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## **A serum protein factor mediates maturation and apoB-association of HCV particles in the extracellular milieu**

**Short title:** HCV virion maturation occurs post-secretion

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## Abstract

**BACKGROUND & AIMS:** Hepatitis C virus (HCV) particles detected in sera from infected patients display heterogeneous forms of low-buoyant densities (<1.08), underscoring their lipidation *via* association with apoB-containing lipoproteins, which was proposed to occur during assembly or secretion from infected hepatocytes. However, the mechanisms inducing this association remain poorly-defined and, intriguingly, most HCVcc particles secreted from cultured cells exhibit higher density (>1.08) and are not/poorly apoB-associated.

**METHODS:** We produced HCVcc particles of Jc1 or H77 strains from Huh-7.5 hepatoma cells cultured in 10%-fetal calf serum (FCS) standard conditions vs. in serum-free or human serum (HS) conditions before analyzing their density profiles comparatively to patient-derived virus. We also characterized serum-free medium-produced wild-type and Jc1/H77 HVR1-swapped mutant HCVcc particles incubated with either serum type or with purified lipoproteins.

**RESULTS:** Compared to serum-free or FCS conditions, production with HS redistributed most HCVcc infectious particles to low-density (<1.08) or very-low-density (<1.04) ranges. In addition, short-time incubation with HS was sufficient to shift HCVcc physical particles to low-density fractions, in time- and dose-dependent manners, which increased their specific infectivity, promoted apoB-association and induced neutralization-resistance. Moreover, compared to Jc1, we detected higher levels of H77 HCVcc infectious particles in very-low-density fractions, which could unambiguously be attributed to strain-specific features of HVRI sequence. Finally, all three lipoprotein classes, *i.e.*, very-low-density, low-density and high-density lipoproteins, could synergistically induce low-density shift of HCV particles; yet, this required additional non-lipid serum factor(s) that include albumin.

**CONCLUSIONS:** The association of HCV particles with lipids may occur in the extracellular milieu. The lipidation level depends on serum composition as well as on HVRI-specific properties. These simple culture conditions allow production of infectious HCV particles resembling those of chronically-infected patients.

## Lay summary

- HCV particles may associate to apoB and acquire neutral lipids after cell egress, and hence, low-buoyant density.
- The hypervariable region 1 (HVR1) is a major viral determinant of E2 that controls this process.
- Besides lipoproteins, specific serum factors including albumin promote extracellular maturation of HCV virions.
- HCVcc production *in vitro* with media of defined serum conditions allow production of infectious particles resembling those of chronically-infected patients.

## Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases worldwide. Although recent direct antiviral agents (DAA) can now cure most patients, it remains major challenges in basic, translational and clinical research<sup>1</sup>. As DAAs are only curative, the development of a protective vaccine remains an important goal; yet, this requires deeper knowledge of HCV particles structure. Indeed, HCV virion exhibits unusually heterogeneous particles in morphology, size and properties<sup>2</sup>, as immunocapture of its surface proteins revealed particles of 50-80nm without symmetrical arrangement<sup>3-6</sup>. HCV particles harbor two envelope glycoproteins, E1 and E2, inserted on a membranous envelope that surrounds a nucleocapsid, composed of a core protein multimer and RNA[+] viral genome; yet, the organization of the virion surface remains elusive and there is currently no clear model of HCV particles topology. A remarkable feature of HCV is the particularly low buoyant-density of virions, coined lipo-viro-particles (LVP)<sup>6</sup>. In sera from infected patients and experimentally-infected chimpanzees, most HCV particles are found in densities between 1.00-1.10<sup>6-8</sup>, owing to their particular lipid composition, distinct from other enveloped viruses. Indeed, patient-derived HCV particles contain neutral lipids such as triglycerides or cholesteryl esters and are associated with circulating apolipoproteins such as apoC-I, apoE and apoB<sup>6,7,9,10</sup>; the latter one being the major structural and non-exchangeable component of low-density (LDL) and very-low-density lipoproteins (VLDL). Importantly, injection of HCV particles in experimentally-infected animals revealed that the inoculum of low-density is the most infectious<sup>11</sup>.

A major bottleneck of HCV characterization is the lack of *in vitro*-infectivity of patient-derived particles<sup>1,12</sup>, which prevents unravelling the properties of virus/lipid interaction. To overcome this, several experimental cellular models have been designed, including cell culture-grown HCV (HCVcc) particles that are usually produced from Huh-7.5 hepatoma cells<sup>8,13</sup>. While HCVcc particles have heterogeneous density profile, from 1.00 to >1.17, their specific infectivity in low-density fractions is poor<sup>5,8,14,15</sup>, which reflects incomplete virion lipidation. Indeed, while HCVcc particles are associated with apoC-I<sup>16</sup> and apoE<sup>17</sup>, their association with apoB is variably detected<sup>4,5,12,17-19</sup>.

The lipidation difference between HCVcc and patient-derived particles has been attributed to a defective lipoprotein-metabolism pathway in Huh-7 cells, preventing the formation of fully lipidated VLDLs<sup>12,20-22</sup>. HCVcc particles grown in primary human hepatocytes (PHH), which produce normal VLDLs, have higher specific infectivity owing to the viral RNA peak that coincides with the fractions of highest infectivity at densities of 1.10-1.11<sup>20</sup>, which are above those of patient-derived HCV. Likewise, HCV particles grown in PHH-xenograft mouse models that release normal human apoB and apoE levels display coincident infectivity and viral RNA peaks at 1.06-1.11<sup>8,21</sup>, which, again, are higher than for patient-derived HCV.

While HCVcc studies have tremendously advanced the knowledge of HCV and host-virus interactions, culminating with new DAAs regimens that cure most patients, many aspects of HCV biology remain ill-defined because of lack of models fully mimicking the conformation of authentic HCV particles. Furthermore, as lipoprotein-association is thought to shape their surface and induce neutralization resistance<sup>4,6,7,14,23</sup>, elucidating their structure would improve the design of rational vaccine candidates.

Here, by studying HCVcc production *in vitro* with media of defined serum conditions, we show how HCVcc particles can be fully converted to apoB-containing, low- or very-low-density particles, which augments their specific infectivity and inhibits their neutralization by antibodies. We reveal that besides lipoproteins, specific serum factors are required to allow extracellular maturation of HCV virions, hence indicating that a major determinant that imprints lipidation of particles is serum itself, likely after cell egress.

## **Materials and Methods**

### **Human serum (HS), lipoprotein-deficient serum and lipoproteins.**

The origin and characteristics of normal, consisting in pools of >4 specimen, or patient-derived HS samples are detailed in the Supplementary Material and Methods.

Lipoprotein-deficient serum (LPDS) samples, corresponding to HS fractions of density >1.24, were prepared from HS by a two-step ultracentrifugation procedure (Supplementary Material and Methods) and exhibited over 100-fold reduced apoB levels (Supplementary Figure 1B).

Commercial sources (Supplementary CTAT Table) of purified VLDLs, LDLs, HDLs, apoE and human serum albumin (HSA) (fraction V) were used for reconstitution experiments, at amounts corresponding to that of 10% HS (Supplementary Figure 1C), *i.e.*, 0.038mg/mL cholesterol-VLDL, 0.093mg/mL cholesterol-LDL, 0.047mg/mL cholesterol-HDL, 0.057mg/mL apoE, and 4.5mg/mL HSA, respectively.

### **HCVcc production and characterization.**

Methods for production of HCVcc from electroporated HCV RNAs (see plasmids description in Supplementary Material) and characterization of viral particles were described previously<sup>14</sup>. Electroporated Huh-7.5 cells were grown in serum-free medium (OptiMEM, Invitrogen) only or in media supplemented with 10% fetal calf serum (FCS) or HS. For incubation experiments, HCVcc particles grown in OptiMEM were incubated with OptiMEM vs. with FCS, HS or serum factors at 37°C. Intracellular infectivity was determined as described previously<sup>14</sup> from HCVcc producer cells following four freeze/thaw cycles. Buoyant-density analysis of HCV particles

was performed by iodixanol density-gradient fractionation (Supplementary Materials and Methods). Neutralization assays were performed as described previously<sup>14</sup>.

### **Co-immunoprecipitation assays.**

100µl of HCVcc supernatants or patient' sera were precleared for 1h at 4°C with protein A/G agarose beads and incubated overnight at 4°C with 15µl goat anti-apoB (AB742, Millipore) or appropriate control IgGs under continuous agitation. The immune complexes were incubated for 2h at room-temperature with 40µl of protein A/G agarose beads. The complexes were washed three times in PBS and were re-suspended in Tri-Reagent for RNA extraction or eluted in RIPA buffer for wester-blot analysis and core quantification.

### **Statistical analysis.**

Significance values were determined by applying the non-parametric Friedman test using the GraphPad Prism-6 software. All data are represented as means ± SEM (standard error of the mean). The statistical analyses compare serum vs. no serum condition, unless otherwise indicated in the figure legends. P-values >0.05 were considered statistically not significant (ns) and the following denotations were used: \*\*\*\*, P≤0.0001; \*\*\*, P≤0.001; \*\*, P≤0.01; \*, P≤0.05.

## **Results**

### **Serum composition of culture media dictates HCV particle density.**

HCV particles isolated from HCV-infected patient sera exhibit low buoyant densities, with most viral RNAs detected at densities <1.08 (Figure 1A), reflecting their lipidation state<sup>6-8</sup>. Since HCVcc is typically produced from Huh-7.5 cells grown with 10% FCS and has higher density, we hypothesized that the species origin and composition of serum used in culture media (Supplementary Figure 1A) may influence virion density. Whereas infectious HCV particles produced for 72h in serum-free media appeared poorly lipidated (Figure 1B), with <10% infectivity in fractions below 1.08 (see examples of raw density profiles in Supplementary Figure 2), 38% of the infectivity of HCVcc particles grown in FCS-containing media were shifted to low-density fractions, yet with <8% infectivity detected below 1.04 (Figure 1B). Strikingly, HCVcc produced with 10% HS (from a pool of normal HS) exhibited >80% infectivity at densities <1.08 and ca. 60% at density <1.04 (Figure 1B). Importantly, the density shifts of infectivity correlated with profiles of viral RNA and core in gradients (Figure 1C; Supplementary Figure 2A-C). Indeed, compared to serum-free HCVcc production that raised poor levels of HCV RNAs in low-density fractions (ca. 10% in densities <1.08), the quantities of low-density RNA were increased by ca. 7-fold upon virus production in 10% HS-containing media (Figure

1C). Of note, although most infectious particles shifted to low density, a large part of HCV RNAs could still be detected in densities >1.08 (Figure 1C); they could represent non-infectious particles such as e.g., RNA-containing exosomes<sup>24</sup>. Similar low-density shift of HCV particles was obtained when using different pools of normal HS or shorter production times (data not shown), suggesting that HCV virions become lipidated when produced in the presence of serum, with a major effect of HS.

We extended these results obtained with genotype 2a (Jc1 strain) to H77 strain (of genotype 1a) HCVcc (Figure 2; Supplementary Figure 2D-F). Interestingly, the infectivity of H77 HCVcc grown with serum shifted to lower densities than Jc1 particles. Indeed, compared to Jc1, H77 infectious particles were detected in higher proportions in densities <1.08 [60% (H77) vs. 38% (Jc1)] upon FCS production, and, strikingly, peaked at lower densities: 1.02-1.09 (H77) vs. 1.06-1.09 (Jc1) and 1.00-1.03 (H77) vs. 1.03-1.06 (Jc1) upon FCS and HS production, respectively (Figures 2A vs. 1B). Likewise, compared to serum-free production, the amounts of H77 physical particles produced in serum-containing medium increased by >10-fold in low-density fractions, with higher increases of viral RNAs and core for particles produced in HS than for FCS (Figure 2B; Supplementary Figure 2E-F) and for H77 than for Jc1 particles (Figures 2B vs. 1C). Altogether, these results suggested that HCV-specific sequences influence the lipidation level of viral particles.

### **Lipidation of HCV particles occurs post-egress and enhances specific infectivity.**

The high-density profile of HCVcc particles produced in serum-free conditions suggested that the extracellular milieu imprints virion composition. Accordingly, when we investigated Jc1 and H77 intracellular particles, we found that they had identical density profiles with almost no infectivity in fractions <1.08 whether the virus-producer culture media contained no serum or up to 50% HS (Figures 1D and 2C; Supplementary Figure 3C,F,I). This indicated that acquisition of the low-density profile of HCV occurs at a post-assembly step, i.e., during secretion, as proposed elsewhere<sup>15</sup>, and/or after secretion in the extracellular milieu.

To test the latter possibility, we incubated similar inputs of Jc1 and H77 physical particles ( $5 \times 10^7$  GE) produced in serum-free conditions in media containing 10% HS or FCS vs. no serum for 6h at 37°C (Figure 3; Supplementary Figure 4A,D for raw data examples). We found that this incubation procedure could reproduce the patterns observed in the HCVcc production protocol (Figure 3 vs. Figures 1 and 2). Indeed, Jc1 or H77 viruses incubated with HS shifted >90% of infectivity in densities <1.08 (Figure 3A,B), which correlated with a shift of physical particles in low-density fractions (Figure 3C; Supplementary Figure 4A,B,D,E ). Again, more marked shifts of both infectious and physical particles were detected for incubation in HS- rather FCS-containing media, implying the presence of species-specific lipidation factor(s). Likewise, a stronger shift was observed for H77 compared to Jc1 particles (Figure 3A-C), with

50% vs. 20% of infectivity in densities <1.04, respectively. Note that similar differences were obtained when using smaller Jc1 inputs to normalize Jc1 vs. H77 infectivity differences (ca. 6-fold) or when using HS from different individuals (Supplementary Figure 5). Finally, we found that HS-incubation of HCV particles produced from PHH could also induce low-density shifts of both infectious and physical particles (Supplementary Figure 6), suggesting that maturation of HCV may occur after egress from infected hepatocytes.

Importantly, purified intracellular particles incubated with serum-containing media readily shifted to low-density fractions, again with more marked shifts for HS rather than FCS and for H77 virus rather than Jc1 (Figure 4A,B; Supplementary Figure 4C,F), suggesting that HCV particles are initially produced as no or poorly lipidated forms that are progressively matured during secretion<sup>15</sup> or, as indicated by our results, at a post-egress step. To corroborate these results, we incubated extracellular or intracellular viral particles purified from fractions of densities of 1.08-1.14 (Figure 5A,B) with HS-containing vs. serum-free media before subjecting them to a second density-gradient analysis. We found that incubation with HS readily shifted infectious and physical particles to low-density fractions (Figure 5C,D).

Altogether, these results indicated that incubation of poorly lipidated viral particles change virion structure and/or composition, inducing low-density shift and suggesting lipid acquisition after virion egress.

While displacement of HCV particles to low-density fractions upon HS-incubation resulted in strongly reduced infectivity in fractions of density 1.08-1.14 (Figures 3-5), the levels of viral RNAs and core in these fractions remained abundant (Figure 3C; Figure 5C,D; Supplementary Figure 4A,D), reflecting that most viral RNAs and/or particles in these densities are not infectious. In contrast, the comparison of the shifts of infectious vs. physical particles in low-density fractions suggested that HS-incubation of Jc1 and H77 viruses increased more readily the infectivity than the viral RNA quantities (Figure 3B vs. 3C). Accordingly, the ratio of infectivity to viral RNAs calculated in each fraction steadily increased once viruses acquired lower densities (Supplementary Figure 7), with respectively 14-fold and 25-fold mean augmentations for HS-incubated Jc1 and H77 particles compared to serum-free incubation in fractions below <1.04 (Figure 3D), and likewise for fractions of densities 1.04-1.08. This suggested that lipidation modifies the specific infectivity of HCV particles upon their shift to low-densities and render the particles more infectious.

### **Time and serum dose-dependent HCV virions lipidation induce apoB-association and neutralization escape.**

The higher infectivity detected in low-density fractions for viral particles produced for 72h with HS-containing media compared to viruses incubated for 6h with HS (Figure 3 vs. Figures 1



and 2) suggested that virion lipidation is a time-dependent process. Upon incubation with HS for different times, we found that Jc1 and H77 particles (Figure 6A,B; Supplementary Figure 8 for examples of raw data of infectivity, core and RNA) progressively shifted to densities of 1.04-1.08, reaching a transient maximum at ca. 4-6h incubation, after which the infectious particles in these densities decreased and steadily accumulated in the very-low-density fractions (<1.04). This suggested that HCV lipidation is a continuous process that may progressively occur or proceed after secretion from producer cells.

Next, we determined whether serum concentration influences the lipidation levels of HCVcc. As shown in Figure 6C-E (Supplementary Figure 9 for raw data), incubation of HCVcc particles with increasing HS doses gradually shifted infectivity and physical particles, reaching nearly 100% infectivity in low-density fractions (<1.08) with 50% HS. These results suggested that lipid acquisition depends on serum concentration and underscored a limiting serum factor(s) responsible for lipidation of HCV particles at a post-egress step.

Since HS-incubated HCVcc particles reached densities close to those of HCV-infected patients (Figure 1A), we wondered if the former viruses were associated to apoB, as observed *in vivo*<sup>6,7,9,10</sup>. When we quantified viral components after co-immuno-precipitation assays with apoB antibodies, we found a strong enrichment of viral RNAs (Figure 6F) or core (Figure 6G) for Jc1 and H77 particles incubated with HS. Note that the levels of co-immuno-precipitated HCVcc RNAs were comparable to those of patient-derived virus (Figure 6F). Conversely, E2 immuno-precipitation of HS-incubated HCV particles, via a FLAG tag inserted at E2 amino-terminus (Jc1 FLAG-E2 virus<sup>5,25</sup>), allowed apoB co-immuno-precipitation (Supplementary Figure 10. Altogether, this indicated that the low-density profile of HCVcc incubated with HS reflects their association with apoB, suggesting that upon HS-incubation, virus particles interact with apoB-containing lipoproteins in a manner similar to patient-derived HCV.

Finally, we found that while Jc1 and H77 particles produced in serum-free conditions were readily inhibited by the 3/11<sup>26</sup> and AR3A<sup>27</sup> E2 neutralizing antibodies, with IC<sub>70</sub> of ca. 2 µg/ml, they were strongly resistant to neutralization after HS treatment (Figure 6H). Specifically, such antibodies neutralized less than 40-70% of HCVcc particles, even for antibody concentrations higher than 20 µg/ml. This indicated that acquisition of low-density shift correlates with HCV neutralization escape.

### **HVRI sequence of HCV E2 glycoprotein determines lipidation of viral particles.**

Since Jc1 and H77 viruses displayed different lipidation levels and rates (Figure 6A,B), we sought to investigate which viral determinant could influence virion lipidation. We found that swapping the H77 HVRI sequence in Jc1 virus (Figure 7A) induced stronger low-density shift of the resulting Jc1\_HVR1\_H77 recombinant particles compared to parental Jc1 virus, with an increase of over 5-6 fold of both infectious [Figure 7B,C (left panels); Supplementary Figure

11B] and physical [Figure 7D (left panel); Supplementary Figure 11C] particles detected in densities <1.04 upon HS-incubation. Interestingly, the low-density shift of this recombinant virus was higher than that of parental H77 virus itself, with >2-fold more infectious particles detected in such fractions [80% vs. 40%; Figure 7B,C (left vs. right panels); Supplementary Figure 11B vs. 11E], correlating with the stronger shift of physical particles [6-7 fold vs. 2.5-fold; Figure 7D (left vs. right panel); Supplementary Figure 11C,F]. Conversely, compared to parental H77 virus, swapping the Jc1 HVRI sequence decreased the low-density shift of the resulting H77\_HVR1\_Jc1 recombinant particles upon HS-incubation, as shown by reduction of ca. 40-fold of the percentage of infectious particles detected in fractions of densities <1.04 [Figure 7B,C (right panels); Supplementary Figure 11E]. Note that similar inputs of mutant and parental physical particles were used in these assays (Supplementary Figure 11A). Altogether, these results suggested that HVRI is a key viral determinant regulating the lipidation level of viral particles in an HCV strain-dependent manner.

### **HSA is a serum factor that promotes HCV particle lipidation by apoB-containing lipoproteins.**

That HS more efficiently shifted density of viral particles compared to FCS (Figures 1-4) suggested that either serum type differs in HCV-specific lipidation factor(s). Indeed, when we quantified the lipoproteins composition of sera used in our experiments, we found that compared to HS, FCS displayed 6-7 folds lower amounts of both HDLs and LDLs, and no VLDLs (Supplementary Figure 1A). While this difference may partially explain a better maturation of viral particles in HS, as suggested in Figure 6C-E, we sought to characterize serum factors, including lipoproteins, that modulate HCV lipidation.

To determine which lipoprotein class induces lipidation of viral particles, we depleted lipoproteins from HS to generate a LPDS with >100-fold decreased apoB levels despite normal HSA levels (Supplementary Figure 1B), indicating effective lipoproteins removal. Next, we incubated Jc1 HCVcc produced in serum-free conditions with LPDS vs. with different lipoprotein types, *i.e.*, VLDL, LDL or HDL, before density-gradient analyses (Figure 8A). We found that incubation with LPDS only did not grossly change the lipidation profile of viral particles (Figure 8B,C), suggesting that serum lipoproteins are likely the source of virion lipidation. Likewise, incubation of HCVcc with purified VLDLs, LDLs or HDLs at physiological concentrations did not significantly change their lipidation profiles, as shown by peaks of infectivity that remained in the 1.10-1.11 range (Figure 8B), underscoring a serum protein component required for virus lipidation by either lipoprotein type. Accordingly, we found that HCVcc incubation with LPDS added to lipoproteins shifted viral particles to low-densities, with 55-70% of infectivity detected below 1.08 (Figure 8B,C). Furthermore, incubation of HCVcc particles with LPDS and combinations of HDL and VLDL or LDL induced full low-density shifts,

similar to that detected with HS-incubation (Figure 8B-D). Of note, similar results were obtained with H77 HCVcc particles (data not shown).

Finally, we sought to unravel serum factors promoting HCV lipidation. We found that the blocking of serum lipoprotein-modification factors such as cholesteryl ester transfer protein (CETP) and lecithin-cholesterol acyltransferase (LCAT) did not change HS-induced virion shift to low-density (Supplementary Figure 12), thus ruling out their involvement in HCV lipidation. Likewise, apoE did not allow lipidation by either of the three lipoproteins type, alone or in combination (Supplementary Figure 13), indicating that ApoE is likely not the factor of LPDS that stimulates extracellular virion lipidation. Interestingly, we found that purified HSA, a protein abundant in HS and LPDS (Supplementary Figure 1B), added to VLDL or LDL induced HCV low-density shift, though to lower extents than LPDS (Figure 8B,C). Of note, in contrast to LPDS, HSA (Figure 8B,C) did not allow virion lipidation by HDL.

Altogether, these results suggested that lipoproteins are the main source of HCV lipidation, which requires additional serum component(s) that include HSA.

## Discussion

*In vivo*, HCV particles are associated to apoB, the non-exchangeable and major structural component of VLDLs and LDLs. This may explain the particularly low buoyant-density of patient-derived HCV LVPs, below 1.08 (Figure 1A), which is significantly lower than other enveloped viruses<sup>2</sup>. Indeed, the lipidomics of purified HCVcc particles confirmed that their lipid content is related to that of serum lipoproteins<sup>5</sup>. Several studies proposed that HCV production from hepatocytes depends on cellular factors controlling VLDL-biosynthesis, suggesting that virion interaction with serum lipoproteins begins at the steps of viral assembly and secretion processes during which immature particles are converted to mature virions by lipidation and incorporation of some apolipoproteins<sup>4-10,12,14-19</sup>. Yet, while there is ample evidence that the exchangeable apolipoprotein apoE is necessary to promote envelopment, lipidation and/or release of viral particles, some studies suggested that apoB may not be required for these initial steps (reviewed in<sup>2</sup>). This is intriguing because apoB is essential for VLDL-biogenesis<sup>28</sup> and because patient-derived HCV is readily immuno-precipitated with apoB antibodies<sup>7,9</sup> (Figure 6F). One orthodox explanation is that Huh-7.5 hepatoma cells that efficiently support HCVcc production<sup>8,13</sup> lack many properties of hepatocytes, such as ability to produce normal VLDLs<sup>12,20-22</sup>. That Huh-7.5 cells produce under-lipidated, VLDL-like particles would thus explain why the HCVcc particles they produce have higher buoyant-density and lower specific infectivity than HCV produced *in vivo*<sup>6-8</sup> or from PHH<sup>8,20,21</sup>. However, when HepG2 hepatoma

cells, thought to represent more mature hepatocytes than Huh-7 cells, are induced to produce normal, apoB-containing VLDLs, they yield infectious particles that are biophysically and biochemically similar to those produced from Huh-7.5 cells<sup>29</sup>.

Since this and other evidence<sup>2</sup> showed that there is no clear correlation between the ability of HCV-infected cells to secrete apoB or VLDLs and their capacity to produce LVPs, we thought to investigate whether LVP formation could derive from a post-secretion interaction between extracellular HCV particles and VLDLs or other lipoproteins. Our results indicate that while Huh-7.5 cells grown in serum-free medium produce immature HCV particles of density >1.08, their maturation to fully-lipidated, apoB-containing infectious virions of low-density is rapidly induced once they encounter in the extracellular milieu appropriate serum conditions with physiologic concentrations of lipoproteins. Importantly, our results do not disrepute results of others<sup>12,15,19</sup> proposing that, *in vivo* as well as in metabolically-adapted hepatoma cells upon prolonged (*ca.* 21 days) cultures in HS, HCV maturation may occur during transit through the cell secretory pathway, where virions could associate or fuse with apoB-containing lipoproteins precursors and/or luminal, apoE-containing lipid droplets<sup>28</sup>. Yet, our report shows that an alternative factor of lipid-imprinting of HCV is the immediate environment of the extracellular milieu and/or serum characteristics.

Our data suggest that HCV lipidation requires interaction(s) with defined serum lipoproteins but also involves additional, presumably non-lipid serum factor(s) such as HSA (Figure 8). We found that all principal lipoprotein classes, *i.e.*, VLDL, LDL and HDL, induce low-density shift of HCVcc particles *in vitro*, upon short-time incubation in the presence of LPDS, with a synergistic effect when either VLDL or LDL was combined with HDL. These results are consistent with a stronger lipidation induced by HS in contrast to FCS that contains >6-fold lower concentrations of either lipoprotein type (Supplementary Figure 1A). This questions the nature of interactions between lipoproteins and viral particles, and their outcome in terms of virion morphology. As HCV LVPs incorporate different lipoprotein components, they may indeed be formed by transfer of lipids, through spontaneous release/exchange or *via* fusion (“single-particle” model), or, alternatively, by virion association with lipoproteins (“two-particle” model)<sup>2</sup>.

Although direct evidence by electron microscopy is missing in favor of the latter model<sup>3-6</sup>, perhaps owing to its transient or unstable state, our results argue for it since lipidation of cell-free viral particles upon short-time HS-treatment induced their association with apoB, a non-exchangeable apolipoprotein (Figure 6F,G; Supplementary Figure 10). Other arguments for this model are that *in vivo*, HCV particles associate with apoB48-containing chylomicrons<sup>10</sup>, although HCV is not produced by enterocytes, and that they exhibit rapid though transient postprandial shifts in buoyant-density upon lipid-rich diet in HCV-infected patients<sup>30</sup>. Yet, these

facts do not exclude the nonexclusive possibility that viral particles may mimic lipoproteins and thus, exchange or uptake lipids<sup>31</sup>. Indeed, previous reports indicated that apolipoproteins can be exchanged or transferred from cells, lipoproteins or serum itself to infectious particles<sup>16,32,33</sup>. Thus, as LVPs contain specific apolipoproteins subsets<sup>4-6</sup>, this may regulate exchange and/or transfer of lipids with/from lipoproteins, which could modify their inner neutral lipid content, as suggested by the lipidation state and infectivity of viral particles that are altered upon treatment with lipoprotein lipase and/or hepatic triglyceride lipase<sup>34</sup>. Hence, our HS-incubation kinetics and dose-dependence assays (Figure 6), suggesting that lipidation of infectious particles is progressive, also argue for the “single-particle” model of HCV virion, sharing with lipoprotein particle(s) its envelope that is continuously remodeled upon interactions with lipoproteins. Since HCV lipidation is induced by different lipoprotein types (Figure 8), we surmise that the mode of lipoprotein association or lipidation varies accordingly, which would be consistent with coexistence of both HCV morphologic models.

Importantly, we reveal that HCV lipidation by lipoproteins requires additional serum factor(s), since incubation with lipoproteins could induce low-density shift of HCV virions only in presence of LPDS. We were unable to block HS-induced lipidation of viral particles by inhibitors of CETP and LCAT that promote lipid transfer to lipoproteins and maturation (Supplementary Figure 12), hence discarding these candidate factors. Likewise, apoE could not mediate low-density shift by lipoproteins (Supplementary Figure 13). Yet, we found that HSA, added to VLDL or LDL (though not HDL), induces HCV partial lipidation. This indicates that HSA is a serum co-factor mediating LVP formation, perhaps by bridging apoB-containing lipoproteins and virions<sup>35</sup> or by favoring lipid efflux<sup>36</sup>. Of note, additional serum factors, present in LPDS (Figure 8), seem required to induce full virion lipidation.

Our results reveal that the HVRI domain of the E2 glycoprotein accounts for the differential lipidation sensitivity of Jc1 vs. H77 viruses, consistent with its previously proposed roles in HCV lipidation process<sup>14,25,37,38</sup> and recruitment and/or conformation of apolipoproteins such as apoC1<sup>16</sup> and apoE<sup>25</sup> on virions. While HVR1-deletion shifts HCV particles to densities of 1.10-1.16<sup>14,37,39</sup> corresponding to those of the non-lipidated particles of our study, it is interesting that simple HVR1-swaps between H77 and Jc1 HCVcc reconstituted the differential density profiles of either parental virus. This suggests that HVR1 organizes the HCV lipidation process in a strain-specific manner, which raises different and fascinating scenarios as for reasons why HCV is lipidated *in vivo*. First, in agreement with previous reports suggesting that lipidated viruses exhibit strain-dependent usage of different lipoprotein receptors as entry factors<sup>38</sup>, including SR-BI<sup>14,21,38,39</sup>, LDLr<sup>38</sup> and VLDLr<sup>40,41</sup>, HVR1 plasticity and evolution may dictate the set of receptors used during entry steps by driving both lipidation and apolipoprotein display. Second, as shown by neutralization resistance induced by low-density shift of HS-

treated, HCV particles (Figure 6H), lipidation may contribute to shielding cross-neutralizing epitopes<sup>4,6,7,14,23</sup>, perhaps in concert with the alternative role of HVR1 in concealing CD81-binding site<sup>14</sup> and accessibility to antibodies that target virus-CD81 interaction<sup>39,42</sup>. Accordingly, it is possible that lipidation allows viral particles to access, following a first interaction with lipoprotein receptors, to a protected environment where they can subsequently and safely interact with CD81 in a putative neutralizing antibody-free sanctuary and where further steps of cell penetration may proceed. A third scenario, consistent with the latter, is that usage of lipoprotein receptors increases HCV specific infectivity. Indeed, lipidation of viral particles, which shift them to low/very-low densities, raised their specific infectivity (Figure 3D). This could provide a positive selection for eliciting HVR1 variants that drive full lipidation. The varying specific infectivity according to virion density could thus reflect the differential efficiency of cell entry pathways depending on the lipidation status of the particles, which would influence binding and usage of specific lipoprotein receptors. Finally, it is also possible that lipidation increases the stability and/or infectivity of particles by protecting them from degradation<sup>12</sup>.

In conclusion, our results suggest that HCV particles acquire neutral lipids from the extracellular milieu, which requires both lipoproteins and serum protein factors as well as HVR1 determinants. Overall, these findings allow developing simple and useful culture conditions to produce infectious HCV particles that resemble those retrieved from chronically-infected patients, which will facilitate structural and functional investigations but also the rationale design of a much-needed vaccine.

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Author names in bold designate shared co-first authorship



## Figures legends

### **Figure 1: Serum conditions dictate the density of extracellular HCV particles.**

Density-gradient analysis of viral particles from HCV-infected patients (**A**) displaying percentages of HCV RNAs in each fraction (left panels) or after grouping in 3 categories of density (right panels). The HCV genotypes (Gt) and viral loads (log[copies]/mL) in patient-sera are indicated. Density-gradient analysis of extracellular (**B, C**) or intracellular (**D**) Jc1 HCVcc viral particles produced for 72h without serum vs. with FCS or HS, as indicated. The results display the mean values of infectivity or HCV RNAs in each fraction from different experiments (left panels) (see representative examples of individual experiments in Supplementary Figure 2A-C). The values from different gradients regrouped in 3 categories of density (right panels) display the mean percentages of infectivity or the fold-increase of percentages of HCV RNAs relative to serum-free conditions (see the mean percentages of infectivity or HCV RNAs in each fraction from different experiments in Supplementary Figure 3A-C).

### **Figure 2: Lipidation of HCV particles is HCV genotype-independent.**

Density-gradient analysis of extracellular (**A, B**) and intracellular (**C**) H77 HCVcc particles produced for 72h without serum vs. with FCS or HS, as indicated. The results display the mean values of infectivity or HCV RNAs in each fraction from different experiments (left panels) (see representative examples of individual experiments in Supplementary Figure 2D-F). The values from different gradients regrouped in 3 categories of density (right panels) display the mean percentages of infectivity or the fold-increase of percentages of HCV RNAs relative to serum-free conditions (see the mean percentages of infectivity or HCV RNAs in each fraction from different experiments in Supplementary Figure 3G-I).

### **Figure 3: Lipidation of HCV particles occurs in the extracellular medium and increases their specific infectivity.**

Density-gradient analysis of serum-free produced Jc1 (red) or H77 (blue) extracellular HCVcc particles ( $5 \times 10^7$  GE) incubated for 6h at 37°C with or without serum. The results display the mean values of infectivity (A) or the analysis by categories for infectivity (B), viral RNAs (C), and specific infectivity (D). See representative examples of individual experiments and of the mean percentages of infectivity in each fraction from different experiments in Supplementary Figure 4A,B,D,E.

### **Figure 4: Lipidation of intracellular HCV particles.**

Density-gradient analysis of serum-free produced Jc1 (red) or H77 (blue) intracellular HCVcc particles incubated for 6h with or without serum. The results display the mean values of

infectivity (A) or the analysis by categories for infectivity (B). See the mean percentages of infectivity in each fraction from different experiments in Supplementary Figure 4C,E.

**Figure 5: High-density HCV particles shift to low-densities upon lipidation.**

Serum-free produced Jc1 extracellular (left panels) or intracellular (right panels) HCVcc particles were layered on a first density-gradient (A, B). Fractions of densities 1.08-1.14, containing ca. 80% of infectivity, were pooled and incubated for 6h with or without serum before being layered on secondary density-gradients and performing infectivity analyses, which are displayed by categories of density (C, D) for infectivity, HCV RNAs, and core, as indicated. nd, not detectable.

**Figure 6: Lipidation of secreted HCV particles is time- and dose-dependent, and induces apoB-association.**

(A, B) Serum-free produced Jc1 (red) and H77 (blue) extracellular HCVcc particles were incubated for different times with 10% HS. The results display representative examples (A) or analysis by categories of densities (B) for infectivity. The results of HCVcc production for 48h and 72h in HS-containing medium are included here for sake of comparison (dotted lines). See representative examples of individual experiments in Supplementary Figure 8. (C-E) Serum-free produced Jc1 extracellular HCVcc particles were incubated with different HS concentrations. The results display representative examples (C) or analysis by categories of densities for infectivity (D) and viral RNAs (E). See representative examples of individual experiments in Supplementary Figure 9. Co-immunoprecipitation analysis of viral particles from sera of HCV-infected patients and from serum-free produced Jc1 or H77 HCVcc particles incubated for 6h with or without human serum (F, G). The results show the levels of RNAs (F) or core protein (G) co-precipitated with apoB relative to control antibodies. Serum-free produced Jc1 (red) or H77 (blue) extracellular HCVcc particles were incubated with HS (dotted lines) or without HS (plain lines) for 6h and were mixed with different concentrations of AR3A (top panel) or 3/11 (bottom panel) neutralizing monoclonal antibodies (H). The results show the mean percentages of neutralization relative to absence of antibody.

**Figure 7: An HVR1 determinant of HCV E2 regulates lipidation levels of HCV particles.**

Schematic representation of recombinant genomes generated by swapping HVRI domains between Jc1 and H77 HCVcc sequences (A). Density-gradient analysis of serum-free produced Jc1 vs. Jc1\_HVRI\_H77 (left panels) or H77 vs. H77\_HVRI\_Jc1 (right panels) extracellular HCVcc particles ( $5 \times 10^7$  GE) incubated for 6h with or without serum (B-D). The results display representative examples (B) or analysis by categories for infectivity (C) and

viral RNAs (**D**). See representative examples of individual experiments in Supplementary Figure 11.

**Figure 8: Lipoproteins and serum protein(s) are required for lipidation of HCV particles.** Density-gradient analysis of serum-free produced Jc1 extracellular HCVcc particles incubated for 6h without serum vs. with the indicated serum factors at concentrations equivalent to 10% HS. Illustration of the method used to analyze and represent the data (**A**). For each condition, the boxes represent the densities for which infectivity values were above 15% of total infectivity (**B**). The black diamonds in each box represent the density of the peak infectivity. The infectivity values from different gradients are regrouped by categories of density (**C, D**).

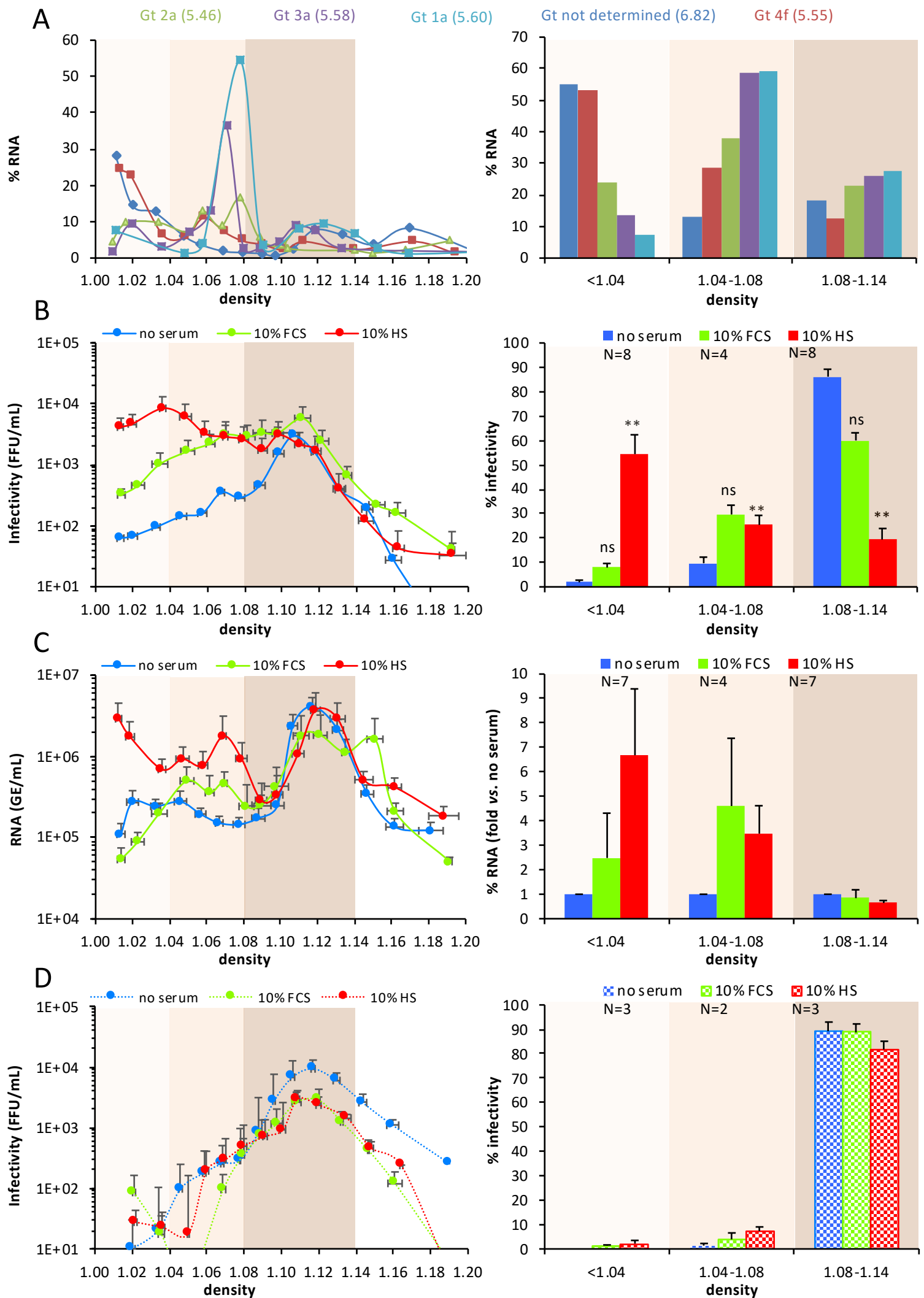


Figure 1

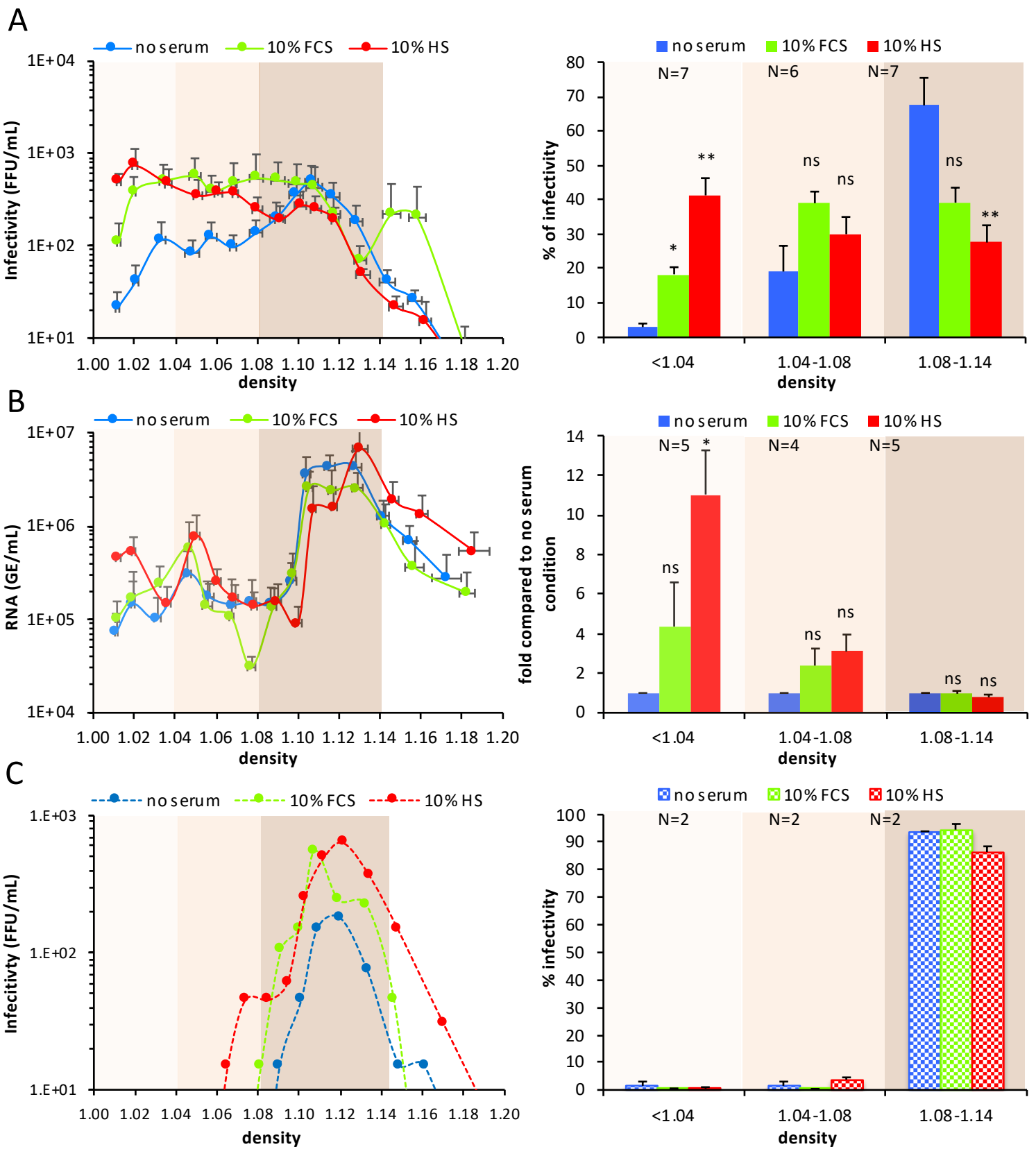


Figure 2

### JC1 virus

### H77 virus

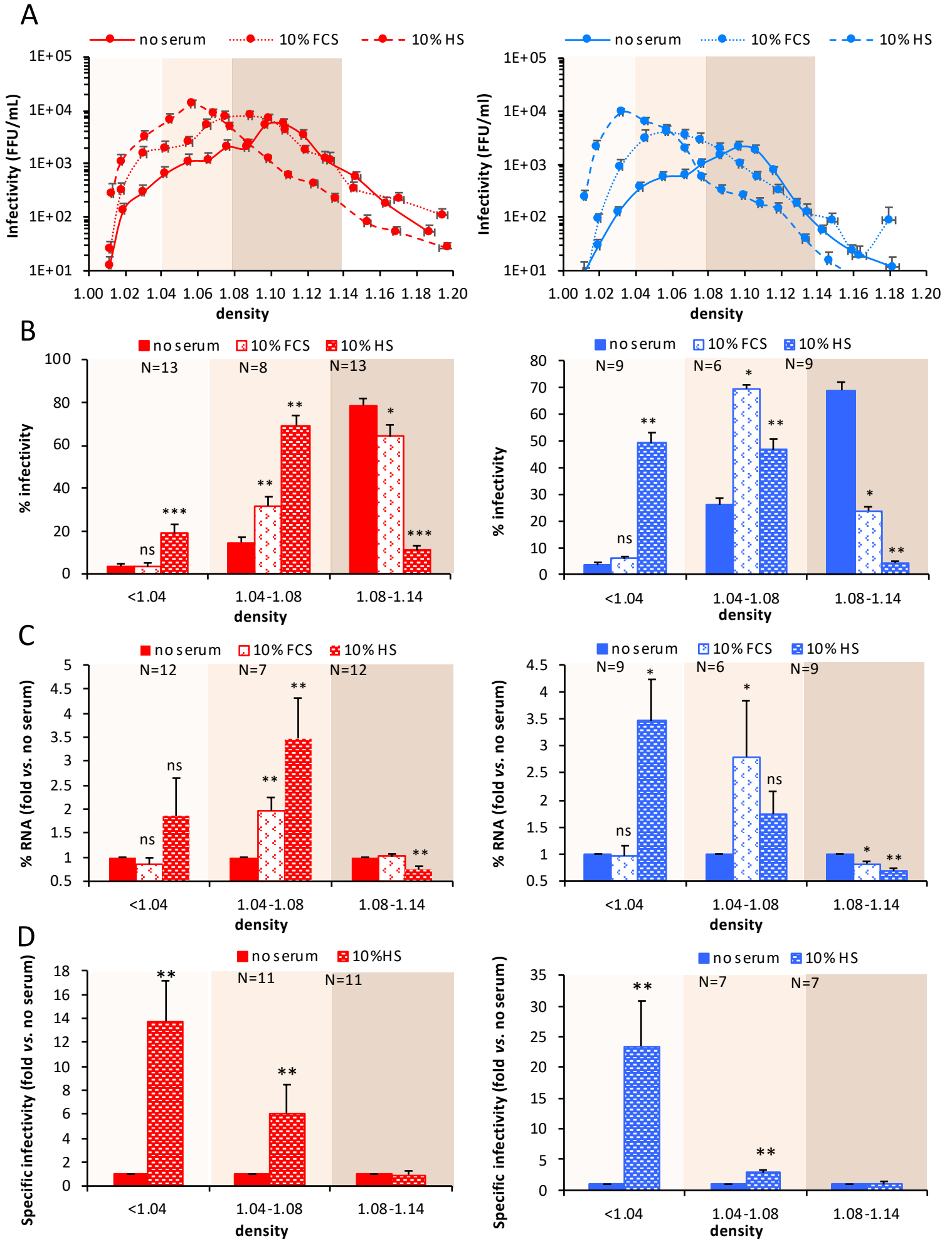


Figure 3

### JC1 intracellular virus

### H77 intracellular virus

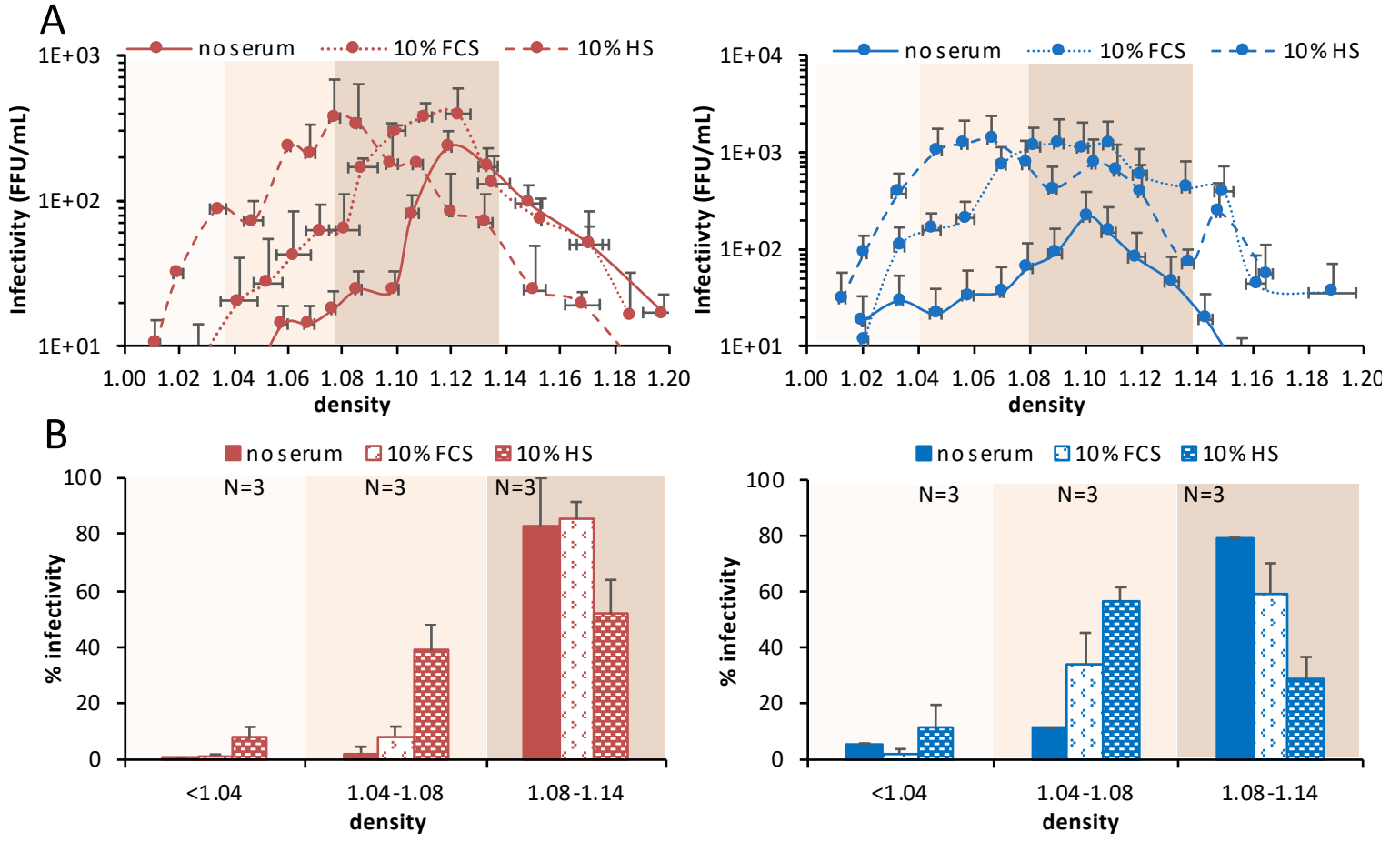
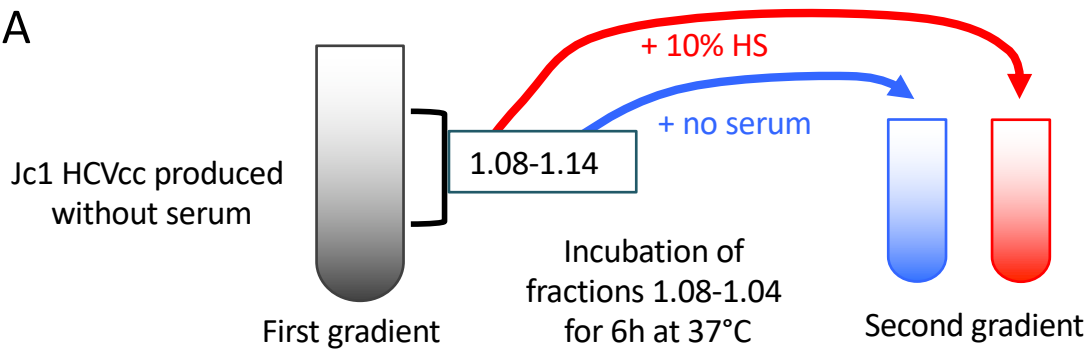
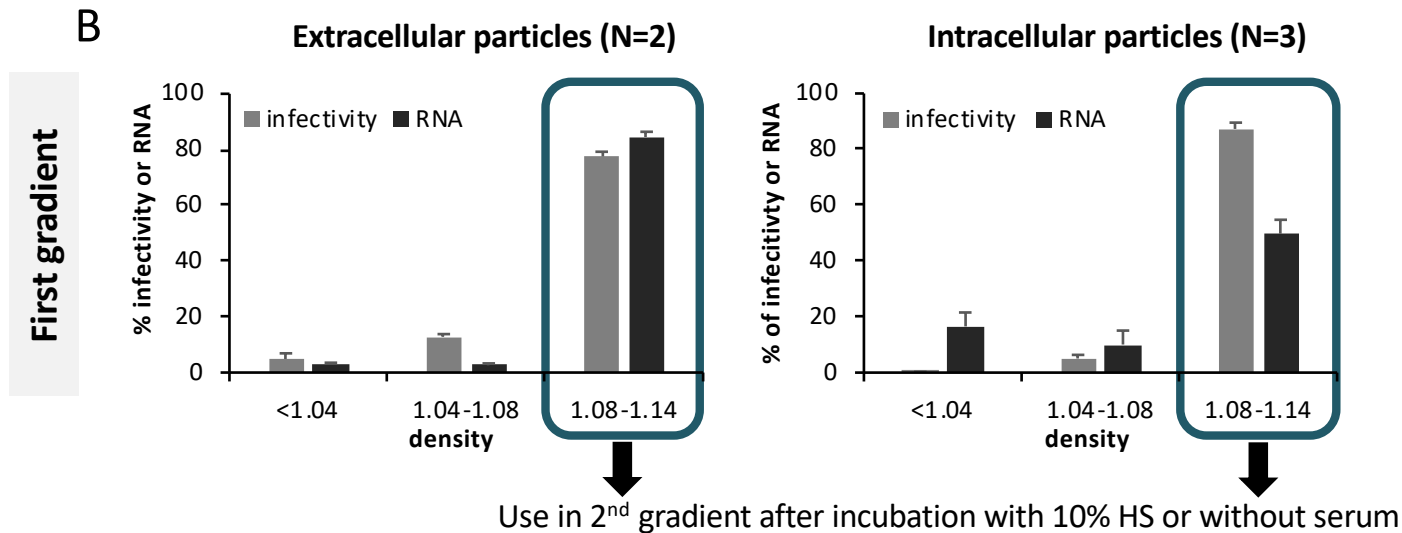


Figure 4

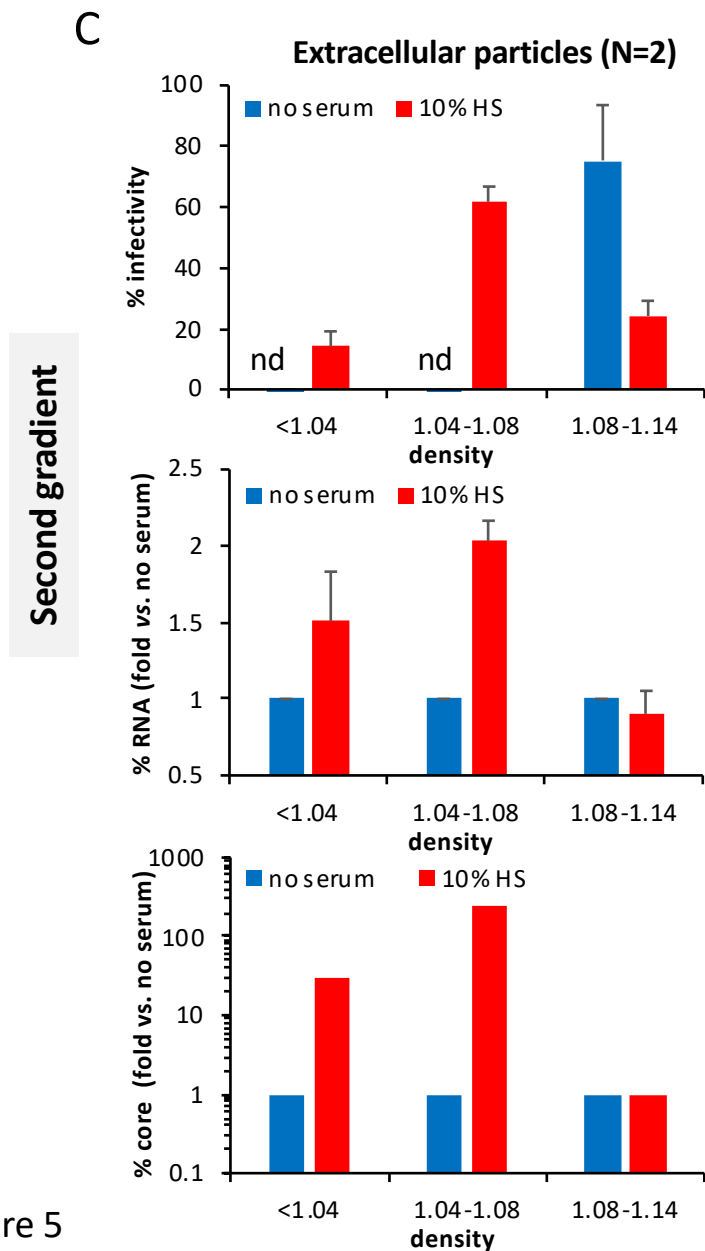
**A**



**B**



**C**



**D**

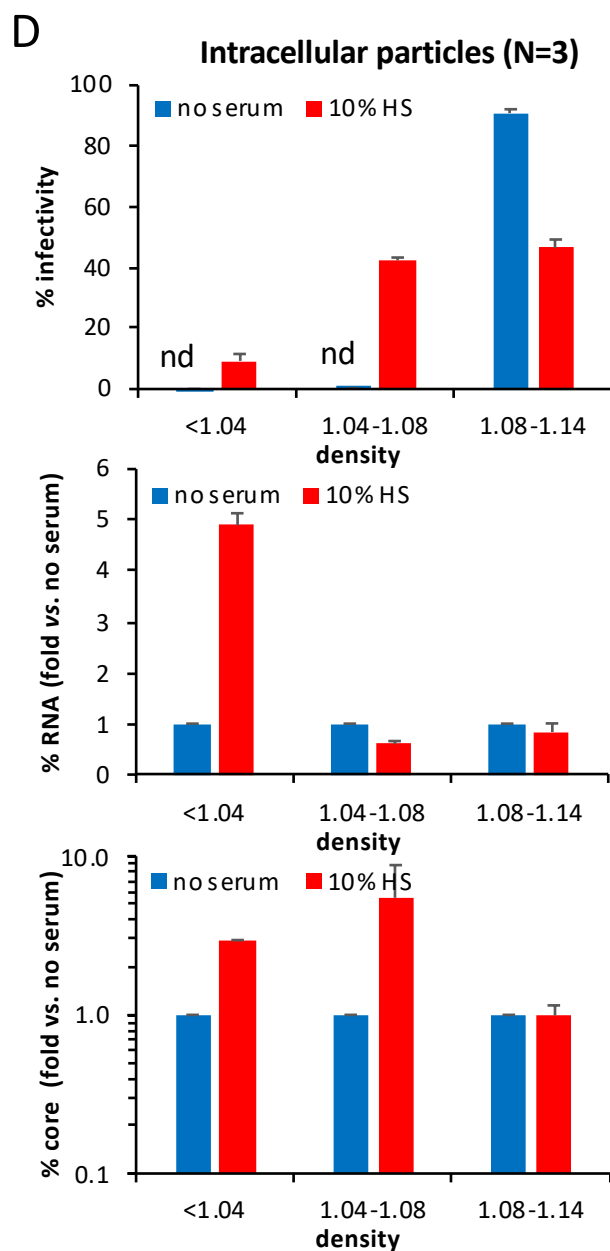


Figure 5



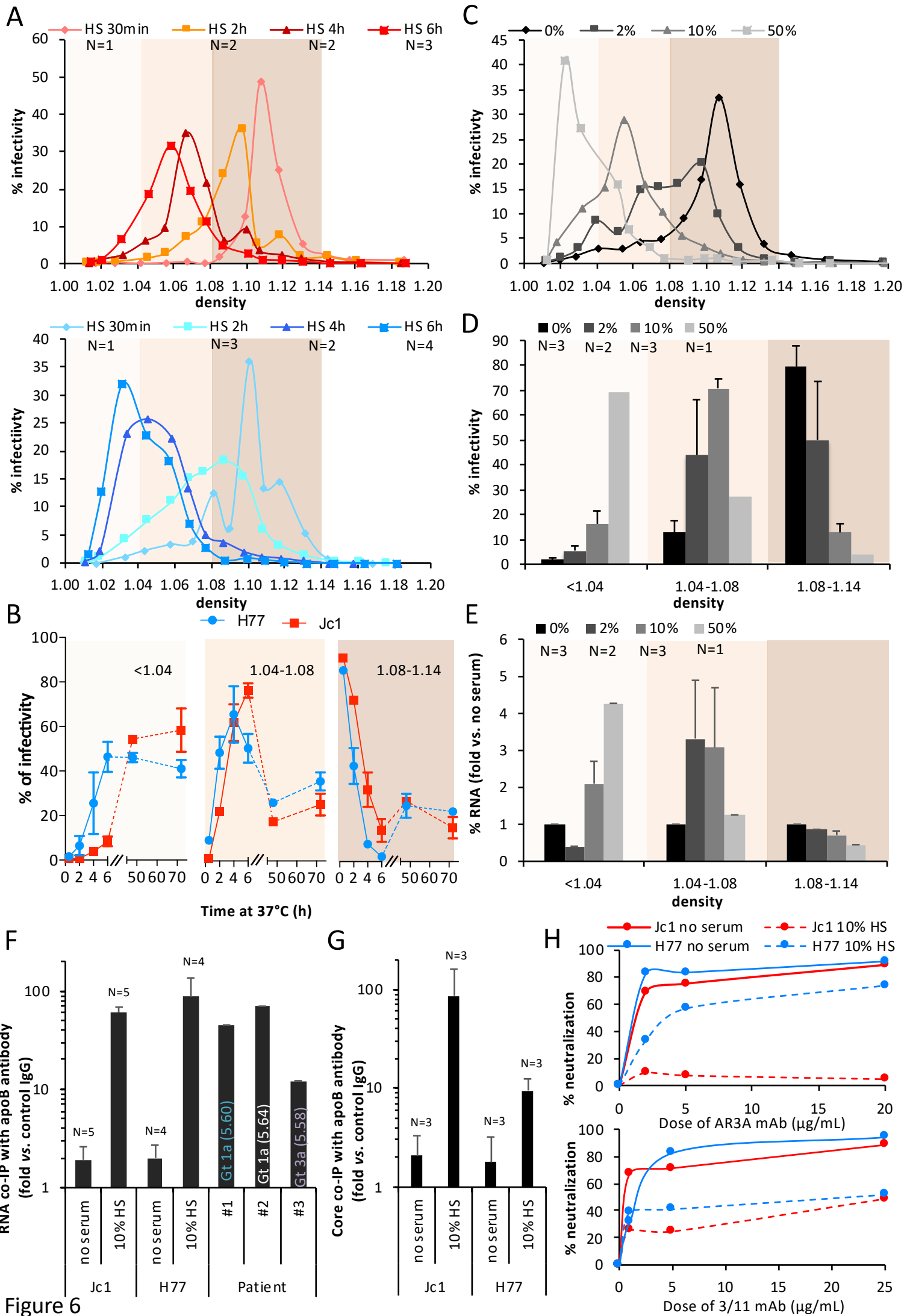
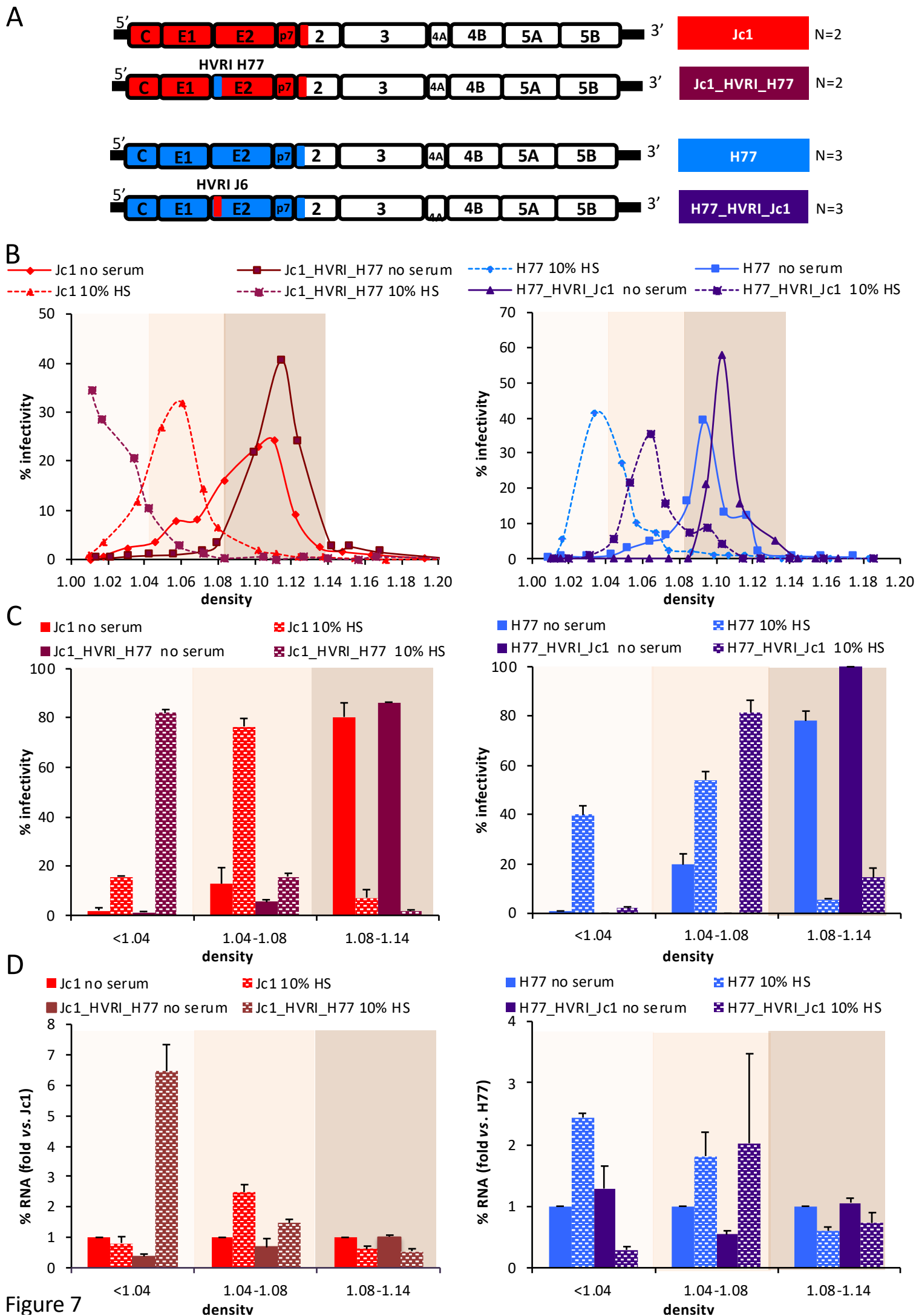


Figure 6



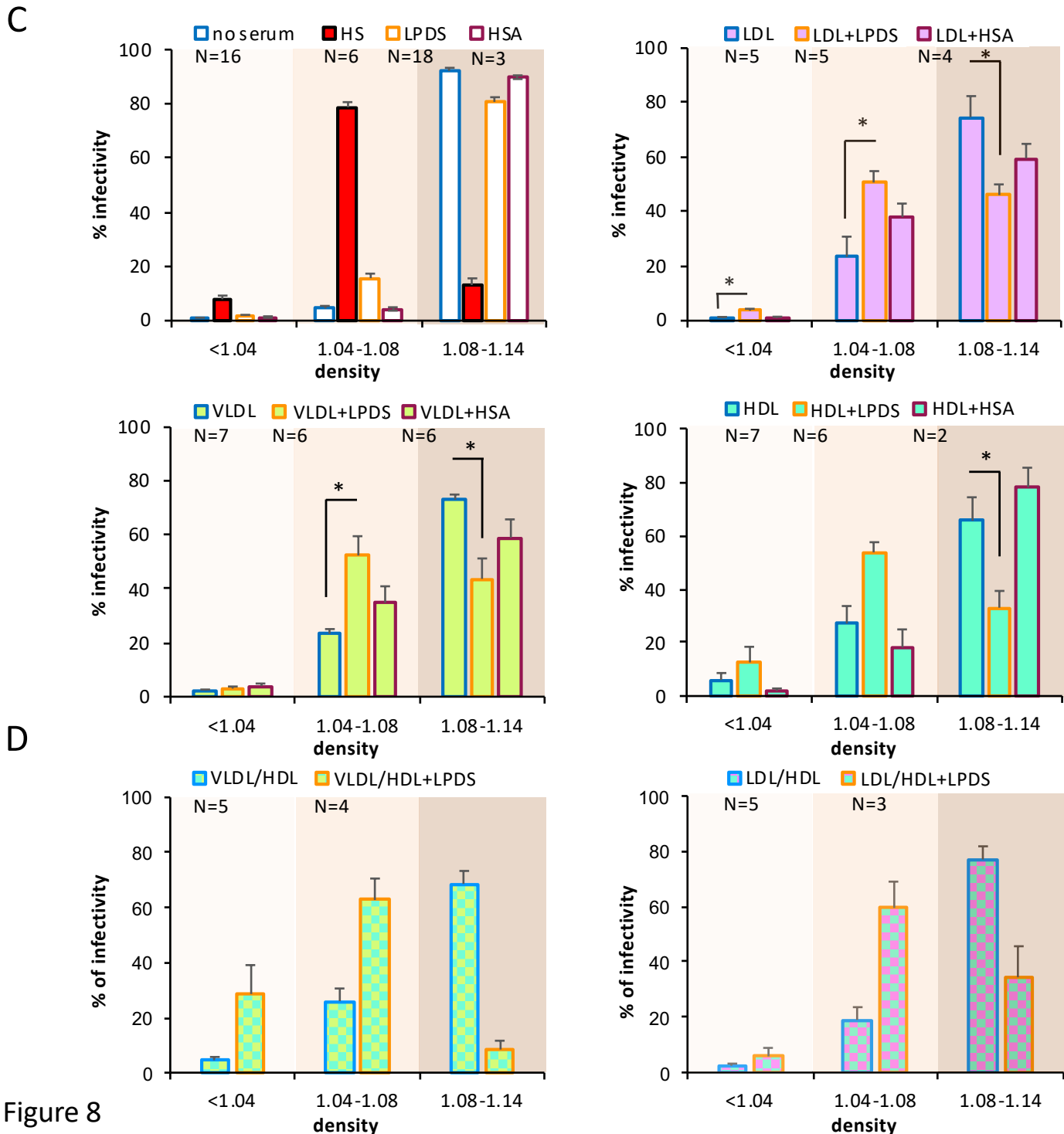
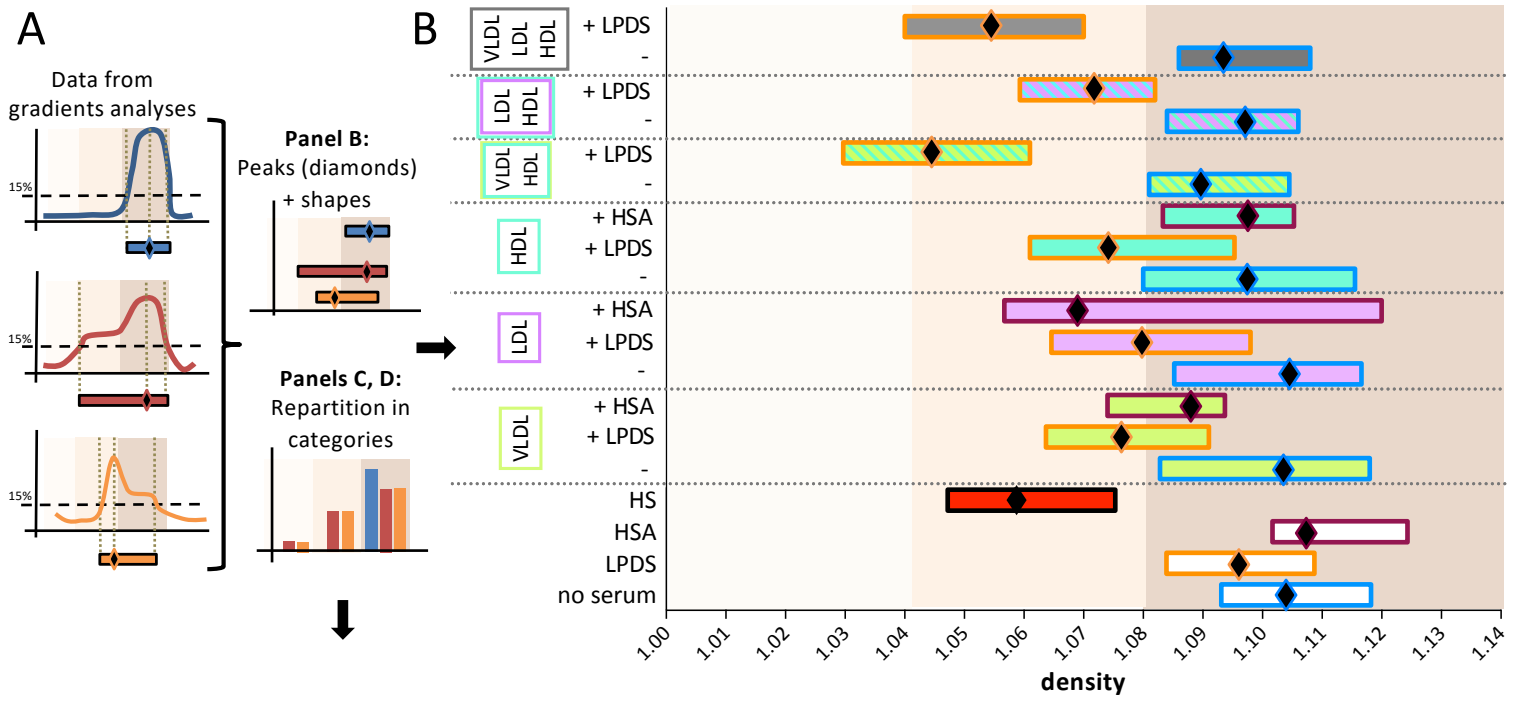


Figure 8

