Amoebae, Giant Viruses, and Virophages Make Up a Complex, Multilayered Threesome

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Viral infection had not been observed for amoebae, until the Acanthamoeba polyphaga mimivirus (APMV) was discovered in 2003. APMV belongs to the nucleocytoplasmatic large DNA virus (NCLDV) family and infects not only A. polyphaga, but also other professional phagocytes. Here, we review the Megavirales to give an overview of the current members of the Mimiviridae and Marseilleviridae families and their structural features during amoebal infection. We summarize the different steps of their infection cycle in A. polyphaga and Acanthamoeba castellani. Furthermore, we dive into the emerging field of virophages, which parasitize upon viral factories of the Megavirales family. The discovery of virophages in 2008 and research in recent years revealed an increasingly complex network of interactions between cell, giant virus, and virophage. Virophages seem to be highly abundant in the environment and occupy the same niches as the Mimiviridae and their hosts. Establishment of metagenomic and co-culture approaches rapidly increased the number of detected virophages over the recent years. Genetic interaction of cell and virophage might constitute a potent defense machinery against giant viruses and seems to be important for survival of the infected cell during mimivirus infections. Nonetheless, the molecular events during co-infection and the interactions of cell, giant virus, and virophage have not been elucidated, yet. However, the genetic interactions of these three, suggest an intricate, multilayered network during amoebal (co-)infections. Understanding these interactions could elucidate molecular events essential for proper viral factory activity and could implicate new ways of treating viruses that form viral factories.

Keywords: Acanthamoeba polyphaga mimivirus (APMV), virophage, nucleocytoplasmatic large DNA virus (NCLDV), mimivirus, pathogen defense

INTRODUCTION TO GIANT VIRUSES

The discovery of giant viruses in the early 2000s led to a mind shift in the field of virology with respect to the potential origins of viruses (La Scola et al., 2003; Raoult et al., 2004). Originally, viruses were thought of as submicroscopic particles with a self-evident denial that viruses might exist, whose size would be large enough to be resolved with a simple light microscope (Lwoff, 1957; Raoult, 2013). Due to this mindset, the large, gram-positive particles in an Acanthamoeba polyphaga population were at first erroneously classified as bacteria (Birtles et al., 1997; La Scola et al., 2003; Raoult et al., 2007). Only the absence of ribosomal DNA in the presumed bacterium, led to the discovery and definition of the A. polyphaga mimivirus (APMV) in 2003 (La Scola et al., 2003).
The acronym mimivirus (for mimicking microbe) reflects the resemblance to bacteria upon gram staining. At the same time, the discovery of APMV was the first ever report of a virus infecting amoebae. Amongst other features that are detailed below, APMV is unusual as it contains a large genome of 1.14 Mbp, thereby even surpassing the genome size of some bacterial species (Raoult et al., 2004). APMV particles are characterized by an up to 700 nm large capsid (Figure 1A), which is well above the resolution of a simple light microscope. Once it was established that giant DNA viruses of amoebae exist, many more such viruses, belonging to the nucleoplasmatic large DNA viruses (NCLDV) were found in the environment, as well as within a wide range of host organisms from humans, monkeys, and oysters (Boughalmi et al., 2013a; Dornas et al., 2014; Andrade et al., 2015). Ex vivo studies of human cell lines revealed that APMV is capable of infecting myeloid and mononuclear blood cells and interferes with the type I Interferon system (Silva et al., 2014). In addition, a distantly APMV-related NCLDV family member has been shown to productively infect T-lymphocytes under laboratory conditions (Popgeorgiev et al., 2013). In 2008, a small particle called Sputnik 1 (La Scola et al., 2008) was discovered in *A. polyphaga*, which parasitizes viral factories of giant viruses. Due to the functional similarity to bacteriophages in mediating lateral gene transfer, Sputnik was classified as a virophage (La Scola et al., 2008). Here, we will review the expanding family of virophages and discuss the implications for giant virus reproduction inside amoebae.

### THE DIVERSE FAMILIES OF GIANT VIRUSES THAT INFECT AMOEBAE

The discovery of APMV sparked the interest in giant viruses and spawned a contemporary research field of its own (La Scola et al., 2003). Up until today, two giant virus families belonging to the NCLDV have been described that primarily infect amoebae: the *Mimiviridae* and the *Marseilleviridae* (Figure 1B). The latter has the *A. polyphaga* marseillevirus (APMaV) as founding member, which was discovered in 2009 (Boyer et al., 2009; Colson et al., 2013). In the last decade, nine additional viruses have been associated with the *Marseilleviridae* group (Colson et al., 2017). The *Acanthamoeba castellani* lausannevirus (ACLaV) was discovered by incubating water from the Seine river in France with *A. castellani*, a close relative of *A. polyphaga* (Thomas et al., 2011). ACLaV is the first known giant virus to encode histone-like proteins, which could point towards a DNA packaging mechanism similar to eukaryotes (Thomas et al., 2011). The Cannes 8 virus (Ca8V) (La Scola et al., 2010) and the Senegal virus (SNGV) (Lagier et al., 2012) have been isolated using similar co-culture methods and are grouped with the *Marseilleviridae*. The icosahedral capsid of the *Marseilleviridae* is between 190 and 250 nm in diameter (Colson et al., 2013). Like the genome of the *Mimiviridae*, the 370,000 bp dsDNA genome is encased in a lipid bilayer and encodes about 450 proteins (Boyer et al., 2009; La Scola et al., 2010; Thomas et al., 2011; Lagier et al., 2012). Both, *Mimiviridae* and *Marseilleviridae*, share only nine core genes with all NCLDVs (Figure 1C) and 180 genes are shared with at least two of the NCLDV families (Yutin et al., 2009; Yutin and Koonin, 2012). Based on the discovery of APMV and its complex genome, it was suggested to incorporate viruses into the tree of life by defining them as capsid-encoding organisms contrary to the ribosome-encoding organisms, which are represented by eukarya, bacteria, and archaea (Raoult and Forterre, 2008).

### APMV—THE BEST STUDIED GIANT VIRUS OF AMOEBAE

APMV was the first giant virus to be discovered (La Scola et al., 2008) and confronted the scientific community with features never observed in a virus before. Its capsid size and genetic complexity with many genes usually found in eukaryotic and prokaryotic cells challenged the Lwoff’s characteristics of a virus (Raoult et al., 2004; Raoult and Forterre, 2008). The AT-rich 1.14 Mbp APMV genome features an impressive number of 979 protein-encoding genes in a dense arrangement (Raoult et al., 2004; Legendre et al., 2011). Several of its genes are only found in giant viruses of amoebae and code for virus-atypical proteins involved in DNA repair, protein folding, tRNA synthesis and translation, and more (Raoult et al., 2004). In addition, the APMV genome displays some plasticity and encodes self-splicing introns, inteins, and a specific set of mobile genetic elements called transpovirons (Desnues et al., 2012). Furthermore, the genome contains many genes likely acquired via horizontal gene transfer, paralogous genes, and so called ORFans, genes that encode proteins with unknown function (Suhre, 2005; Filée et al., 2007; Moreira and Brochier-Armanet, 2008; Forterre, 2010). Many of these genes are shared with the poxviruses, phycodnaviruses, and other NCLDVs (Filée et al., 2007). ORFans represent roughly 50% of genes and about 40% of the APMV proteome, which results in a high number of factors with unknown functions that might act during viral replication and morphogenesis (Renesto et al., 2006). Alike “classical” viruses, APMV genes are partly under the control of early and late stage-specific promoters (Raoult et al., 2004; Suhre et al., 2005).

The APMV particles possess remarkable structural features, separating them from the classical structures of viruses (Figure 1A). In its center, the viral DNA, mRNAs and proteins are packed into the core compartment (Xiao et al., 2009; Kuznetsov et al., 2013) and enclosed by a lipid membrane. Among the pre-packed proteins are 12 enzymes involved in transcription, five in DNA repair, two in RNA modification, and five in protein modification (Renesto et al., 2006). The central compartment is surrounded by an approximately 340 nm-large lipid bilayer and a secondary bilayer directly underneath an icosahedral capsid. This is comprised of major capsid proteins and features a five-branch proteinaceous structure, the “stargate,” at one vertex (Kuznetsov et al., 2013). The capsid itself is covered by a compact layer of about 120–140 nm long, heavily glycosylated fibrils, which potentially facilitate the attachment of APMV to its host cells (Rodrigues et al., 2015).

As of now, only four fiber associated proteins (FAP1-4) have been functionally associated with either fibril biosynthesis or as components of the fibrils (Sobhy et al., 2015). FAP1 is an aryl
alcohol oxidase, which catalyzes the degradation of lignin or lignin derivatives. This suggests that APMV might also be able to infect lignin-containing algae (Klose et al., 2015; Rodrigues et al., 2015). However, the fibrils and associated proteins are not essential for the productive infection of amoebae: during long-term intramoebal culture (150 generations), the responsible genes are lost (Boyer et al., 2011; Rodrigues et al., 2015). This indicates that the genomic complexity of APMV might be maintained to allow for a broad host range. If so, only a subset of its diverse molecular tools would come in use to enter and infect individual hosts.

**INFECTION CYCLES OF GIANT VIRUSES IN AMOEBAE**

Even though the replication cycle of most giant viruses differ in aspects like nuclear involvement, duration, assembly, and release of the viral progeny, key steps in the infection appear to be conserved, as summarized recently (Colson et al., 2017). For example, all known giant viruses enter the host cell by phagocytosis and release their DNA into the cytosol in a similar manner (Ghigo et al., 2008). Furthermore, viral replication takes place in specialized endoplasmic reticulum (ER)-derived compartments that are found in the cytosol and are called viral factories (Xiao et al., 2009; Mutsafi et al., 2010; Kuznetsov et al., 2013).

After uptake, the virus resides in a de-novo phagosome. Subsequently, the phagosomal and viral membranes fuse, which allows the release of the viral core, that contains the genome, proteins, and mRNAs into the cytosol (Zauberman et al., 2008; Mutsafi et al., 2014). Alike the well-described poxvirus (Broyles, 2003), the structural integrity of the viral core seems to be maintained until viral factories arise (Claverie et al., 2009; Mutsafi et al., 2014). Intriguingly, recent experiments suggest that viral transcription might be initiated already before the release of the viral core (Mutsafi et al., 2014). Once in the cytosol, replication of the viral genome begins immediately and the expression of early stage genes leads to the formation of early viral factories (Suzan-Monti et al., 2007; Mutsafi et al., 2013, 2014). The replication cycle is confined to the cytosol, again a trait shared with the poxvirus (La Scola et al., 2003; Claverie et al., 2009). This also suggests that giant viruses (like the poxvirus) must carry transcription complexes to initiate transcription immediately after infection (Resch et al., 2007; Claverie et al., 2009). In later stages of infection, these viral factories merge into one large cytosolic compartment for replication and capsid assembly (Suzan-Monti et al., 2007; Mutsafi et al., 2014). It should be noted that viral factories are not chaotic, but rather appear to feature distinct assembly lines for their progeny. The viral
factory is made up of functional regions playing discrete roles in replication, capsid assembly, DNA packaging, and attachment of fibrils (Suzan-Monti et al., 2007; Mutsafi et al., 2014). In the outermost layer of the viral factory, the internal membrane layers of APMV are assembled from host-derived membrane vesicles, which are thought to rupture, thereby forming open single-layer membrane sheets (Mutsafi et al., 2013). Capsid assembly occurs around these membrane sheets and is scaffolded by the major capsid protein L425 (Mutsafi et al., 2013). Upon capsid formation, the genome is deposited into the empty viral particle through a transient interstice distal from the “stargate” structure (Zauberman et al., 2008). There is little evidence for a nuclear stage of giant viruses. However, the nuclei of A. polyphaga and A. castellani exhibit transient changes in their morphology during the early stages of infection with members of the Marseilleviridae family (Arantes et al., 2016). This indicates that nuclear host factors might play a role in the APMV replication, a notion that is supported by a two-fold decrease of the nuclear size in infected A. polyphaga cells (Colson et al., 2017). This might be due to a substantial redistribution of nuclear factors for viral replication, transcription or other processes (Colson et al., 2017). Albeit indirectly, this scenario is supported by data on the cytoplasmic replication of the Vaccinia virus (a poxvirus), to which mimivirus replication bears similarities (Mutsafi et al., 2010) and for which the involvement of nuclear enzymes has been demonstrated (Oh and Broyles, 2005).

VIROPHAGES AS PARASITES OF THE MEGAVIRALES

The description of Megavirales infection of amoebae was followed by the discovery of the fascinating virophage Sputnik in 2008 (La Scola et al., 2008). Sputnik was found infecting the viral factories of the mamavirus, a close relative of APMV (La Scola et al., 2008). Replication of the Sputnik virophages inside APMV-infected A. castellani cells is deleterious to APMV replication and results in abortive DNA replication and disruption of capsid biogenesis (La Scola et al., 2008). There is an ongoing discussion on the classification of virophages, that are denoted in several articles as satellite viruses (Krupovic and Cvirkaite-Krupovic, 2011; Blanc et al., 2015; Koonin and Krupovic, 2017). Satellite viruses are characterized by their dependency on factors of a helper virus. However, the Sputnik genomes itself encodes factors involved in viral replication (La Scola et al., 2008), suggesting that Sputnik can be classified as a virus, rather than a defective viral particle or sub-viral agent (Fischer, 2011; Desnues and Raoul, 2012).

All known members of the virophage family parasitizing on giant viruses are categorized into the large virus-dependent or -associated (Lavida)-viridae family that is divided into the Sputnikvirus and Mavirus genera (Krupovic et al., 2016). At the species level, the Sputnikvirus genus can be differentiated into the APMV-dependent Sputnik virophage and the APMV-dependent Zamilon virophage (Table 1), while Mavirus genus contains only the Cafeteria roenbergensis virus (CroV)-dependent mavirus (Krupovic et al., 2016).

Virophage replication has been extensively studied in particular for Sputnik, Zamilon and mavirus. Studies on amoebae infected with different mimiviruses revealed that Sputnik virophages can parasitize mimiviruses from all Mimiviridae lineages but apparently not the Marseilleviridae lineages (Gaia et al., 2013). Sputnik replicates inside mamavirus-infected A. castellani cells within the viral factories, nonetheless, with different kinetics as the mamavirus and at multiple hot spots inside the factory (La Scola et al., 2008). In APMV's viral factories, Sputnik infection results in the emergence of newly generated particles 6h post infection with a concomitant decrease of infective APMV particles (Ogata and Claverie, 2008). The 18,343-kilobase circular dsDNA genome of Sputnik possesses 21 partly overlapping open-reading frames (ORFs) encoding for several factors involved in DNA replication (La Scola et al., 2008). Interestingly, four of the ORFs are strongly homologous to APMV-encoded genes (La Scola et al., 2008; Gaia et al., 2013). Since Sputnik virophages encode a lambda-type integrase, the molecular tools for genomic integration are present (La Scola et al., 2008). Indeed, an integration of the Sputnik genome into the genome of the Lentille virus, a relative of APMV, could be observed experimentally (Desnues et al., 2012). There is no indication of Sputnik genome integration into the host cell genome, in line with the lack of indications for a nuclear phase.

The Zamilon virophage (belonging to the Sputnikvirus genus) was discovered together with the Mont1 mimivirus in soil samples from Tunisia (Boughalmi et al., 2013a; Gaia et al., 2014). The 60 nm-wide, spherical virophage carries a 17,276 bp dsDNA genome encoding 20 genes. Although Zamilon shares 76% of its genomic sequence with Sputnik, Zamilon can only infect lineages B and C (Gaia et al., 2014). Furthermore, the tv_L8 protein, encoded in the transposvirus of the Montv mimivirus, shares significant homology with the ORF8-encoded protein of Zamilon (Gaia et al., 2014). This suggests that an exchange of genetic material can in principle occur between the giant virus and the Zamilon virophage within co-infected amoebae, although this has not been observed experimentally so far.

The Maverick-related virus (mavirus), lonely member of the Mavirus genus, parasitizes the viral factories of CroV that infects the marine heterotrophic nanoflagellate C. roenbergensis (Fischer et al., 2010; Fischer and Suttle, 2011). Although this review is predominantly concerned with infection of amoebae, mavirus is included here for its unique features for a virophage. Its 19,063 bp circular genome possesses 20 ORFs including a retroviral integrase, an unusual, protein-primed DNA polymerase, plus four additional proteins, all of which are also found conserved in Maverick/Polinton (MP) retroelements (Fischer and Suttle, 2011; Krupovic et al., 2014, 2016). Additionally, the termini of the mavirus genome consist of long terminal repeats similar to those found in MP retroelements (Yutin et al., 2013; Krupovic et al., 2016). Both findings suggest that these retroelements might have originated from mavirus genome integration events in mavirus co-infected cells (Fischer and Suttle, 2011; Krupovic et al., 2016). Nonetheless, this hypothesis for the origins of MP retroelements remains to be tested experimentally. Fischer and Hackl (2016) succeeded to monitor the integration of mavirus into the C. roenbergensis genome by co-infection with a low multiplicity of infection of CroV. Intriguingly, genes in the
| Family | L³ | Name | Place discovered | Size [nm] | References | GenBank Acc. No. | Genome size [kbp] | No. of ORFs (predicted) | GC% | Virophage | References |
|--------|----|------|------------------|----------|------------|-----------------|-----------------|----------------------|------|-----------|------------|
| Mimiviridae | A | A. polyphaga mimivirus | Bradford, England | 750 | La Scola et al., 2003 | NC_014649.1 | 1,182 | 979 | 28 | Sputnik | La Scola et al., 2008 |
| | A | A. polyphaga mamavirus | Paris, France | 750 | Colson et al., 2011 | JF801956.1 | 1,192 | 1023 | 28 | Sputnik | La Scola et al., 2008 |
| | A | Hrudkovirus | Tunisia | 520 | Boughalmi et al., 2013a | KF493731.1 | 1,155 | 992 | 28 | No report | |
| | A | Nemerovirus | Belo Horizonte, Brazil | 620 | Boratto et al., 2015 | KT599914.1 | 1,299 | 1003 | 28 | No report | |
| | A | Samba virus | Negro River, Brazil | 570 | Campos et al., 2014; Assis et al., 2015 | KH959826.2 | 1,181 | 971 | 28 | Rio Negro | Campos et al., 2014 |
| | A | Amazonian virus | Negro River, Brazil | 620 | Assis et al., 2015 | KM982403 | 1,180 | 979 | 27 | No report | |
| | A | Kroon virus | Lagoa Santa, Brazil | 620 | Assis et al., 2015 | KM982402.1 | 1,222 | 944 | 27 | No report | |
| | A | Oyster virus | Florianópolis, Brazil | 570 | Assis et al., 2015 | KM982401.1 | 1,200 | 948 | 27 | No report | |
| | A | Pointe-Rouge 1 virus | Marseille, France | 390 | La Scola et al., 2010 | LN871174.1 | 1,151 | 27 | No report | |
| | A | Longchamps virus | Marseille, France | 450 | La Scola et al., 2010 | LN871173.1 | 1,104 | 27 | No report | |
| | A | Fauteuil virus | Marseille, France | 600 | La Scola et al., 2010 | LN871163.1 | 1,181 | 27 | No report | |
| | A | Terra virus | Marseille, France | 370 | La Scola et al., 2010; Yoosuf et al., 2014b | KF527228.1 | 1,167 | 890 | 28 | Sputnik | Gaia et al., 2013 |
| | A | Pointe-Rouge 2 virus | Marseille, France | 500 | La Scola et al., 2010 | LN871172.1 | 1,163 | 27 | No report | |
| | A | Lactour virus | Marseille, France | 450 | La Scola et al., 2010 | CXOL000000000.1* | 1,181 | 27 | No report | |
| | A | Lentille virus | Marseille, France | 500 | La Scola et al., 2010 | AFYO00000000.1* | 1,193 | 807 | 27 | Sputnik | La Scola et al., 2010 |
| | A | Shirakomae virus | Nagano, Japan | 450 | Takeumura et al., 2016 | AP017645.4 | 1,183 | 996 | 27 | No report | |
| | A | Kasai virus | Tokyo, Japan | 450 | Takeumura et al., 2016 | AP017644.1 | 1,183 | 996 | 27 | No report | |
| | A | Bombay virus | Mumbai, India | 435 | Chatterjee et al., 2016b | KU761889.1 | 1,182 | 898 | 28 | No report | |
| B | A. polyphaga moumouivirus | South-East France | 420 | La Scola et al., 2010; Yoosuf et al., 2012 | NO020104.1 | 1,021 | 930 | 27 | Sputnik | Gaia et al., 2013 |
| B | Monme virus | Jeddah, Saudi Arabia | 390 | La Scola et al., 2010 | JN85994-6001* | 1,015 | 27 | No report | |
| B | Saudi moumouivirus | Jeddah, Saudi Arabia | 500 | Bajrai et al., 2016 | KY110734.1 | 1,046 | 868 | 26 | No report | |
| B | Goulette virus | Las Cruces, Chile | 700 | Boughalmi et al., 2013c | KC000857.2 | 1,017 | 979 | 25 | No report | |
| C | Megavirus chileniss | Las Cruces, Chile | 700 | Arslan et al., 2011 | JN258408.1 | 1,259 | 1120 | 25 | No report | |
| C | LBA111 virus | Tunisia | 550 | Saadi et al., 2013a | JX885207.1 | 1,231 | 1183 | 25 | No report | |
| C | Couido 11 virus | Saint-Raphael, France | 450 | La Scola et al., 2010; Yoosuf et al., 2014a | JX975216.1 | 1,246 | 1166 | 25 | Sputnik | Gaia et al., 2013 |

(Continued)
| Family   | Lineage | Name                          | Place discovered | Size [nm] | References                                                                 | GenBank Acc. No. | Genome size [kbp] | No. of ORFs (predicted) | GC% | Virophage | References           |
|----------|---------|-------------------------------|------------------|-----------|----------------------------------------------------------------------------|--------------------|--------------------|------------------------|------|------------|-----------------------|
| C        |         | Courdo 7 virus                | France           | 400       | La Scola et al., 2010                                                      | JN859990.3        | 1,000              |                        |      |            | Gaia et al., 2013      |
| C        |         | Terra1 virus                  | Marseille, France| 420       | La Scola et al., 2010; Yoo et al., 2014b                                   | KF527229.1        | 1,234              | 1055                   | 25   |            | Gaia et al., 2013      |
| C        |         | Shan virus                    | Marseille, France| 640       | Saad et al., 2013b                                                         | LN868520.1        | 1,259              |                        |      |            | No report              |
| C        |         | Courdol5 virus                |                  | 400       | La Scola et al., 2010                                                      | LN868542.1        | 0,922              |                        |      |            | Gaia et al., 2013      |
| C        |         | Powai Lake megavirus          | Mumbai, India    | 425       | Chatterjee et al., 2016a                                                   | KU677344.1        | 1,209              | 996                    | 25   |            | No report              |
| C        |         | Bus virus                     |                  | 400       | La Scola et al., 2010                                                      | LN868509.1        | 1,229              |                        |      |            | Gaia et al., 2013      |
| C        |         | Avenue 9 virus                |                  | 400       | Boughalmi et al., 2013c                                                    | LN867403.1        | 1,214              |                        |      |            | No report              |
| C        |         | Montpellier 3 virus           | Montpellier, France| 370    | La Scola et al., 2010                                                      | LN868518.1        | 1,243              |                        |      |            | Gaia et al., 2013      |
| C        |         | Mont1 virus                   | Tunisia          | 500       | Boughalmi et al., 2013c                                                    |                   |                    |                        |      |            | Zamilon               |

*Genomes with separate available contigs or only raw sequencing data.

aLineage.
mavirus genome possess promoter sequences similar to the late stage promoter of CroV (Fischer and Hackl, 2016). As a consequence, re-infection of C. roenbergensis carrying the integrated mavirus genome with CroV resulted in inhibition of CroV DNA replication, concomitantly with an increased survival of C. roenbergensis (Fischer and Hackl, 2016).

Other virophages have been discovered by metagenomic analysis of water samples [e.g., the Organic Lake virophage (Yau et al., 2011), the Yellowstone Lake virophages (Zhou et al., 2013, 2015)]. However, the viral and cellular host for these remain to be determined (Krupovic et al., 2016), unlike the situation of the Rio Negro virophage that has the Samba virus as viral host (Campos et al., 2014).

OUTLOOK

Since the discovery of its first member APMV in 2003, new giant viruses are discovered continuously in samples from all over the world and added to the Megavirales family. The addition of virophages as parasites of giant viruses, their high abundance in the environment, and the genetic interactions between cell, giant virus, and virophage, suggest an intricate, multilayered network during amoebal co- and super-infections. Future studies of these dynamic interactions could elucidate the inner mechanics of viral factories.

AUTHOR CONTRIBUTIONS

All authors designed the mini review. JD: generated the figures and drafted the text; MH and CH: wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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