Dengue virus capsid protein interacts specifically with very low-density lipoproteins

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Supplementary Material

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Supplementary Introduction

**Dengue mosquito vectors spreading to temperate regions**

Dengue virus (DENV) causes the most important arthropod-borne viral disease in humans.\(^1\) Human beings are both the normal reservoir and the death-end hosts of the urban-endemic DENV.\(^1,2\) Its disease vectors are *Aedes aegypti* and *Aedes albopictus* mosquitoes.\(^3,4\) These tropical and sub-tropical arthropods transmit the disease between humans,\(^5\) causing serious outbreaks mainly in developing nations.\(^4,6,7\) The World Health Organization (WHO) estimates 2.5 billion people to live at risk areas, with approximately 50 million DENV infections occurring annually, causing more than 20 thousand deaths.\(^1,2\) For example, in Brazil, a Country where the disease is well-established, 1 million people have been infected in 2011 alone, leading to over 15,000 cases of the severe dengue hemorrhagic fever. Once infected, no vaccine or specific treatments are currently available.\(^2,8,9\) It is thus easy to imagine the extent and social impact of these outbreaks.\(^4,7\) Additionally, these mosquitoes are spreading to more temperate climates all over the world, due to the increasing movement of people and goods and to global warming.\(^1,10,11\) They already reached significant areas within USA (28 of the 50 states, mainly in southern and eastern regions).\(^12\) In Europe, *Aedes aegypti* is now found in Amsterdam (The Netherlands)\(^10\) and in Madeira (Portugal),\(^13\) while *Aedes albopictus* already occupies significant areas in France, Italy, Spain, Switzerland, Croatia and other Balkan countries.\(^14\) Conditions for a dengue outbreak in Europe are therefore now set, with two recent events clearly demonstrating such risk: in the summer of 2010, two independent dengue imported cases caused DENV autochthonous transmissions in Nice (France) and Korčula (Croatia), regions where the *Aedes albopictus* population is established.\(^15,16\) Local cases of dengue transmission have also occurred in Miami, Florida, in 2011. More recently, over 2,000 autochthonous infection cases were diagnosed from October 2012 to January 2013 on the Portuguese island of Madeira. It should be mentioned that several *Aedes* spp. eradication attempts in the affected European countries have been unsuccessful so far and, in fact, the mosquitoes continue to expand their distribution territory.\(^11\)
**Similarities between very low-density lipoproteins and lipid droplets**

Lipoproteins circulate in the bloodstream as part of the blood plasma and have key physiological functions, mainly on lipid homeostasis.\(^{17,18}\) They are essentially classified in four major types, according to their density and function:\(^{17,18}\) chylomicra, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Mainly formed in the liver, VLDL distribute the lipids from there to all over the body. Interestingly, they are quite similar to hepatic lipid droplets (LDs) in terms of structure and lipid composition (Table S1).\(^{17-19}\) Although both of these structures are endoplasmic reticulum (ER) membrane derived (which explains their similarities), VLDL formation occurs on the luminal side whereas LDs are formed on the cytosolic side of the ER membrane.\(^{17-19}\) The main differences between these structures are directly caused by these different formation pathways. LDs are responsible for the lipid homeostasis inside the cell, in contrast to VLDL, which are exported to the extracellular medium.\(^{17-19}\) LDL are generated from VLDL through delipidation, deproteinization and enzymatic metabolization in the bloodstream,\(^{17,18}\) rendering them increasingly different from LDs (Table S1). A key difference between LDL and VLDL is their protein composition: LDL main protein is apolipoprotein (apo) B100, while VLDL also have significant amounts of apo E, C-I, C-II and C-III.\(^{17,18}\) There are specific similarities between VLDL apoE and LDs protein perilipin 3. Despite their different physiological functions and locations, these proteins are structurally similar (Figure S1),\(^{20}\) having also sequence similarities, as further discussed in the article. All these similarities between VLDL and LDs point to the possibility of DENV C being able to interact with VLDL. Our working hypothesis involves the formation of lipoviroparticles (LVP) by DENV (as observed for other *Flaviviridae*)\(^{21-23}\) through the interaction of DENV C with emerging VLDL during viral assembly.
Table S1 – LDL, VLDL, LDs and LVP structure and composition.

Accepted structure, physical properties, biochemical composition and main proteins of low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), lipid droplets (LDs) and lipoviroparticles (LVP). The drawings are merely representative and are not at the same scale. Roughly, all these systems are constituted by a core of neutral lipids (mainly triglycerides and cholesteryl esters; colored yellow), surrounded by a monolayer of polar lipids (mainly phospholipids and cholesterol; colored brown) where specific proteins are embedded.\textsuperscript{17-19,22-24} However, they have different lipid compositions (in %), giving them different densities. Their biochemical and protein compositions are explained by its formation pathway and also by its function. All these systems are ER-derived.\textsuperscript{17-19,22,23} LDL, VLDL and LVP are extracellular structures that have similar proteins;\textsuperscript{17,18,22,23} apoB100 (black) is present in all of them, VLDL and LVP also contain apoE (red) and apoCs (dark green). LDs main proteins are perilipins 1, 2 and 3 (violet, grey and light green).\textsuperscript{19} LVPs, as chimerical structures related with virions and with VLDL, also contain viral envelope proteins (E; cyan) and a nucleocapsid in its core, composed by viral genomic RNA (orange) condensed with the capsid protein (C; blue). As it can be observed, VLDL are very similar to LDs and LVP in terms of lipid composition.\textsuperscript{17-19,22-24}

<table>
<thead>
<tr>
<th>Lipid system</th>
<th>LDL</th>
<th>VLDL</th>
<th>LDs</th>
<th>LVP\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>![Structure LDL]</td>
<td>![Structure VLDL]</td>
<td>![Structure LDs]</td>
<td>![Structure LVP]</td>
</tr>
<tr>
<td>Density (g/mL)</td>
<td>1.019 – 1.063</td>
<td>0.930 – 1.006</td>
<td>–</td>
<td>~1.0</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>18 – 25</td>
<td>30 – 80\textsuperscript{b}</td>
<td>100 – 1000\textsuperscript{c}</td>
<td>20 – 80</td>
</tr>
<tr>
<td>Cholesterol (%)</td>
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<td>20</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Triglycerides (%)</td>
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<td>20</td>
<td>20</td>
<td>45</td>
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<td>20</td>
<td>10</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Proteins</td>
<td>ApoB100</td>
<td>ApoB100, ApoE, ApoC-I, ApoC-II, ApoC-III</td>
<td>Perilipin 1, Perilipin 2, Perilipin 3</td>
<td>ApoB100, ApoE, ApoC-I, C-II, C-III, Envelope, Capsid</td>
</tr>
</tbody>
</table>

\textsuperscript{a} LVP are formed during HCV infection and they may be a common feature among flaviviruses.\textsuperscript{21,23}
\textsuperscript{b} Premature VLDL (pre-VLDL) and VLDL in pathological conditions can exceed 200 nm in diameter.\textsuperscript{17,18}
\textsuperscript{c} LDs larger than 1 μm usually occur in adipocytes, in fatty acid induced cells or pathogen-infected cells.\textsuperscript{19,25,26}
Figure S1 – Structural similarity between apoE N-terminus and perilipin 3 C-terminus.

The available structures of apoE N-terminus (a) (PDB ID: 2KC3) and perilipin 3 C-terminus (b) (PDB ID: 1SZI) superimpose in space (c, d) through its common four-helix-bundle motif,\textsuperscript{27} as previously reported.\textsuperscript{20} This evidence, in addition to the sequence similarity shown in Figure 6 of the article, reinforces the similarities between these two proteins and between VLDL and LDs.
DENV C structure-function relationships

Flaviviruses capsid proteins are structural proteins encoded by the viral genome that are present in the virions conjugated with the genomic RNA, forming the nucleocapsid.28,29 DENV C is a protein essential for virus assembly, ensuring the specific encapsidation of the DENV viral genome.28-31 This protein, with 100 amino acid residues, presents a homodimer α-helical fold (Figure S2a), with each monomer containing four α-helices (α1 to α4) connected by short loops, as determined by NMR spectroscopy (PDB ID: 1R6R).31 DENV C is a very basic protein due to its 26 Arg and Lys residues and only 3 negatively-charged residues. This is related with its RNA-binding function. DENV C also contains a 21 amino acid-hydrophobic segment required for the maturation and assembly of the viral particles.32 Most of its positively-charged residues are concentrated in one face of the dimer, formed by the α4 helices of each monomer (α4-α4’; Figure S2b, brown), while the opposite face, formed by α1-α1’ and α2-α2’ helices, is largely composed by non-charged and by non-polar residues (Figure S2b, orange and grey, respectively). This asymmetric charge distribution in DENV C led to the proposal that the α4-α4’ region would interact with viral RNA, whereas α2-α2’ would bind to molecular components of the viral membrane.31 However, the structure of the first twenty residues was neither solved for this protein nor for the very similar West Nile virus (another member of Flaviviridae family) capsid protein.31,33 Recently, it was found that DENV C interacts with intracellular lipid droplets (LDs) through a potassium- and LDs protein(s)-dependent mechanism, being this interaction essential for the viral replication and encapsidation to occur.34,35 The unstructured N-terminus, the α1, and the α2-α2’ regions of DENV C were proved to be involved in this interaction.35,36 Taking into account the similar results obtained in the present study, it is reasonable to hypothesize that these are the most likely DENV C regions to directly interact with VLDL. Interestingly, the hepatitis C virus capsid protein, which is found in the core of HCV LVP,22,23 was also found to interact with LDs during HCV replication,37 reinforcing the parallelisms between the function of these capsid proteins and between DENV and HCV.
Figure S2 –DENV C structure and charge distribution.

DENV C is an α-helical homodimer (a) that presents an unusual charge distribution (b) (PDB ID: 1R6R). In (a) a ribbon view of the protein is presented, colored by secondary structure, with α-helices in blue and loops (random-coils) in red. The first 20 residues of DENV C (before the α1 helix) are unstructured\textsuperscript{31,36} and are not shown in this figure. In (b) the protein surface is shown in the same views as in (a), colored according to the electrostatic properties of the amino acids. Hydrophobic residues are colored grey, polar non-charged residues are colored orange, negatively-charged residues are colored yellow and positively-charged residues are colored brown. Relevant for the protein function, the α2-α2’ interface is highly hydrophobic (the grey region in (b); middle) and the α4-α4’ region is mainly positively-charged (brown-colored regions in (b); right), suggesting an interaction with membrane components and with the viral genomic RNA, respectively, through this two regions.\textsuperscript{31}
Supplementary Results

**AFM supplementary results**

Figure S3 shows the force rupture histograms for the interaction between DENV C and oxidized LDL (a) and for control experiment with a non-functionalized AFM tip with LDL (b). The interactions between DENV C and oxidized LDL are of the same order of those observed for LDL (Figure 1b of the article), with 12.7% of (un)binding events for oxidized LDL vs. 12.1% for LDL. In terms of the force values, and having in mind also the results achieved for the adhesion events of the LDL control experiment (Figure S3b), we can conclude that the large majority of the forces measured correspond to values below 35 pN, considered to be unspecific interactions.

![Force histograms for DENV C with oxidized LDL and control experiment for LDL](image)

**Figure S3 – Interaction of DENV C with oxidized LDL and control experiment for LDL.**

The force histogram of the interaction between DENV C and oxidized LDL, in 20 mM Tris-HCl buffer, pH 7.4, with 1 mM EDTA, 1 mM EGTA and 100 mM KCl, is shown in (a). The control force histogram obtained with a non-functionalized tip interacting with LDL in the same buffer is shown in (b). The visible peak of a maximum of approximately 18 pN corresponds to the unspecific interactions between the unmodified tip and LDL, which match in force with the experimental situation showed on Figure 1b of the article (these panels are presented with the same xx and yy scales as those from Figure 1).
**NMR supplementary results**

In Figure S4, the \((^{15}\text{N},^{1}\text{H})\)-HSQC NMR spectra from DENV C protein in the absence (red) and presence (green) of VLDL are shown superimposed and scaled.

![Figure S4 – \((^{15}\text{N},^{1}\text{H})\)-HSQC spectra of DENV C protein in the absence and presence of VLDL.](image)

The DENV C protein \((^{15}\text{N},^{1}\text{H})\)-HSQC spectra (at 500 μM of concentration; pH 7.4) show a clear decrease in all peak intensities of the DENV C protein residues when in the presence of VLDL (green), compared to the condition without VLDL (red), as highlighted by the horizontal and vertical spectral cross sections presented at the chart top and right edges. Moreover, as can be seen either in the whole spectra or in the representative insert, there is no significant chemical shift perturbation (CSP) between spectra in the different conditions.
Additionally to the sequence alignment of the apoE N-terminus (first 220 residues) with perilipin 3 (TIP47) C-terminus (last 220 residues), presented in Figure 6 of the article, we also performed an alignment between the sequences of the apoE N-terminus with perilipin 2 (formerly known as adipose differentiation-related protein, or ADRP) C-terminus (last 238 residues), which is shown in Figure S5. Nevertheless, there are still more similarities between the sequences aligned in Figure 6 than those present in Figure S5. Perilipin 2 and perilipin 3 are very similar. In fact, they are homologous proteins, and perilipin 2 may also contribute (at a lower extent) for the DENV C-LDs binding. In agreement with those previous results for the interaction with LDs, apoE presents a higher similarity with perilipin 3 than with perilipin 2.

**Figure S5 – Sequence similarities between apolipoprotein E N-terminus and perilipin 2 C-terminus.**

The sequences of apoE N-terminus (first 220 residues) and perilipin 2 C-terminus (last 238 residues) show several similar regions that could be involved in similar functions/interactions. Equal residues present in both of the aligned sequences are labeled red, and stereochemically and/or functionally identical residues are labeled black. In the sequence consensus, “h”, “+” and “-” stand respectively for hydrophobic, positively-charged and negatively-charged similar residues in the same aligned position.
Supplementary References


