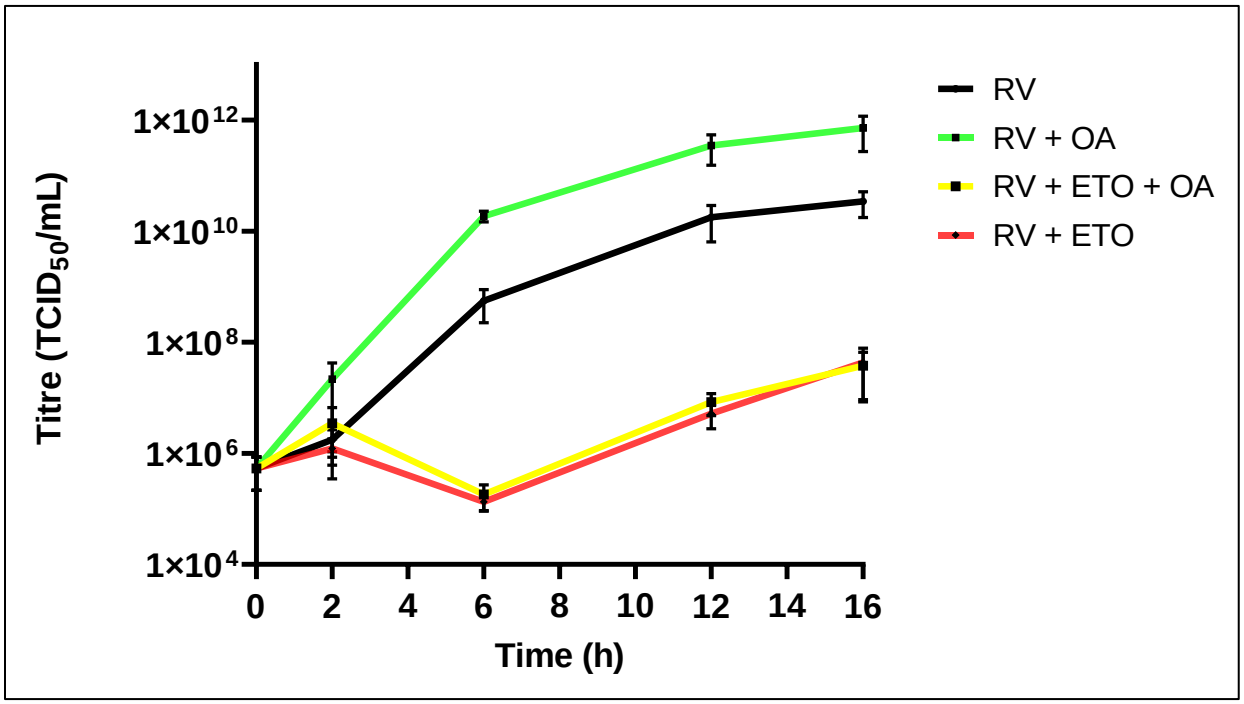


**Figure 3.9. The viral replication kinetics of rotavirus (RV) infection during fatty acid (FA) β-oxidation inhibition in HEK293 cells.** HEK293 cells were treated with 60 µM ETO for 16 h post infection. This was done in the presence or absence of 50 µM OA supplementation. $TCID_{50}$ were used to determine RV titers at various time points post infection. Cells were counted before seeding and again prior to infection. Values depict the mean of three biological replicates. Standard deviation is indicated by error bars.

## Chapter 4: Discussions, concluding remarks and future perspectives

Since it was first shown that rotavirus (RV) viroplasms recruit and co-localize with host lipid droplets (LDs), studies investigating the effect of RV lipidome modulation of host cell LDs and fatty acid (FA) β-oxidation are increasing. The aim of our study was to investigate the effect of oleic acid (OA) (18:1) supplementation on LDs, FA β-oxidation, and subsequently rotavirus (RV) replication. Specific objectives included:

**(1)** Investigating the effect of OA supplementation on LDs during RV infection

**(2)** Investigating the effect of OA supplementation on FA β-oxidation during RV infection

**(3)** Investigate the effect of LD and FA β-oxidation modulation on RV infection

### 4.1. Objective 1: Investigate the effect of oleic acid supplementation on lipid droplets during rotavirus infection

#### 4.1.1. Effect of oleic acid supplementation on lipid droplets and subsequent impact on rotavirus replication

Lipid droplets (LDs) are shown to be essential organelles in host cells that play important roles in the storage of a variety of neutral lipids including triacylglycerides (TAG) (Krahmer et al., 2009; Murphy et al., 2009; Onal et al., 2017). During starvation, the cell promotes the lipolysis of LDs for the release of fatty acids (FAs) which are then directed for degradation by FA β-oxidation to generate energy for cellular function (Carmen & Víctor, 2006; Gao et al., 2014; Marcinkiewicz et al., 2006). Different extracellular FAs including oleic acid (OA) are shown to promote the biogenesis of LDs, while the presence of excess amounts of FAs results in negative feedback that promotes reduced production of intracellular FAs (Rohwedder et al., 2014). The excess amounts of intracellular/extracellular FAs are converted to TAG for storage in LDs (Rohwedder et al., 2014). A variety of viruses have been shown to associate with LDs and promote the growth and biogenesis of LDs through increasing the overall total lipid/FA content of LDs (Monson et al., 2021). This was also evident in our study. The provision of HEK293 cells with extracellular OA resulted in an overall increase in TAG content of isolated LDs (**Figure 3.3A**). The presence of excess intracellular/extracellular FAs induce a negative feedback mechanism that redirects FA metabolism to promote the conversion of free FAs to TAG for storage in LDs. The redirection and storage of excess FAs in LDs leads to an increase in the size and number of LDs as to accommodate more FAs (Krahmer et al., 2013) (**Figure 4.1**). Lipid droplet growth/enrichment due to supplied OA also had a complementary effect to RV infection, the observed increase in overall

lipid content of TAG in LDs correlated with an increase in RV propagation over time (**Table 3.8; Figure 3.7**). The apparent increase in LDs (**Figure 4.1**) may provide platform for more LDs to be recruited by viroplasms to create more viroplasm-LD complexes that promote viral propagation (Crawford & Desselberger, 2016). This may provide insight into a direct mechanism which show how OA directly affect cellular lipidome, LDs and subsequently RV infection.

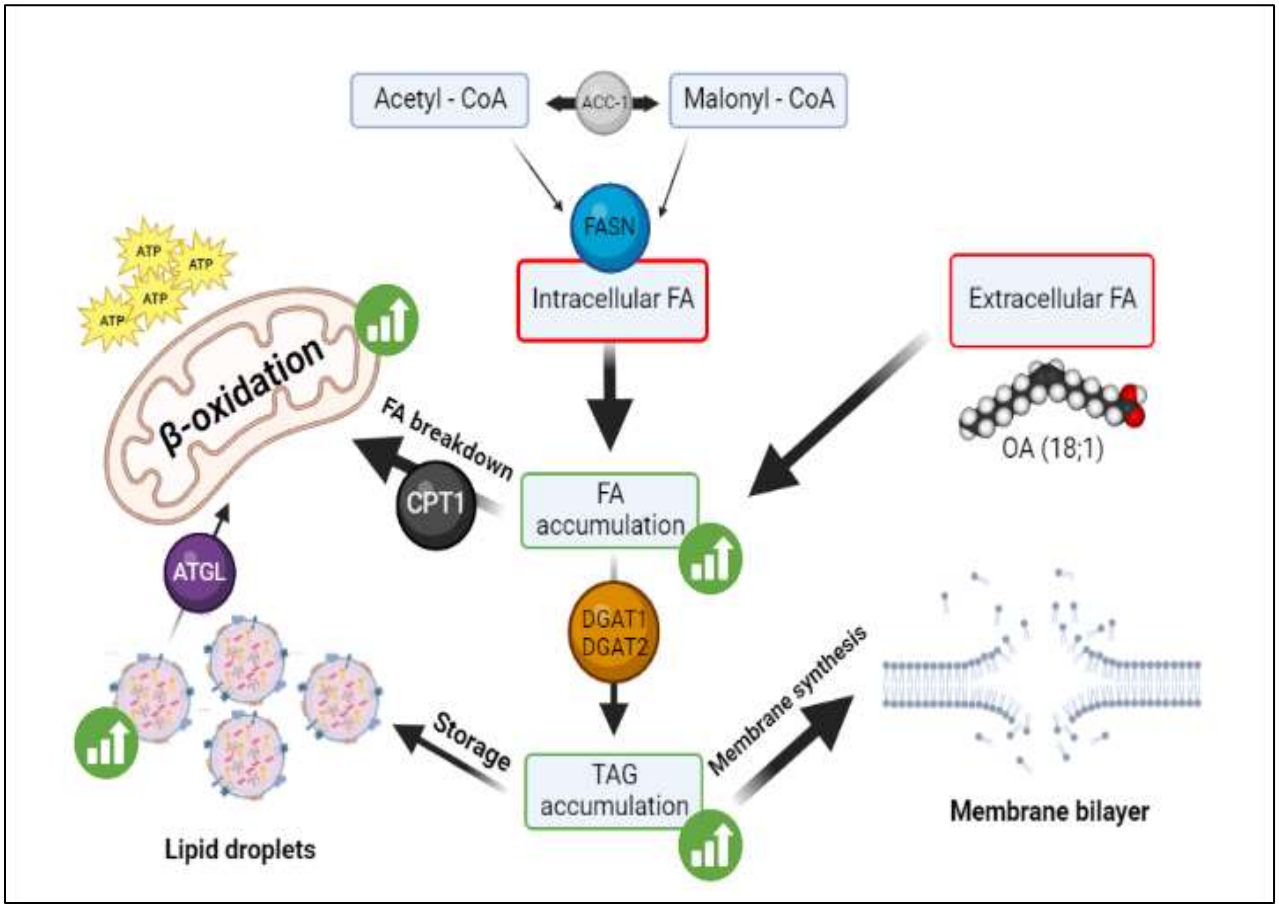


**Figure 4.1. An overview of the effect of oleic acid (OA) supplementation on lipid droplet formation (LD) and fatty acid (FA) β-oxidation.** The intracellular lipogenesis and external supply of OA leads to the accumulation of excess FAs in host cells. The accumulation of excess FAs intracellularly results in the breakdown of FAs by FA β-oxidation while also promoting the conversion of FAs to TAG for storage in LDs or synthesis of membranes. Host cells use the mechanisms of increased FA β-oxidation and LD biogenesis to prevent host lipotoxicity while producing intracellular ATP (Ahowesso et al., 2015; Brasaemle & Wolins, 2012; Guo et al., 2009b; Ojuka et al., 2016; Sander, 2019; Stone et al., 2009). Designed with BioRender.

### 4.1.2. Impact of rotavirus infection on host cell lipid droplet biogenesis

Previous studies have shown that viroplasms recruit LDs for the successful propagation of RV in host cells and the presence of more LDs are advantageous for the formation of viroplasm-LD complexes for RV infection (Cheung et al., 2010; Gaunt et al., 2013b). Rotavirus is one of the pathogens that modulate cell total lipid content and promote the enrichment or biogenesis of LDs (Chueng et al., 2010; Gaunt et al., 2013b; Sander et al., 2022). We have shown that OA supplementation promotes the enrichment and growth of LDs by the increase of total TAG content while subsequently increasing RV replication (**Figure 3.3A; Figure 3.7**). It is important to note that independently, RV infection promotes the enrichment/growth of host cell LDs through increasing the total TAG content in isolated LDs (Criglar et al., 2020, 2022) (**Figure 3.4A; Figure 4.2**). Rotavirus infection in host cells, thus alters the host cell lipidome by channeling different FAs towards conversation to TAG for LD biogenesis. The increase in total host LDs is advantageous for RV infections as that allows for formation of more viroplasm-LD complexes that leads to an overall increase in viral load (**Figure 4.2**).

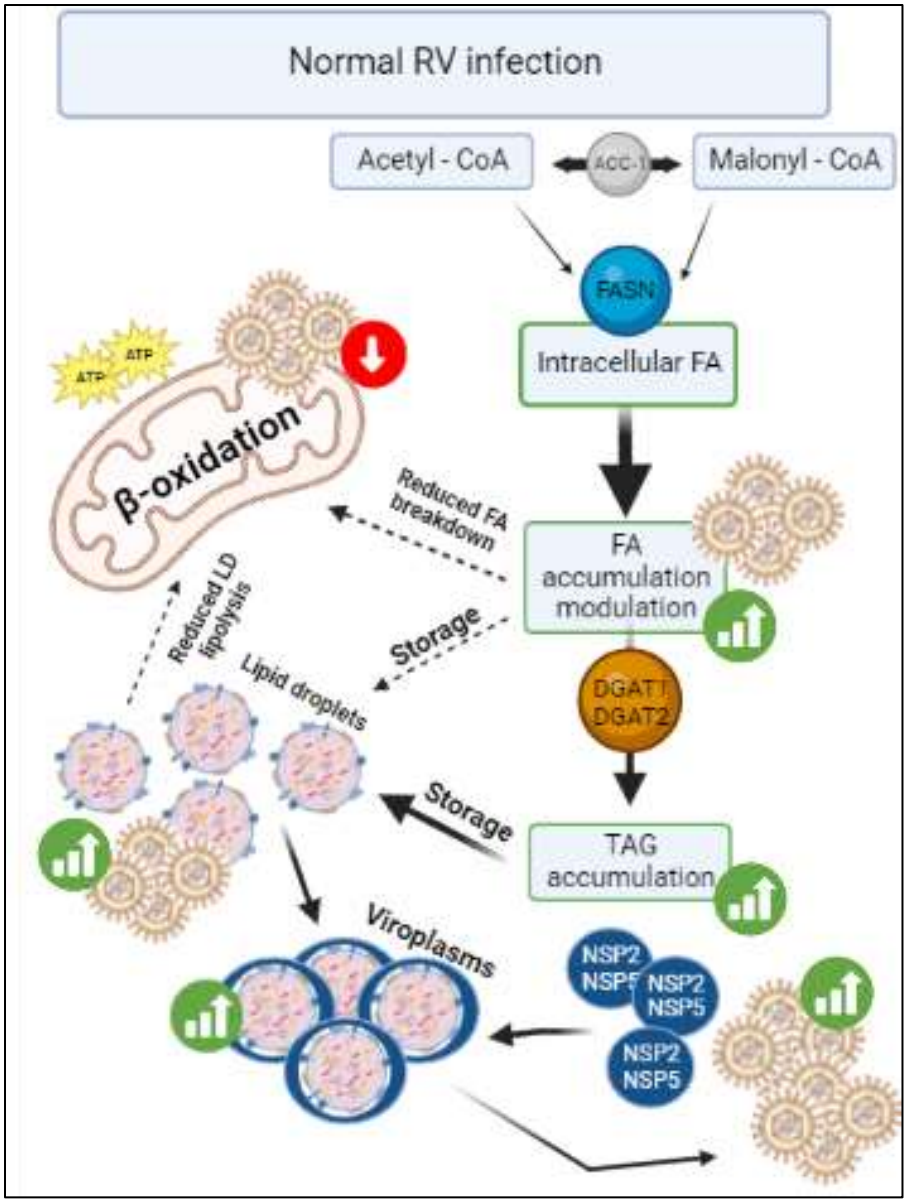


**Figure 4.2. An overview of the effect of rotavirus (RV) infection on lipid droplet (LD) formation and overall, fatty acid (FA) β-oxidation.** Rotavirus infections in host cells modulate cell lipidome that lead to the accumulation of a variety of FAs in addition to the intracellular generated FAs. Rotavirus infections reduce FA β-oxidation (also reducing ATP production) which also leads to FA accumulation. The host cell promotes conversion of accumulated FAs to TAG for the biogenesis of LDs. An increase in LD biogenesis increases the formation of more viroplasm-LD complex which are advantageous for increased viral load (Dickman et al., 2000; Eichwald et al., 2004; Gaunt, Zhang, et al., 2013a; Lever & Desselberger, 2016; Monson et al., 2021; Sanchez & Lagunoff, 2015; Sander, Kemp, et al., 2022). Designed with BioRender.

## 4.2. Objective 2: Investigate the effect of oleic acid supplementation on fatty acid β-oxidation during rotavirus infection

### 4.2.1. Effect of oleic acid supplementation on fatty acid β-oxidation and subsequent impact on rotavirus replication

Fatty acid (FA) β-oxidation is one of the key metabolic processes that maintain both host cell FA and energy homeostasis through breaking down excess FAs and providing cellular energy for host cells during starvation (Houten & Wanders, 2010). In our study, we also show that the increase in total cell TAG content during OA supplementation correlates with an increase in FA β-oxidation depicted by an increase in the overall host cell oxygen consumption rate (OCR) (**Figure 3.5A**). As we have shown, cells may reduce the overall excess free FAs by storage in LDs (increase TAG in LDs) and increased FA degradation by FA β-oxidation to prevent cell lipotoxicity (Ahowesso et al., 2015) (**Figure 3.3A; Figure 3.5A**). Thus, we show that excess FAs in host cells induce the accumulation of free FAs that are stored in LDs and also broken down with FA β-oxidation (**Figure 4.1**).

### 4.2.2. Impact of rotavirus infection on host cell fatty acid β-oxidation

Interestingly, we further show that RV infections reduce host cell FA β-oxidation as observed by a reduction in overall host cell OCR (**Figure 3.6A**). This further suggests the direct mechanism used by RV to modulate host cell lipidome and LD biogenesis. The important role of LDs in the formation of viroplasms-LD complexes during RV replication cause RV to reduce the breakdown of FA by β-oxidation and instead promoted an accumulation of free FAs that will be later stored in LDs (Crawford & Desselberger, 2016; Dickman et al., 2000; Olofsson et al., 2008; Sander, Kemp, et al., 2022). This directed accumulation of FAs in LDs thus results in more LDs present in host cells to interact with viroplasms and intern promote RV propagation (**Figure 4.2**). Studies have also shown intact RV and some viral NSPs in host cell mitochondria, a site for β-oxidation (Altenburg et al., 1980; Holloway et al., 2015). The physical association of RV and the mitochondria may suggest the mechanism RV use to modulate overall β-oxidation (Altenburg et al., 1980) **(Figure 1.12)**.

## 4.4. Objective 3: Investigate the effect of lipid droplets and fatty acid β-oxidation modulation on rotavirus infection.

### 4.4.1. Association between lipid droplets and fatty acid β-oxidation

The common and most important link between LDs and FA β-oxidation during RV infections are the lipids that are stored in LDs and, if required for energy production, are broken down by FA β-oxidation (Gao et al., 2014; Jie et al., 2015). Our study shows that the fragmentation of LDs into smaller micro-droplets by treatment with (3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP)) reduces host cell LDs indicated by the overall reduction in total TAG content of isolated LDs (**Figure 3.3A**). The apparent reduction in LDs and total TAG content thus reduce the TAG to produce available FAs for FA β-oxidation. This correlates with the observed reduction in the overall FA β-oxidation in HEK293 cells when treated with IBMX and ISP (**Figure 3.5A**). The reverse process is also shown, the reduction or inhibition of FA β-oxidation by inhibiting carnitine palmitoyltransferase 1 (CPT1) with etomoxir (ETO), results in the overall reduction in host FA β-oxidation indicated by reduced host OCR (**Figure 3.5A**). The reduction of host cell FA β-oxidation induces an accumulation of free FAs which cause the cell to respond by storing these free FAs in LDs (Ducharme & Bickel, 2008). Our study also supported the redirection of free FAs to LD storage during FA β-oxidation inhibition by ETO treatment, depicted by the detection of elevated TAG content in isolated LDs (**Figure 3.3A**). The supply of host cells with excess extracellular OA still shows the same trend during the modulation of both processes and the interactions between both processes remain the same although with slightly elevated values due presence of extracellular FA supplementation (**Figure 3.3A; Figure 3.5A**).

### 4.4.2. Impact of LD and FA β-oxidation modulation on RV infection

Previous studies have shown that the fragmentation of LDs and inhibition of FA β-oxidation to have deleterious effects on a variety of viruses (Cheung et al., 2010; Rasmussen et al., 2011). Our study also shows that the reduction and fragmentation of LDs by (IBMX and ISP) reduces the overall available LDs required for the formation of viroplasms-LD complexes. This correlated with the subsequent reduction in the production of infectious RV progeny over time (**Table 3.9; Figure 3.8; Figure 4.3**). Although we have shown that RV infection reduces FA β-oxidation (**Figure 3.6A**), interestingly the treatment of host cells with ETO also shows to have the similar deleterious effect on RV propagation (**Table 3.10; Figure 3.9; Figure 4.2**). We also observed that, OA supplementation during host cell treatment with ETO rescues the deleterious effect of the inhibit on RV propagation overtime (**Table 3.10; Figure 3.9**). Although this inhibitory effect results in reduced FA breakdown that may promote the growth and enrichment of LDs necessary

viroplasms-LD complex formation (**Figure 3.3A; Figure 4.2**), the direct inhibition of FA β-oxidation is not advantageous for viral propagation. It important to note that ETO is an irreversible inhibitor for the CPT1 transporter (**Figure 1.11**) that causes a direct reduction of FA transportation into the host mitochondria (Divakaruni et al., 2018; Raud et al., 2018). The data thus suggest, RV does not necessarily promote the overall inhibition or reduction of FA β-oxidation but may still require the process to either generate energy to keep host cells viable, to drive its replication or generate specific metabolites that RV may require for its propagation.

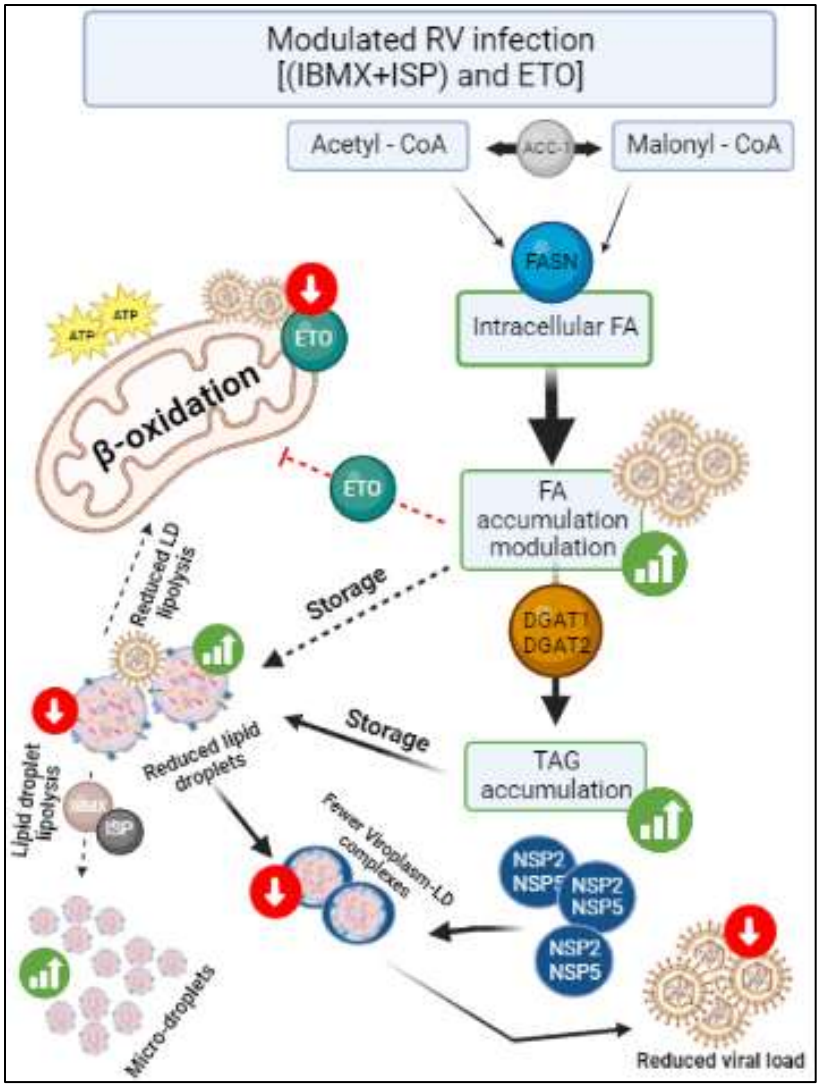


**Figure 4.3. An overview of the effect of rotavirus (RV) infection on lipid droplet (LD) formation and overall, fatty acid (FA) β-oxidation in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX) and isoproterenol (ISP) or etomoxir (ETO) treatment.** Although RV infection promotes FA accumulation for TAG production for subsequent LD biogenesis, IBMX and ISP treatment reduce host TAG content by fragmenting LDs. The reduction in observed LDs reduce the available TAG available for FA β-oxidation (reduce β-oxidation and ATP production) while also reducing the available LDs required to form viroplasm-LD complexes that are needed for viral replication. This results in the reduction of the overall viral yield. Although RV infection also reduce FA β-oxidation, treatment of host cell with ETO inhibits the complete entry of FAs in the mitochondria observed to also be deleterious for RV infection (Brasaemle & Wolins, 2012; Crawford & Desselberger, 2016; Dickman et al., 2000; Gaunt et al., 2013a; Monson et al., 2021; Pike et al., 2011; Rasmussen et al., 2011). Designed with BioRender.

### 4.5. Overview of the effect of oleic acid supplementation on lipid formation, β-oxidation and subsequently RV infection

Oleic acid supplementation directly promotes viral propagation by increasing the growth and enrichment of LDs in hosts. The enrichment of LDs also results in an increased biogenesis of LDs that will create a conducive environment for viroplasms-LD complex formation and overall increase in viral propagation (Gaunt et al., 2013a). Upon viral infection, RV hijacks host machinery to promote viral propagation (Criglar et al., 2022; Dai et al., 2020; López & Arias, 2012). In this study we show that RV infections simultaneously promotes the accumulation and storage of FA in LDs while reducing FA β-oxidation. An accumulation of LDs promotes viral replication by increasing the interaction with viroplasms. The RV-induced reduction in FA β-oxidation also causes a reduction in mitochondrial based energy production (Dickman et al., 2000) (**Figure 4.2**). The process of LD biogenesis and FA β-oxidation are essential for RV propagation, as RV particles are observed in close proximity with organelles with LDs and the mitochondria during RV infection (Altenburg et al., 1980; Crawford & Desselberger, 2016; Lever & Desselberger, 2016). During RV infection, the reduction of LDs by induced lipolysis causes a reduction in formation of viroplasms-LD complexes that subsequently hinder viral propagation (**Figure 4.3**). RV infection negatively affects FA β-oxidation, but we also observe chemically induce inhibition of β-oxidation to be unfavorable for viral propagation (**Figure 4.2**). Thus, RV may require the process of FA β-oxidation in a different mechanism that remains unclear. The trend observed in the reduction of viral load during treatment with different LD fragmenting compounds and FA β-oxidation inhibitors remain the same also during OA supplementation.

### 4.6. Concluding remarks and future perspectives

Our study has shown that both LDs and FA β-oxidation are interconnected in the network of FA metabolism in host cell during provision of exogenous OA and during RV infection. The supplementation of HEK293 cells with OA has shown to have an up regulatory effect on LD biogenesis, FA β-oxidation and subsequently increased viral load, indicating an important role of OA in viral propagation. In correlation with previous studies, our study has also shown that RV infection upregulates the enrichment of LDs while also reducing the overall FA β-oxidation which may subsequently lead to reduced energy productions of host cells (Dickman et al. 2000; Cheung et al 2010). This provides insight that RV may not necessarily modulate host lipidome by channeling FAs to FA β-oxidation for energy production but instead promote the storage of FAs for LD enrichment, even though inhibition by ETO did not promote viral replication. Furthermore, we have further elucidated the importance of both LDs and FA β-oxidation for RV propagation by chemical modulations of these processes. The fragmentation of LDs by (IBMX and ISP) showed

to affect multiple targets in host cells including the reduction of host TAG content in LDs, reduced FA β-oxidation and subsequently reduced RV replication. The FA β-oxidation inhibition by treatment with ETO also affected the two associated processes, by reducing the overall FA β-oxidation, increasing TAG content in LDs and subsequently reduced RV replication.

The study's objective was to highlight the possible mechanisms in which FA supplementation affects host LD formation and RV propagation during infections in the presence of FA β-oxidation inhibitors and LD fragmenting compounds, however we have noted a variety of limitations to the study including:

1. It has been shown that concentrations of ETO > 5 μM induce acute production of reactive oxygen species (ROS) as evidence of severe oxidative stress in T-cells (O'Connor et al., 2018) was previously observed. In addition high concentration (> 100 μM) of ETO may lead to off-target effects (Divakaruni et al., 2018; Raud et al., 2018). Although the concentration used for ETO in the study is less than 100 μM, it is possible that off-target effects for ETO in HEK293 cells were observed. Therefore, in future, concentrations < 5 μM should be tested and/or an alternative inhibitor of β-oxidation should be explored.
2. The production of ROS is known to affect viral replication (Sander et al., 2022). For RV specifically, ROS has been shown to affect viral pathogenesis (Gac et al., 2010; Guerrero & Acosta, 2016; Patra et al., 2020). The effect of ETO-induced ROS on RV replication, specifically in the context of this study, is however unknown.
3. The LD fragmentation compounds IBMX and ISP, when used in combination, have previously been shown to induce LD fragmentation in M104 cells (Cheung et al., 2010). The effectiveness of these compounds to induce LD fragmentation in HEK293 cells, specifically at the concentrations used in this study should be evaluated in-depth.

For future perspectives, most studies have shown that the targeting of LDs may serve as a therapeutic site for the reduction of RV infections (Cheung et al., 2010; Gaunt et al., 2013a; Marcinkiewicz et al., 2006). The phenomena of RV infections modulating and reducing host cell FA β-oxidation may be a point of interest, although the direct mechanism in which RV modulates this process remains unclear. The emergence of intact RV particles and viral NSPs being found in and associating with the mitochondria would need to be better studied and understood. This will provide better insight of the beneficial potential of the mitochondria for RV infections, thus providing another opportunity for a therapeutic target site for remedying RV infections.

Abaandou, L., Quan, D., & Shiloach, J. (2021). Affecting hek293 cell growth and production performance by modifying the expression of specific genes. *Cells*, *10*(7). https://doi.org/10.3390/cells10071667

Ahowesso, C., Black, P. N., Saini, N., Montefusco, D., Chekal, J., Malosh, C., Lindsley, C. W., Stauffer, S. R., & DiRusso, C. C. (2015). Chemical inhibition of fatty acid absorption and cellular uptake limits lipotoxic cell death. *Biochemical Pharmacology*, *98*(1). https://doi.org/10.1016/j.bcp.2015.09.004

Altenburg, B. C., Graham, D. Y., & Kolb Estes, M. (1980). Ultrastructural study of rotavirus replication in cultured cells. *Journal of General Virology*, *46*(1). https://doi.org/10.1099/0022-1317-46-1-75

Ando, T., Imamura, H., Suzuki, R., Aizaki, H., Watanabe, T., Wakita, T., & Suzuki, T. (2012). Visualization and measurement of ATP levels in living cells replicating hepatitis C virus genome RNA. *PLoS Pathogens*, *8*(3). https://doi.org/10.1371/journal.ppat.1002561

Arnold, M., Patton, J. T., & McDonald, S. M. (2009). Culturing, storage, and quantification of rotaviruses. In *Current Protocols in Microbiology* (Issue SUPPL. 15). https://doi.org/10.1002/9780471729259.mc15c03s15

Boström, P., Andersson, L., Rutberg, M., Perman, J., Lidberg, U., Johansson, B. R., Fernandez-Rodriguez, J., Ericson, J., Nilsson, T., Borén, J., & Olofsson, S. O. (2007). SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nature Cell Biology*, *9*(11). https://doi.org/10.1038/ncb1648

Bozza, P. T., & Viola, J. P. B. (2010). Lipid droplets in inflammation and cancer. *Prostaglandins Leukotrienes and Essential Fatty Acids*, *82*(4–6). https://doi.org/10.1016/j.plefa.2010.02.005

Bozza, P. T., Yu, W., Penrose, J. F., Morgan, E. S., Dvorak, A. M., & Weller, P. F. (1997). Eosinophil lipid bodies: Specific, inducible intracellular sites for enhanced eicosanoid formation. *Journal of Experimental Medicine*, *186*(6). https://doi.org/10.1084/jem.186.6.909

Brasaemle, D L. (2007). Thematic review series: adipocyte biology. The perilipin family of

structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of Lipid Research*, *48*(0022-2275 (Print)).

Brasaemle, Dawn L., & Wolins, N. E. (2012). Packaging of fat: An evolving model of lipid droplet assembly and expansion. In *Journal of Biological Chemistry* (Vol. 287, Issue 4). https://doi.org/10.1074/jbc.R111.309088

Burrone, O. R., Afrikanova, I., Vascotto, F., & Fabbretti, E. (1999). Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo. *Journal of General Virology*, *80*(2), 333–339. https://doi.org/10.1099/0022-1317-80-2-333

Campagna, M., Eichwald, C., Vascotto, F., & Burrone, O. R. (2005). RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle. *Journal of General Virology*, *86*(5). https://doi.org/10.1099/vir.0.80598-0

Carmen, G. Y., & Víctor, S. M. (2006). Signalling mechanisms regulating lipolysis. In *Cellular Signalling* (Vol. 18, Issue 4). https://doi.org/10.1016/j.cellsig.2005.08.009

Cheung, W., Gill, M., Esposito, A., Kaminski, C. F., Courousse, N., Chwetzoff, S., Trugnan, G., Keshavan, N., Lever, A., & Desselberger, U. (2010). Rotaviruses Associate with Cellular Lipid Droplet Components To Replicate in Viroplasms, and Compounds Disrupting or Blocking Lipid Droplets Inhibit Viroplasm Formation and Viral Replication. *Journal of Virology*, *84*(13), 6782–6798. https://doi.org/10.1128/jvi.01757-09

Choudhary, V., Golden, A., & Prinz, W. A. (2016). Keeping FIT, storing fat: Lipid droplet biogenesis. *Worm*, *5*(2), e1170276. https://doi.org/10.1080/21624054.2016.1170276

Clark, B., & Desselberger, U. (1988). Myristylation of rotavirus protein. *Journal of General Virology*, *69*(10). https://doi.org/10.1099/0022-1317-69-10-2681

Cohen, J., Laporte, J., Charpilienne, A., & Scherrer, R. (1979). Activation of rotavirus RNA polymerase by calcium chelation. *Archives of Virology*, *60*(3–4). https://doi.org/10.1007/BF01317489

Crawford, S. E., & Desselberger, U. (2016). Lipid droplets form complexes with viroplasms and are crucial for rotavirus replication. In *Current Opinion in Virology* (Vol. 19, pp. 11–15). Elsevier B.V. https://doi.org/10.1016/j.coviro.2016.05.008

Crawford, S. E., Ramani, S., Tate, J. E., Parashar, U. D., Svensson, L., Hagbom, M., Franco, M. A., Greenberg, H. B., O'Ryan, M., Kang, G., Desselberger, U., & Estes, M. K. (2017).

Rotavirus infection. *Nature Reviews Disease Primers*, *3*. https://doi.org/10.1038/nrdp.2017.83

Criglar, J. M., Crawford, S. E., Zhao, B., Smith, H. G., Stossi, F., & Estes, M. K. (2020). A Genetically Engineered Rotavirus NSP2 Phosphorylation Mutant Impaired in Viroplasm Formation and Replication Shows an Early Interaction between vNSP2 and Cellular Lipid Droplets. *Journal of Virology*, *94*(15). https://doi.org/10.1128/jvi.00972-20

Criglar, J. M., Estes, M. K., & Crawford, S. E. (2022). Rotavirus-Induced Lipid Droplet Biogenesis Is Critical for Virus Replication. *Frontiers in Physiology*, *13*. https://doi.org/10.3389/fphys.2022.836870

Dai, X., Hakizimana, O., Zhang, X., Kaushik, A. C., & Zhang, J. (2020). Orchestrated efforts on host network hijacking: Processes governing virus replication. In *Virulence* (Vol. 11, Issue 1). https://doi.org/10.1080/21505594.2020.1726594

Desselberger, U. (2014). Rotaviruses. *Virus Research*, *190*, 75–96. https://doi.org/10.1016/j.virusres.2014.06.016

Desselberger, U., Richards, J., Tchertanov, L., Lepault, J., Lever, A., Burrone, O., & Cohen, J. (2013). Further characterisation of rotavirus cores: Ss(+)RNAs can be packaged in vitro but packaging lacks sequence specificity. *Virus Research*, *178*(2). https://doi.org/10.1016/j.virusres.2013.09.034

Díaz, G. C., Tapanes, N. de la C. O., Câmara, L. D. T., & Aranda, D. A. G. (2014). Glycerol conversion in the experimental study of catalytic hydrolysis of triglycerides for fatty acids production using Ni or Pd on Al2O3 or SiO2. *Renewable Energy*, *64*. https://doi.org/10.1016/j.renene.2013.11.006

Dickman, K. G., Hempson, S. J., Anderson, J., Lippe, S., Zhao, L., Burakoff, R., & Shaw, R. D. (2000). Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *279*(4 42-4). https://doi.org/10.1152/ajpgi.2000.279.4.g757

Divakaruni, A. S., Hsieh, W. Y., Minarrieta, L., Duong, T. N., Kim, K. K. O., Desousa, B. R., Andreyev, A. Y., Bowman, C. E., Caradonna, K., Dranka, B. P., Ferrick, D. A., Liesa, M., Stiles, L., Rogers, G. W., Braas, D., Ciaraldi, T. P., Wolfgang, M. J., Sparwasser, T., Berod, L., … Murphy, A. N. (2018). Etomoxir Inhibits Macrophage Polarization by Disrupting CoA Homeostasis. *Cell Metabolism*, *28*(3), 490-503.e7.

https://doi.org/10.1016/j.cmet.2018.06.001

Dormitzer, P. R., Sun, Z.-Y. J., Blixt, O., Paulson, J. C., Wagner, G., & Harrison, S. C. (2002). Specificity and Affinity of Sialic Acid Binding by the Rhesus Rotavirus VP8* Core. *Journal of Virology*, *76*(20). https://doi.org/10.1128/jvi.76.20.10512-10517.2002

Dormitzer, P. R., Sun, Z. Y. J., Wagner, G., & Harrison, S. C. (2002). The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. *EMBO Journal*, *21*(5). https://doi.org/10.1093/emboj/21.5.885

Ducharme, N. A., & Bickel, P. E. (2008). Minireview: Lipid droplets in lipogenesis and lipolysis. In *Endocrinology* (Vol. 149, Issue 3). https://doi.org/10.1210/en.2007-1713

Dvorak, A. M., Morgan, E., Schleimer, R. P., Ryeom, S. W., Lichtenstein, L. M., & Weller, P. F. (1992). Ultrastructural immunogold localization of prostaglandin endoperoxide synthase (cyclooxygenase) to non-membrane-bound cytoplasmic lipid bodies in human lung mast cells, alveolar macrophages, Type II pneumocytes, and neutrophils. *Journal of Histochemistry and Cytochemistry*, *40*(6). https://doi.org/10.1177/40.6.1316915

Eaton, S. (2002). Control of mitochondrial β-oxidation flux. In *Progress in Lipid Research* (Vol. 41, Issue 3). https://doi.org/10.1016/S0163-7827(01)00024-8

Eichwald, C., Rodriguez, J. F., & Burrone, O. R. (2004). Characterization of rotavirus NSP2/NSP5 interactions and the dynamics of viroplasm formation. *Journal of General Virology*, *85*(3), 625–634. https://doi.org/10.1099/vir.0.19611-0

Estes, M. K., Graham, D. Y., Gerba, C. P., & Smith, E. M. (1979). Simian rotavirus SA11 replication in cell cultures. *Journal of Virology*, *31*(3). https://doi.org/10.1128/jvi.31.3.810-815.1979

Fei, W., Shui, G., Zhang, Y., Krahmer, N., Ferguson, C., Kapterian, T. S., Lin, R. C., Dawes, I. W., Brown, A. J., Li, P., Huang, X., Parton, R. G., Wenk, M. R., Walther, T. C., & Yang, H. (2011). A role for phosphatidic acid in the formation of “supersized” Lipid droplets. *PLoS Genetics*, *7*(7). https://doi.org/10.1371/journal.pgen.1002201

Gac, M., Bigda, J., & Vahlenkamp, T. W. (2010). Increased mitochondrial superoxide dismutase expression and lowered production of reactive oxygen species during rotavirus infection. *Virology*, *404*(2), 293–303. https://doi.org/10.1016/j.virol.2010.05.018

Gao, J., Liao, R., Yu, Y., Zhai, H., Wang, Y., Sack, R., Peters, A. H. F. M., Chen, J., Wu, H.,

Huang, Z., Hu, M., Qi, W., Lu, C., Atadja, P., Oyang, C., Li, E., Yi, W., & Zhou, S. (2014). Absolute quantification of histone PTM marks by MRM-based LC-MS/MS. *Analytical Chemistry*, *86*(19). https://doi.org/10.1021/ac502333a

Gaunt, E. R., Cheung, W., Richards, J. E., Lever, A., & Desselberger, U. (2013). Inhibition of rotavirus replication by downregulation of fatty acid synthesis. *Journal of General Virology*, *94*(PART 6), 1310–1317. https://doi.org/10.1099/vir.0.050146-0

Gaunt, E. R., Zhang, Q., Cheung, W., Wakelam, M. J. O., Lever, A. M. L., & Desselberger, U. (2013a). Lipidome analysis of rotavirus-infected cells confirms the close interaction of lipid droplets with viroplasms. *Journal of General Virology*, *94*(PART7), 1576–1586. https://doi.org/10.1099/vir.0.049635-0

Gaunt, E. R., Zhang, Q., Cheung, W., Wakelam, M. J. O., Lever, A. M. L., & Desselberger, U. (2013b). Lipidome analysis of rotavirus-infected cells confirms the close interaction of lipid droplets with viroplasms. *Journal of General Virology*, *94*(PART7), 1576–1586. https://doi.org/10.1099/vir.0.049635-0

Graham, F. L., Smiley, J., Russell, W. C., & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *Journal of General Virology*, *36*(1). https://doi.org/10.1099/0022-1317-36-1-59

Guerrero, C. A., & Acosta, O. (2016). Inflammatory and oxidative stress in rotavirus infection. *World Journal of Virology*, *5*(2), 38. https://doi.org/10.5501/wjv.v5.i2.38

Guo, Y., Cordes, K. R., Farese, R. V., & Walther, T. C. (2009a). Lipid droplets at a glance. *Journal of Cell Science*, *122*(6), 749–752. https://doi.org/10.1242/jcs.037630

Guo, Y., Cordes, K. R., Farese, R. V, & Walther, T. C. (2009b). Lipid droplets at a glance. *Journal of Cell Science*, *122*, 749–752. https://doi.org/10.1242/jcs.037630

Guo, Y., Walther, T. C., Rao, M., Stuurman, N., Goshima, G., Terayama, K., Wong, J. S., Vale, R. D., Walter, P., & Farese, R. V. (2008). Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. *Nature*, *453*(7195). https://doi.org/10.1038/nature06928

Haselhorst, T., Fleming, F. E., Dyason, J. C., Hartnell, R. D., Yu, X., Holloway, G., Santegoets, K., Kiefel, M. J., Blanchard, H., Coulson, B. S., & Von Itzstein, M. (2009). Sialic acid dependence in rotavirus host cell invasion. *Nature Chemical Biology*, *5*(2).

https://doi.org/10.1038/nchembio.134

Heaton, N. S., & Randall, G. (2010). Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host and Microbe*, *8*(5). https://doi.org/10.1016/j.chom.2010.10.006

Hendrik Rudolf. (2021, April 21). *Cytotoxicity Testing According to EN ISO 10993-5: Avoiding Pitfalls When Choosing a Laboratory*. Johner Institude.

Holloway, G., Johnson, R. I., Kang, Y., Dang, V. T., Stojanovski, D., & Coulson, B. S. (2015). Rotavirus NSP6 localizes to mitochondria via a predicted N-terminal α-helix. *Journal of General Virology*, *96*(12). https://doi.org/10.1099/jgv.0.000294

Houten, S. M., & Wanders, R. J. A. (2010). A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation. In *Journal of Inherited Metabolic Disease* (Vol. 33, Issue 5). https://doi.org/10.1007/s10545-010-9061-2

Hu, L., Crawford, S. E., Czako, R., Cortes-Penfield, N. W., Smith, D. F., Le Pendu, J., Estes, M. K., & Prasad, B. V. V. (2012). Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen. *Nature*, *485*(7397). https://doi.org/10.1038/nature10996

Huang, P., Xia, M., Tan, M., Zhong, W., Wei, C., Wang, L., Morrow, A., & Jiang, X. (2012). Spike Protein VP8* of Human Rotavirus Recognizes Histo-Blood Group Antigens in a Type-Specific Manner. *Journal of Virology*, *86*(9). https://doi.org/10.1128/jvi.05507-11

Jayaram, H., Estes, M. K., & Prasad, B. V. V. (2004). Emerging themes in rotavirus cell entry, genome organization, transcription and replication. *Virus Research*, *101*(1). https://doi.org/10.1016/j.virusres.2003.12.007

Kassan, A., Herms, A., Fernández-Vidal, A., Bosch, M., Schieber, N. L., Reddy, B. J. N., Fajardo, A., Gelabert-Baldrich, M., Tebar, F., Enrich, C., Gross, S. P., Parton, R. G., & Pol, A. (2013). Acyl-CoA synthetase 3 promotes lipid droplet biogenesis in ER microdomains. *Journal of Cell Biology*, *203*(6). https://doi.org/10.1083/jcb.201305142

Kavsan, V. M., Iershov, A. V., & Balynska, O. V. (2011). Immortalized cells and one oncogene in malignant transformation: Old insights on new explanation. In *BMC Cell Biology* (Vol. 12). https://doi.org/10.1186/1471-2121-12-23

Kerner, J., & Hoppel, C. (2000). Fatty acid import into mitochondria. In *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (Vol. 1486, Issue 1).

https://doi.org/10.1016/S1388-1981(00)00044-5

Korenaga, M., Wang, T., Li, Y., Showalter, L. A., Chan, T., Sun, J., & Weinman, S. A. (2005). Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *Journal of Biological Chemistry*, *280*(45). https://doi.org/10.1074/jbc.M506412200

Kory, N., Farese, R. V., & Walther, T. C. (2016). Targeting Fat: Mechanisms of Protein Localization to Lipid Droplets. In *Trends in Cell Biology* (Vol. 26, Issue 7). https://doi.org/10.1016/j.tcb.2016.02.007

Krahmer, N., Farese, R. V., & Walther, T. C. (2013). Balancing the fat: Lipid droplets and human disease. In *EMBO Molecular Medicine* (Vol. 5, Issue 7). https://doi.org/10.1002/emmm.201100671

Krahmer, N., Guo, Y., Farese, R. V., & Walther, T. C. (2009). SnapShot: Lipid Droplets. In *Cell* (Vol. 139, Issue 5). https://doi.org/10.1016/j.cell.2009.11.023

Kuerschner, L., Moessinger, C., & Thiele, C. (2008). Imaging of lipid biosynthesis: How a neutral lipid enters lipid droplets. *Traffic*, *9*(3). https://doi.org/10.1111/j.1600-0854.2007.00689.x

Lam, T. K. T., Pocai, A., Gutierrez-Juarez, R., Obici, S., Bryan, J., Aguilar-Bryan, L., Schwartz, G. J., & Rossetti, L. (2005). Hypothalamic sensing of circulating fatty acids is required for glucose homeostasis. *Nature Medicine*, *11*(3). https://doi.org/10.1038/nm1201

Lever, A., & Desselberger, U. (2016). Rotavirus replication and the role of cellular lipid droplets: New therapeutic targets? *Journal of the Formosan Medical Association*, *115*(6), 389–394. https://doi.org/10.1016/j.jfma.2016.02.004

Liu, M., Mattion, N. M., & Estes, M. K. (1992). Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity. *Virology*, *188*(1). https://doi.org/10.1016/0042-6822(92)90736-9

Lobeck, I., Donnelly, B., Dupree, P., Mahe, M. M., McNeal, M., Mohanty, S. K., & Tiao, G. (2016). Rhesus rotavirus VP6 regulates ERK-dependent calcium influx in cholangiocytes. *Virology*, *499*. https://doi.org/10.1016/j.virol.2016.09.014

López, S., & Arias, C. F. (2012). Rotavirus-host cell interactions: An arms race. In *Current Opinion in Virology* (Vol. 2, Issue 4). https://doi.org/10.1016/j.coviro.2012.05.001

López, T., Rojas, M., Ayala-Bretón, C., López, S., & Arias, C. F. (2005). Reduced expression of the rotavirus NSP5 gene has a pleiotropic effect on virus replication. *Journal of General Virology*, *86*(6). https://doi.org/10.1099/vir.0.80827-0

Lu, X., McDonald, S. M., Tortorici, M. A., Tao, Y. J., Vasquez-Del Carpio, R., Nibert, M. L., Patton, J. T., & Harrison, S. C. (2008). Mechanism for Coordinated RNA Packaging and Genome Replication by Rotavirus Polymerase VP1. *Structure*, *16*(11). https://doi.org/10.1016/j.str.2008.09.006

Mankouri, J., Tedbury, P. R., Gretton, S., Hughes, M. E., Griffin, S. D. C., Dallas, M. L., Green, K. A., Hardie, D. G., Peers, C., & Harris, M. (2010). Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(25). https://doi.org/10.1073/pnas.0912426107

Marcinkiewicz, A., Gauthier, D., Garcia, A., & Brasaemle, D. L. (2006). The phosphorylation of serine 492 of perilipin A directs lipid droplet fragmentation and dispersion. *Journal of Biological Chemistry*, *281*(17). https://doi.org/10.1074/jbc.M600171200

Martín-Acebes, M. A., Blázquez, A. B., Jiménez de Oya, N., Escribano-Romero, E., & Saiz, J. C. (2011). West nile virus replication requires fatty acid synthesis but is independent on phosphatidylinositol-4-phosphate lipids. *PLoS ONE*, *6*(9). https://doi.org/10.1371/journal.pone.0024970

Matthijnssens, J., & Van Ranst, M. (2012). Genotype constellation and evolution of group A rotaviruses infecting humans. In *Current Opinion in Virology* (Vol. 2, Issue 4). https://doi.org/10.1016/j.coviro.2012.04.007

Mattion, N. M., Mitchell, D. B., Both, G. W., & Estes, M. K. (1991). Expression of rotavirus proteins encoded by alternative open reading frames of genome segment 11. *Virology*, *181*(1). https://doi.org/10.1016/0042-6822(91)90495-W

McClain, B., Settembre, E., Temple, B. R. S., Bellamy, A. R., & Harrison, S. C. (2010). X-ray Crystal Structure of the Rotavirus Inner Capsid Particle at 3.8 Å Resolution. *Journal of Molecular Biology*, *397*(2). https://doi.org/10.1016/j.jmb.2010.01.055

McDonald, S. M., & Patton, J. T. (2011). Assortment and packaging of the segmented rotavirus genome. In *Trends in Microbiology* (Vol. 19, Issue 3). https://doi.org/10.1016/j.tim.2010.12.002

McNeal, M. M., & Bernstein, D. I. (2014). Rotaviruses. In *Viral Infections of Humans: Epidemiology and Control* (pp. 713–732). https://doi.org/10.1007/978-1-4899-7448-8_30

Mlera, L., O'Neill, H. G., Jere, K. C., & van Dijk, A. A. (2013). Whole-genome consensus sequence analysis of a South African rotavirus SA11 sample reveals a mixed infection with two close derivatives of the SA11-H96 strain. *Archives of Virology*, *158*(5). https://doi.org/10.1007/s00705-012-1559-5

Monson, E. A., Trenerry, A. M., Laws, J. L., MacKenzie, J. M., & Helbig, K. J. (2021). Lipid droplets and lipid mediators in viral infection and immunity. *FEMS Microbiology Reviews*, *45*(4), 1–20. https://doi.org/10.1093/femsre/fuaa066

Murphy, S., Martin, S., & Parton, R. G. (2009). Lipid droplet-organelle interactions; sharing the fats. In *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (Vol. 1791, Issue 6). https://doi.org/10.1016/j.bbalip.2008.07.004

O'Connor, R. S., Guo, L., Ghassemi, S., Snyder, N. W., Worth, A. J., Weng, L., Kam, Y., Philipson, B., Trefely, S., Nunez-Cruz, S., Blair, I. A., June, C. H., & Milone, M. C. (2018). The CPT1a inhibitor, etomoxir induces severe oxidative stress at commonly used concentrations. *Scientific Reports*, *8*(1), 6289. https://doi.org/10.1038/s41598-018-24676-6

Ohsaki, Y., Suzuki, M., & Fujimoto, T. (2014). Open questions in lipid droplet biology. In *Chemistry and Biology* (Vol. 21, Issue 1). https://doi.org/10.1016/j.chembiol.2013.08.009

Ojuka, E., Andrew, B., Bezuidenhout, N., George, S., Maarman, G., Madlala, H. P., Mendham, A., & Osiki, P. O. (2016). Measurement of β-oxidation capacity of biological samples by respirometry: A review of principles and substrates. In *American Journal of Physiology - Endocrinology and Metabolism* (Vol. 310, Issue 9). https://doi.org/10.1152/ajpendo.00475.2015

Olofsson, S. O., Boström, P., Andersson, L., Rutberg, M., Levin, M., Perman, J., & Borén, J. (2008). Triglyceride containing lipid droplets and lipid droplet-associated proteins. In *Current Opinion in Lipidology* (Vol. 19, Issue 5). https://doi.org/10.1097/MOL.0b013e32830dd09b

Onal, G., Kutlu, O., Gozuacik, D., & Dokmeci Emre, S. (2017). Lipid Droplets in Health and Disease. In *Lipids in Health and Disease* (Vol. 16, Issue 1). https://doi.org/10.1186/s12944-017-0521-7

Orlicky, D. J., Monks, J., Stefanski, A. L., & McManaman, J. L. (2013). Dynamics and Molecular Determinants of Cytoplasmic Lipid Droplet Clustering and Dispersion. *PLoS ONE*, *8*(6), e66837. https://doi.org/10.1371/journal.pone.0066837

Parashar, U. D., Bresee, J. S., Gentsch, J. R., & Glass, R. I. (1998). Rotavirus. *Emerging Infectious Diseases*, *4*(4), 561–570. https://doi.org/10.3201/eid0404.980406

Patra, U., Mukhopadhyay, U., Mukherjee, A., Sarkar, R., & Chawla-Sarkar, M. (2020). Progressive Rotavirus Infection Downregulates Redox-Sensitive Transcription Factor Nrf2 and Nrf2-Driven Transcription Units. *Oxidative Medicine and Cellular Longevity*, *2020*, 1–48. https://doi.org/10.1155/2020/7289120

Patton, J. T., Silvestri, L. S., Tortorici, M. A., Vasquez-Del Carpio, R., & Taraporewala, Z. F. (2006). Rotavirus genome replication and morphogenesis: Role of the viroplasm. In *Current Topics in Microbiology and Immunology* (Vol. 309). https://doi.org/10.1007/3-540-30773-7_6

Patton, J T, Hua, J., & Mansell, E. A. (1993). Location of intrachain disulfide bonds in the VP5* and VP8* trypsin cleavage fragments of the rhesus rotavirus spike protein VP4. *Journal of Virology*, *67*(8). https://doi.org/10.1128/jvi.67.8.4848-4855.1993

Patton, J T, Jones, M. T., Kalbach, A. N., He, Y. W., & Xiaobo, J. (1997). Rotavirus RNA polymerase requires the core shell protein to synthesize the double-stranded RNA genome. *Journal of Virology*, *71*(12). https://doi.org/10.1128/jvi.71.12.9618-9626.1997

Patton, John T. (2001). Rotavirus RNA replication and gene expression. *Novartis Foundation Symposium*, *238*. https://doi.org/10.1002/0470846534.ch5

Piccoli, C., Quarato, G., Ripoli, M., D'Aprile, A., Scrima, R., Cela, O., Boffoli, D., Moradpour, D., & Capitanio, N. (2009). HCV infection induces mitochondrial bioenergetic unbalance: Causes and effects. In *Biochimica et Biophysica Acta - Bioenergetics* (Vol. 1787, Issue 5). https://doi.org/10.1016/j.bbabio.2008.11.008

Piccoli, Claudia, Scrima, R., Quarato, G., D'Aprile, A., Ripoli, M., Lecce, L., Boffoli, D., Moradpour, D., & Capitanio, N. (2007). Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. *Hepatology*, *46*(1). https://doi.org/10.1002/hep.21679

Pike, L. S., Smift, A. L., Croteau, N. J., Ferrick, D. A., & Wu, M. (2011). Inhibition of fatty acid

oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. *Biochimica et Biophysica Acta - Bioenergetics*, *1807*(6), 726–734. https://doi.org/10.1016/j.bbabio.2010.10.022

Ploegh, H. L. (2007). A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum. *Nature*, *448*(7152). https://doi.org/10.1038/nature06004

Prasad, B. V. V., Rothnagel, R., Zeng, C. Q. Y., Jakana, J., Lawton, J. A., Chiu, W., & Estes, M. K. (1996). Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. *Nature*, *382*(6590). https://doi.org/10.1038/382471a0

Rainsford, E. W., & McCrae, M. A. (2007). Characterization of the NSP6 protein product of rotavirus gene 11. *Virus Research*, *130*(1–2). https://doi.org/10.1016/j.virusres.2007.06.011

Ramani, S., Cortes-Penfield, N. W., Hu, L., Crawford, S. E., Czako, R., Smith, D. F., Kang, G., Ramig, R. F., Le Pendu, J., Prasad, B. V. V., & Estes, M. K. (2013). The VP8* Domain of Neonatal Rotavirus Strain G10P[11] Binds to Type II Precursor Glycans. *Journal of Virology*, *87*(13). https://doi.org/10.1128/jvi.03518-12

Rasmussen, A. L., Diamond, D. L., McDermott, J. E., Gao, X., Metz, T. O., Matzke, M. M., Carter, V. S., Belisle, S. E., Korth, M. J., Waters, K. M., Smith, R. D., & Katze, M. G. (2011). Systems Virology Identifies a Mitochondrial Fatty Acid Oxidation Enzyme, Dodecenoyl Coenzyme A Delta Isomerase, Required for Hepatitis C Virus Replication and Likely Pathogenesis. *Journal of Virology*, *85*(22), 11646–11654. https://doi.org/10.1128/jvi.05605-11

Raud, B., Roy, D. G., Divakaruni, A. S., Tarasenko, T. N., Franke, R., Ma, E. H., Samborska, B., Hsieh, W. Y., Wong, A. H., Stüve, P., Arnold-Schrauf, C., Guderian, M., Lochner, M., Rampertaap, S., Romito, K., Monsale, J., Brönstrup, M., Bensinger, S. J., Murphy, A. N., … Berod, L. (2018). Etomoxir Actions on Regulatory and Memory T Cells Are Independent of Cpt1a-Mediated Fatty Acid Oxidation. *Cell Metabolism*, *28*(3). https://doi.org/10.1016/j.cmet.2018.06.002

Rector, R. S., Payne, R. M., & Ibdah, J. A. (2008). Mitochondrial trifunctional protein defects: Clinical implications and therapeutic approaches. In *Advanced Drug Delivery Reviews* (Vol. 60, Issues 13–14). https://doi.org/10.1016/j.addr.2008.04.014

Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent endpoints.

*American Journal of Epidemiology*, *27*(3). https://doi.org/10.1093/oxfordjournals.aje.a118408

Robenek, H., Robenek, M. J., & Troyer, D. (2005). PAT family proteins pervade lipid droplet cores. *Journal of Lipid Research*, *46*(6). https://doi.org/10.1194/jlr.M400323-JLR200

Rohwedder, A., Zhang, Q., Rudge, S. A., & Wakelam, M. J. O. (2014). Lipid droplet formation in response to oleic acid in Huh-7 cells is mediated by the fatty acid receptor FFAR4. *Journal of Cell Science*, *127*(14). https://doi.org/10.1242/jcs.145854

Roingeard, P., & Melo, R. C. N. (2017). Lipid droplet hijacking by intracellular pathogens. In *Cellular Microbiology* (Vol. 19, Issue 1). https://doi.org/10.1111/cmi.12688

Rojo, G., Chamorro, M., Salas, M. L., Viñuela, E., Cuezva, J., & Salas, J. (1998). Migration of Mitochondria to Viral Assembly Sites in African Swine Fever Virus-Infected Cells. *Journal of Virology*, *72*(9). https://doi.org/10.1128/jvi.72.9.7583-7588.1998

Saka, H. A., & Valdivia, R. (2012). Emerging roles for lipid droplets in immunity and host-pathogen interactions. In *Annual Review of Cell and Developmental Biology* (Vol. 28). https://doi.org/10.1146/annurev-cellbio-092910-153958

Sanchez, E. L., & Lagunoff, M. (2015). Viral activation of cellular metabolism. *Virology*, *479–480*, 609–618. https://doi.org/10.1016/j.virol.2015.02.038

Sander, W. J., Fourie, C., Sabiu, S., O'Neill, F. H., Pohl, C. H., & O'Neill, H. G. (2022). Reactive oxygen species as potential antiviral targets. *Reviews in Medical Virology*, *32*(1). https://doi.org/10.1002/rmv.2240

Sander, W. J., Kemp, G., Hugo, A., Pohl, C. H., & O'Neill, H. G. (2022). Rotavirus-Mediated Prostaglandin E2 Production in MA104 Cells Promotes Virus Attachment and Internalisation, Resulting in an Increased Viral Load. *Frontiers in Physiology*, *13*. https://doi.org/10.3389/fphys.2022.805565

Seo, J. Y., & Cresswell, P. (2013). Viperin Regulates Cellular Lipid Metabolism during Human Cytomegalovirus Infection. *PLoS Pathogens*, *9*(8). https://doi.org/10.1371/journal.ppat.1003497

Seo, J. Y., Yaneva, R., & Cresswell, P. (2011). Viperin: A multifunctional, interferon-inducible protein that regulates virus replication. In *Cell Host and Microbe* (Vol. 10, Issue 6). https://doi.org/10.1016/j.chom.2011.11.004

Seo, N., Hyser, J. M., Utama, B., Crawford, S. E., Kim, K. J., Ho, M., & Estes, M. K. (2008). Integrins ⌴ 1 □ 1 and ⌴ 2 □ 1 are receptors for the rotavirus enterotoxin. *Proc Natl Acad Sci USA*, *105*(26), 8811–8818.

Silvestri, L. S., Taraporewala, Z. F., & Patton, J. T. (2004). Rotavirus Replication: Plus-Sense Templates for Double-Stranded RNA Synthesis Are Made in Viroplasms. *Journal of Virology*, *78*(14). https://doi.org/10.1128/jvi.78.14.7763-7774.2004

Stapleford, K. A., & Miller, D. J. (2010). Role of cellular lipids in positive-sense RNA virus replication complex assembly and function. In *Viruses* (Vol. 2, Issue 5). https://doi.org/10.3390/v2051055

Stone, S. J., Levin, M. C., Zhou, P., Han, J., Walther, T. C., & Farese, R. V. (2009). The endoplasmic reticulum enzyme DGAT2 is found in mitochondria-associated membranes and has a mitochondrial targeting signal that promotes its association with mitochondria. *Journal of Biological Chemistry*, *284*(8). https://doi.org/10.1074/jbc.M805768200

Swiech, K., Picanço-Castro, V., & Covas, D. T. (2012). Human cells: New platform for recombinant therapeutic protein production. In *Protein Expression and Purification* (Vol. 84, Issue 1). https://doi.org/10.1016/j.pep.2012.04.023

Tanaka, T., Morishige, J. I., Takimoto, T., Takai, Y., & Satouchi, K. (2001). Metabolic characterization of sciadonic acid (5c,11c,14c-eicosatrienoic acid) as an effective substitute for arachidonate of phosphatidylinositol. *European Journal of Biochemistry*, *268*(18). https://doi.org/10.1046/j.0014-2956.2001.02423.x

Taraporewala, Z. F., Schuck, P., Ramig, R. F., Silvestri, L., & Patton, J. T. (2002). Analysis of a Temperature-Sensitive Mutant Rotavirus Indicates that NSP2 Octamers Are the Functional Form of the Protein. *Journal of Virology*, *76*(14). https://doi.org/10.1128/jvi.76.14.7082-7093.2002

Teimoori, A., Soleimanjahi, H., & Makvandi, M. (2014). Characterization and transferring of human rotavirus double-layered particles in MA104 cells. *Jundishapur Journal of Microbiology*, *7*(6). https://doi.org/10.5812/jjm.10375

Thomas, P., & Smart, T. G. (2005). HEK293 cell line: A vehicle for the expression of recombinant proteins. *Journal of Pharmacological and Toxicological Methods*, *51*(3 SPEC. ISS.). https://doi.org/10.1016/j.vascn.2004.08.014

Torres-Vega, M. A., González, R. A., Duarte, M., Poncet, D., López, S., & Arias, C. F. (2000). The C-terminal domain of rotavirus NSP5 is essential for its multimerization, hyperphosphorylation and interaction with NSP6. *Journal of General Virology*, *81*(3). https://doi.org/10.1099/0022-1317-81-3-821

Trask, S. D., McDonald, S. M., & Patton, J. T. (2012). Structural insights into the coupling of virion assembly and rotavirus replication. In *Nature Reviews Microbiology* (Vol. 10, Issue 3). https://doi.org/10.1038/nrmicro2673

Trask, S. D., Ogden, K. M., & Patton, J. T. (2012). Interactions among capsid proteins orchestrate rotavirus particle functions. In *Current Opinion in Virology* (Vol. 2, Issue 4). https://doi.org/10.1016/j.coviro.2012.04.005

Troeger, C., Khalil, I. A., Rao, P. C., Cao, S., Blacker, B. F., Ahmed, T., Armah, G., Bines, J. E., Brewer, T. G., Colombara, D. V., Kang, G., Kirkpatrick, B. D., Kirkwood, C. D., Mwenda, J. M., Parashar, U. D., Petri, W. A., Riddle, M. S., Steele, A. D., Thompson, R. L., … Reiner, R. C. (2018). Rotavirus Vaccination and the Global Burden of Rotavirus Diarrhea among Children Younger Than 5 Years. *JAMA Pediatrics*, *172*(10). https://doi.org/10.1001/jamapediatrics.2018.1960

Vascotto, F., Campagna, M., Visintin, M., Cattaneo, A., & Burrone, O. R. (2004). Effects of intrabodies specific for rotavirus NSP5 during the virus replicative cycle. *Journal of General Virology*, *85*(11). https://doi.org/10.1099/vir.0.80075-0

Violin, J. D., DiPilato, L. M., Yildirim, N., Elston, T. C., Zhang, J., & Lefkowitz, R. J. (2008). β2-Adrenergic Receptor Signaling and Desensitization Elucidated by Quantitative Modeling of Real Time cAMP Dynamics. *Journal of Biological Chemistry*, *283*(5), 2949–2961. https://doi.org/10.1074/jbc.M707009200

Walczak, C. P., Ravindran, M. S., Inoue, T., & Tsai, B. (2014). A Cytosolic Chaperone Complexes with Dynamic Membrane J-Proteins and Mobilizes a Nonenveloped Virus out of the Endoplasmic Reticulum. *PLoS Pathogens*, *10*(3). https://doi.org/10.1371/journal.ppat.1004007

Walther, T. C., & Farese, R. V. (2009). The life of lipid droplets. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1791*(6), 459–466. https://doi.org/10.1016/j.bbalip.2008.10.009

Walther, T. C., & Farese, R. V. (2012). Lipid droplets and cellular lipid metabolism. *Annual*

*Review of Biochemistry*, *81*. https://doi.org/10.1146/annurev-biochem-061009-102430

Welte, M. A. (2015). Expanding roles for lipid droplets. In *Current Biology* (Vol. 25, Issue 11, pp. R470–R481). https://doi.org/10.1016/j.cub.2015.04.004

Wilfling, F., Wang, H., Haas, J. T., Krahmer, N., Gould, T. J., Uchida, A., Cheng, J. X., Graham, M., Christiano, R., Fröhlich, F., Liu, X., Buhman, K. K., Coleman, R. A., Bewersdorf, J., Farese, R. V., & Walther, T. C. (2013). Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. *Developmental Cell*, *24*(4). https://doi.org/10.1016/j.devcel.2013.01.013

Wolins, N. E., Rubin, B., & Brasaemle, D. L. (2001). TIP47 Associates with Lipid Droplets. *Journal of Biological Chemistry*, *276*(7). https://doi.org/10.1074/jbc.M006775200

Yeager, M., Dryden, K. A., Olson, N. H., Greenberg, H. B., & Baker, T. S. (1990). Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. *Journal of Cell Biology*, *110*(6). https://doi.org/10.1083/jcb.110.6.2133

Yu, W., Bozza, P. T., Tzizik, D. M., Gray, J. P., Cassara, J., Dvorak, A. M., & Weller, P. F. (1998). Co-compartmentalization of MAP kinases and cytosolic phospholipase A2 at cytoplasmic arachidonate-rich lipid bodies. *American Journal of Pathology*, *152*(3), 759–769. http://www.ncbi.nlm.nih.gov/pubmed/9502418

## Annexure A: Ethical clearance

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

**Environmental & Biosafety Research Ethics Committee**

16-Oct-2020

Dear **Mr Tshegofatso Thobane**

Project Title: **Investigating the effects of unsaturated fatty acids (FAs) on lipid droplet formation, fatty acid oxidation, and eicosanoid production during rotavirus infection.**

Department: **Microbial Biochemical and Food Biotechnology Department (Bloemfontein Campus)**

**APPLICATION APPROVED**

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2020/0081/1610**

**Please note the following:**

1. **This ethical clearance is valid for one year from the issuance of this letter.**
2. **If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smithan@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za

Annexure B: Figure Permission

1. Permission for figure 1.2

This Agreement between Mr. Tshegofatso Thobane ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

| | |
|---|---|
| License Number | 5366021244289 |
| License date | Sep 26, 2022 |
| Licensed Content Publisher | Elsevier |
| Licensed Content Publication | Virus Research |
| Licensed Content Title | Rotaviruses |
| Licensed Content Author | Ulrich Desselberger |
| Licensed Content Date | Sep 22, 2014 |
| Licensed Content Volume | 190 |
| Licensed Content Issue | n/a |
| Licensed Content Pages | 22 |
| Start Page | 75 |
| End Page | 96 |
| Type of Use | reuse in a thesis/dissertation |
| Portion | figures/tables/illustrations |
| Number of figures/tables/illustrations | 1 |
| Format | both print and electronic |
| Are you the author of this Elsevier article? | No |
| Will you be translating? | No |
| Title | investigation into the effects of oleic acid supplementation on lipid droplet formation, fatty acid β-oxidation and subsequent Rotavirus replication |
| Institution name | University of the Free State |
| Expected presentation date | Nov 2022 |
| Order reference number | Tshegofatso Thobane |
| Portions | Figure 4 : The rotavirus replication cycle |
| Requestor Location | Mr. Tshegofatso Thobane<br>University of the Free State<br>Free State<br><br>Bloemfontein, Free State 9301<br>South Africa<br>Attn: Mr. Tshegofatso Thobane |
| Publisher Tax ID | ZA 411809848 |
| Total | **0.00 USD** |
| Terms and Conditions | |

**INTRODUCTION**

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

**GENERAL TERMS**

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier's permissions helpdesk here). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for this transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No amendment except in writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

**LIMITED LICENSE**

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com. Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/JSTOR.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com. All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors**: the following clauses are applicable in addition to the above:
**Preprints:**
A preprint is an author's own write-up of research results and analysis. It has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
**Accepted Author Manuscripts:** An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. For **book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

**Elsevier Open Access Terms and Conditions**
You can publish open access with Elsevier in hundreds of open access journals and in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.
**Terms & Conditions applicable to all Open Access articles published with Elsevier:**
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
**Additional Terms & Conditions applicable to each Creative Commons user license:**
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0/.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0/.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0/. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.10

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

2. Permission for figure 1.3, 1.8 and 1.9

CCC | Marketplace™

This is a License Agreement between Tshegofatso Thobane ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Order General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Order General Terms and Conditions below.

| | | | |
|---|---|---|---|
| Order Date | 11-Sep-2022 | Type of Use | Republish in a thesis/dissertation |
| Order License ID | 1267273-1 | Publisher | AMERICAN SOCIETY FOR MICROBIOLOGY. |
| ISSN | 0022-538X | Portion | Image/photo/illustration |

## LICENSED CONTENT

| | | | |
|---|---|---|---|
| Publication Title | Journal of virology | Publication Type | Journal |
| Article Title | Rotaviruses associate with cellular lipid droplet components to replicate in viroplasms, and compounds disrupting or blocking lipid droplets inhibit viroplasm formation and viral replication. | Start Page | 6782 |
| | | End Page | 6798 |
| | | Issue | 13 |
| | | Volume | 84 |
| Author/Editor | AMERICAN SOCIETY FOR MICROBIOLOGY. | URL | https://journals.asm.org/journal/jvi |
| Date | 01/01/1967 | | |
| Language | English | | |
| Country | United States of America | | |
| Rightsholder | American Society for Microbiology - Journals | | |

## REQUEST DETAILS

| | | | |
|---|---|---|---|
| Portion Type | Image/photo/illustration | Distribution | Worldwide |
| Number of images / photos / illustrations | 3 | Translation | Original language of publication |
| Format (select all that apply) | Electronic | Copies for the disabled? | No |
| Who will republish the content? | Academic institution | Minor editing privileges? | No |
| Duration of Use | Life of current edition | Incidental promotional use? | No |
| Lifetime Unit Quantity | More than 2,000,000 | Currency | USD |
| Rights Requested | Main product | | |

## NEW WORK DETAILS

| | | | |
|---|---|---|---|
| Title | Investigation into the effects of oleic acid supplementation on lipid droplet formation, fatty acid β-oxidation and subsequent Rotavirus replication. | Institution name | University of the Free State |
| | | Expected presentation date | 2022-09-11 |
| Instructor name | Prof. Hester O'neill | | |

## ADDITIONAL DETAILS

| | | | |
|---|---|---|---|
| Order reference number | Tshegofatso Thobane | The requesting person / organization to appear on the license | Tshegofatso Thobane |

## REUSE CONTENT DETAILS

| | | | |
|---|---|---|---|
| Title, description or numeric reference of the portion(s) | 10.1128/JVI.01757-09 | Title of the article/chapter the portion is from | Rotaviruses associate with cellular lipid droplet components to replicate in viroplasms, and compounds disrupting or blocking lipid droplets inhibit viroplasm formation and viral replication. |
| Editor of portion(s) | Cheung, Winsome; Gill, Michael; Esposito, Alessandro; Kaminski, Clemens F.; Courousse, Nathalie; Chwetzoff, Serge; Trugnan, Germain; Keshavan, Nandita; Lever, Andrew; Desselberger, Ulrich | Author of portion(s) | Cheung, Winsome; Gill, Michael; Esposito, Alessandro; Kaminski, Clemens F.; Courousse, Nathalie; Chwetzoff, Serge; Trugnan, Germain; Keshavan, Nandita; Lever, Andrew; Desselberger, Ulrich |
| Volume of serial or monograph | 84 | | |
| Page or page range of portion | 6782-6798 | | |
| | | Issue, if republishing an article from a serial | 13 |
| | | Publication date of portion | 2010-07-01 |

3. Permission for figure 1.12

CCC Marketplace

This is a License Agreement between Tshegofatso Thobane ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Order General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

All payments must be made in full to CCC in accordance with the Marketplace Order General Terms and Conditions below.

| | | | |
|---|---|---|---|
| Order Date | 18-Oct-2022 | Type of Use | Republish in a thesis/dissertation |
| Order License ID | 1280332-1 | Publisher | Microbiology Society |
| ISSN | 0022-1317 | Portion | Image/photo/illustration |

## LICENSED CONTENT

| | | | |
|---|---|---|---|
| Publication Title | The Journal of general virology | Rightsholder | Microbiology Society |
| Article Title | Ultrastructural study of rotavirus replication in cultured cells. | Publication Type | Journal |
| Author/Editor | SOCIETY FOR GENERAL MICROBIOLOGY, FEDERATION OF EUROPEAN MICROBIOLOGICAL SOCIETIES. | Start Page | 75 |
| | | End Page | 85 |
| | | Issue | 1 |
| Date | 01/01/1967 | Volume | 46 |
| Language | English | | |
| Country | United Kingdom of Great Britain and Northern Ireland | | |

## REQUEST DETAILS

| | | | |
|---|---|---|---|
| Portion Type | Image/photo/illustration | Distribution | Worldwide |
| Number of Images / Photos / Illustrations | 5 | Translation | Original language of publication |
| Format (select all that apply) | Electronic | Copies for the Disabled? | No |
| Who Will Republish the Content? | Academic institution | Minor Editing Privileges? | No |
| Duration of Use | Current edition and up to 10 years | Incidental Promotional Use? | No |
| Lifetime Unit Quantity | More than 2,000,000 | Currency | USD |
| Rights Requested | Main product, any product related to main product, and other compilations/derivative products | | |

## NEW WORK DETAILS

| | | | |
|---|---|---|---|
| Title | Investigation into the effects of oleic acid supplementation on lipid droplet formation, fatty acid β-oxidation and subsequent Rotavirus replication. | Institution Name | University of the Free State |
| | | Expected Presentation Date | 2022-11-29 |
| Instructor Name | Prof. Trudi O'Neill | | |

## ADDITIONAL DETAILS

| | | | |
|---|---|---|---|
| Order Reference Number | Tshegofatso Thobane | The Requesting Person/Organization to Appear on the License | Tshegofatso Thobane |

## REUSE CONTENT DETAILS

| | | | |
|---|---|---|---|
| Title, Description or Numeric Reference of the Portion(s) | Fig. 2. and Fig. 7. | Title of the Article/Chapter the Portion Is From | Ultrastructural study of rotavirus replication in cultured cells. |
| Editor of Portion(s) | Altenburg, Betty C.; Graham, David Y.; Estes, Mary Kolb | Author of Portion(s) | Altenburg, Betty C.; Graham, David Y.; Estes, Mary Kolb |
| Volume of Serial or Monograph | 46 | Issue, if Republishing an Article From a Serial | 1 |
| Page or Page Range of Portion | 75-85 | Publication Date of Portion | 1980-01-01 |