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PhD THESIS SUMMARY

Production and characterization of novel E2 HCV envelope antigens for vaccine development

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Aim of the study

HCV infection still represents a global health problem. The World Health Organization (WHO) estimates the prevalence of chronic HCV infection at 58 million individuals with another 1.5 million new infections annually (World Health Organization, 2024). Chronic infection leads to associated complications, the most lethal being cirrhosis and hepatocellular carcinoma. Estimates show that approximately 290,000 individuals died from these complications in 2019. HCV prevalence has, however, decreased in recent years with the introduction of direct acting antiviral drugs and increased national screening campaigns. However, there is no vaccine for HCV although the WHO has set as its main targets a 90% reduction in chronic infections and a 65% reduction in HCV-related mortality by 2030.

Thus, developing a vaccine against HCV remains a priority in the field as the most effective way to prevent and control the spread of the virus. HCV RNA genome has a high genetic diversity. This diversity is found from the genotype to isolate quasispiecies level, making it difficult to develop a pan genotypic vaccine that induces cross neutralizing antibodies able to neutralize neutralization-resistant virions. Besides genetic diversity, the virus has several immune evasion strategies, such as interfering directly with innate antiviral immunity, the presence of a glycan shield, and a high degree of conformational flexibility of nAb epitopes, making conserved regions of the envelope inaccessible or diverse in terms of conformation, which in turn generates an inefficient immune response.

Inspired from HIV and RSV reverse vaccinology strategies, we aimed to stabilize a highly potent neutralizing antibodies epitope present in HCV E2. nAbs targeting the conserved 412-423 region in a β-hairpin conformation of the E2 envelope glycoprotein were isolated from both humans (HCV1) and mice (AP33). These antibodies are able to completely neutralize infection, but rarely occur during clinical infection. In order to induce the production of a particular antibody idiotype by vaccination, structure-based design was employed to develop HCV E2 derived antigens. Therefore, the first aim of the present study was to develop novel antigens based on the HCV E2 envelope protein that induce potent and neutralizing AP33-type like antibodies, efficient against highly resistant HCV E2 envelopes. To accomplish that, we aimed to stabilize the 412-423 antigenic site in a β-hairpin loop by introducing a novel disulphide bridge in the context of HVC E2 protein. We also aimed to test the possibility of HCV E2 glycoprotein antigen production in an alternative red microalgal system. This study utilized for the first time *P. purpureum* as an expression platform, a red microalgae species with

a very high intra- and extracellular expression capacity. Thus, we wanted to test the feasibility of vaccine production in *P. purpureum* by characterizing the production capacity, conformation, and antigenicity of the HCV antigen produced, and then to study the immunogenicity along with the neutralizing capacity of the induced antibodies.

Chapter 1. Introduction

Hepatitis C virus is a hepatotropic enveloped RNA virus belonging to the genus Hepacivirus of the family Flaviviridae. It has a positively oriented single-stranded RNA genome. The viral genome displays a continuous reading frame flanked by untranslated regions at the 5' and 3' ends (Brown et al., 1992; Wang et al., 1995). The untranslated regions also contain the internal ribosomal attachment sequence, the site where translation of the viral genome into a single polyprotein begins (Wang et al., 1995). The genome contains between 9024 and 9111 nucleotides, coding for a single polyprotein, which is further cleaved into 10 individual proteins by endogenous and virus-encoded proteases (Alvisi et al., 2011).

The structural proteins are core, E1 and E2. Core represents the viral nucleocapsid protein and has numerous functions such as RNA binding, immune modulation, cell signalling, and oncogenic and apoptotic potential (Kim & Chang, 2013). E1 and E2 are transmembrane glycoproteins that form a non-covalent heterodimer decorating the virus surface. E1 and E2 are strongly glycosylated with 4-5 glycans for E1 and 11 glycans for E2 (Goffard & Dubuisson, 2003). On the virus surface the E1E2 heterodimers appear to interact covalently via disulphide bridges, and intracellularly noncovalently (de la Peña et al., 2022). In 2022, the E1E2 heterodimer crystal was solved, where the N-terminal domain of E1 is observed to adopt a different conformation than previously observed, confirming the necessity of E2 for a proper folding of E1, but inconclusive for classification as a fusion protein (de la Peña et al., 2022). E1 represents a target for neutralizing antibodies (NAbs) having several antigenic regions: the N-terminal end (amino acids 197-207) (Keck et al., 2004; Kong et al., 2015) and a well-conserved region adjacent to the C-terminal end (amino acids 313-328) (Meunier et al., 2008). However, anti-E1 antibodies generated have weak activity in *in vitro* assays and are represented in a much lower titer compared to anti-E2 antibodies (Keck et al., 2004).

The E2 glycoprotein (aa 384-746) is highly post-translationally modified with 11 N-glycans and 9 disulphide bridges (Goffard & Dubuisson, 2003). It has three variable regions (VRs) namely hypervariable region 1 (HVR1), VR2 and VR2 which account for approximately 25% of the E2 protein sequence and contribute significantly to the genetic diversity of HCV. E2 is

responsible for specific interactions with cellular receptors that mediate virus entry into the cell (Meredith et al., 2012).

In the last decade, novel structural data have emerged in which the sE2 (soluble) variant is structurally and antigenically characterized in complex with fragments of human, murine or macaque mAbs (monoclonal antibodies) (Flyak et al., 2018; Khan et al., 2014a; Kong et al., 2013, 2016; Kong, Giang, Nieusma, et al., 2012; Tzarum et al., 2019; Weber et al., 2022). Thus, E2 has been observed to have a central β-sandwich-like ectodomain flanked by a frontal and a distal layer that are stabilized by disulphide bridges (Kong et al., 2013). Alanine-substituted mutagenesis studies together with competition data between different mAbs led to the finding and naming of linear, conformational, neutralizing or non-neutralizing epitope clusters divided by domains (A-E), antigenic regions (AR 1-3), antigenic sites (AS) or epitopes (I, II, III).

The first step in the viral life cycle involves the attachment of the viral particle to cellular co-receptors. The major determinant is the E2 protein, which first facilitates the interaction with the hepatocyte by heparan sulphate proteoglycans recognition (Barth et al., 2003). Cluster of differentiation 81 (CD81), SRB1, claudin-1 (CLDN) and occludin (OCLN) are the cellular factors required for the viral particle to enter the cell (Meredith et al., 2012).

The first factors identified were CD81 and SRB1 ((Pileri et al., 1998 ,Scarselli et al., 2002). SRB1 is a 509-amino-acid (aa) glycoprotein receptor with a major role in the binding of high-density lipoproteins (Acton et al., 1996). It is highly expressed in liver tissue promoting the selective uptake of cholesterol into hepatocytes, thus thought to be a determinant of viral hepatotropism (Scarselli et al., 2002). SRB1 promotes virion internalization by binding to virion associated lipoproteins and HVR1 HCV E2 binding followed by cholesteryl ester transfer which enable the subsequent binding to CD81 co-receptor (Bankwitz et al., 2010). CD81 is a tetraspanin expressed on the surface of most cell types and has roles in adhesion, morphology, proliferation, and differentiation. It has 4 transmembrane domains, 2 short intracellular domains and 2 extracellular loop-like domains (Meredith et al., 2012). The binding of E2 to CD81 represents a subsequent step after SRB1 binding which exposes the E2 binding domain to the large extracellular loop of CD81 (Prentoe et al., 2019). The AS412 region and the front domain are critical determinants in CD81 binding. Recent cryo-microscopy data suggest that AS412 would be in direct contact with the CD81 binding loop in the absence of CD81 and the two elements drastically change their conformation upon binding to CD81. Afterwards, the CD81-SRB1-virion complex migrates to the tight junctions where CLDN1 and OCLN enable the viral entry (Ding et al., 2017). The two tight junction co-receptors do not

interact directly with the viral particle, but most likely form co-receptor complexes with CD81-E2 leading to the virion internalization (Harris et al., 2008).

Antiviral treatments, although very effective, cannot ensure viral eradication. The majority of HCV infections are asymptomatic before liver lesions develop, so many infected individuals are not identified (Walker & Grakoui, 2015). Moreover, individuals who do not respond to antiviral treatment also develop resistance to these drugs, thus spreading incurable forms (Howe et al., 2022). In addition, consequences of HCV infection such as cirrhosis and carcinoma cannot be prevented by antiviral therapy if the infection has advanced (Sanduzzi-Zamparelli et al., 2019). An effective vaccine against all circulating genotypes could lead to a better epidemiologic control of HCV, reducing the costs associated with HCV diagnosis and treatment, and possibly leading to viral eradication.

Spontaneous clearance of infection occurs in approximately 25% of patients with acute infection (Micallef et al., 2006). However, upon re-exposure to the virus, patients can get re-infected. Reinfection is characterized by a reduction in viremia and duration of infection with the emergence of potent neutralizing NAbs, which are able to suppress infection in 80% of (Osburn et al., 2014). These data indicate the induction of an adaptive immune response (IR), which although not 100% protective, protects against chronic infection. Protective IR involves a synergism of adaptive and innate responses, and an ideal vaccine should induce a dual immunologic response (Obaid et al., 2018).

Because E2 mediates attachment to SRB1 and CD81 receptors, it is also the main target of the humoral IR. It is believed that by generating anti-E2 antibodies that specifically target receptor binding sites, infection can be blocked, leading to the emergence of highly neutralizing NAbs, or broadly NAbs (able to neutralize most circulating genotypes and neutralization resistant viral isolates) (Potter et al, 2012; Ströh & Krey, 2020; Yechezkel et al., 2021). The generation of these antibodies is very difficult because the virus has several mechanisms by which it can evade the humoral IR. In acute and chronic infections, humoral RI targets variable regions that are constantly under immunologic selective pressure. Variable regions and glycans increase protein heterogeneity thereby also influencing the immune response (Li et al., 2015). NAbs targeting HVR1 are generally specific to the respective isolate and the virus rapidly develops resistance against them. In addition, the attachment of these antibodies to HVR1 can block other epitopes important in neutralization by sterically inhibiting binding (Bankwitz et al., 2010). Most NAbs bind to E2 by inhibiting CD81 interaction and are highly effective in neutralizing infection regardless of the genotype, as the CD81-binding sequence is highly conserved (Tzarum et al., 2018).

Crystallography studies show a high degree of conformational flexibility in epitope I (412-423) on the front layer and in epitope II on the distal layer, which are highly conserved (Flyak et al., 2020; Gu et al., 2018; Kong et al., 2016; Kong, Giang, Nieusma, et al., 2012; Kong, Giang, Robbins, et al., 2012; Li et al., 2015; Potter et al., 2012). The flexibility of the two layers, suggests the existence of global conformational dynamics. This dynamic appears to influence the different functional states of the virus during cell entry (Tzarum et al., 2020). The property of the envelope to undergo functional movements also affects the IR, leading to very diverse and poorly targeted humoral response. A large number of virions, including flaviviruses, are described as dynamic structures that exist in conformations and arrangements specific to a particular biological role, being characterized by reversible conformational rearrangement of envelope glycoproteins (Dowd & Pierson, 2018). This process termed virus breathing has also been observed in HCV by time- and temperature-dependent neutralization experiments and can provide extremely valuable structural clues in the choice or conceptualization of a potential vaccine (Prentoe et al., 2019; Sabo et al., 2012).

Thus, our goal was to stabilize the conserved 412-423 epitope in order to direct the IR towards an AP33-like humoral response. A library of HCV E2 derived antigens was constructed by the introduction of a disulphide bridge in different positions which are flanking the well-conserved HCV E2 412-423 epitope, thereby stabilizing the hairpin-like loop conformation of the epitope. This library has been tested for expression and antigenicity and the selected candidate was further used in immunogenicity studies.

Alternative HCV antigen production systems

It is estimated that vaccine development, from conceptualization to the distribution phase takes between 5-18 years, with associated costs between 200 to 500 million dollars (Plotkin et al., 2017; Waye et al., 2013). Additionally, developing a production facility can cost between 50-700 million dollars taking on average 7 years (Vidor & Soubeyrand, 2016). Advancing of production and development technologies that are flexible, fast, and low-cost would enable low-income countries to counter a potential epidemic much more efficiently. An alternative production system that combines the productivity of bacterial systems with eukaryotic post translational processing would address some of the current limitations related to cost and accessibility.

Plants present a huge production potential for recombinant proteins. They allow expression of complex proteins without the costs associated with production in insect cells or mammalian cell lines. Plants use solar energy and capture CO2, representing a more environmentally friendly system with extremely low costs and greater feasibility of deployment. Another

advantage of this system is the possibility of oral, biomass delivery of the vaccine (Tacket, 2009), thus avoiding the often costly and laborious antigen purification step. In addition, lyophilization of edible plant material can facilitate its transportation (Rosales-Mendoza, 2020). However, plants display a specific and different glycosylation profile. Although this impediment can be solved by developing transgenic organisms with a glycosylation system close to that of the mammalian cell (Pantazica et al., 2023), the main problem is the low expression yield (Waheed et al., 2016). At the same time, standardization of antigen dose for oral administration is difficult because antigen concentration differs between plant parts (Dobrica et al., 2021).

Microalgae represent an attractive option as an alternative production system for recombinant protein production. This system has many advantages, such as rapid growth rate, ease of cultivation, rapid transformation, but also the ability to allow post-translational modifications and proper folding of complex proteins (Lindh et al., 2014). Microalgae can be cultivated in extremely inexpensive culture media, especially photosynthesizing species that have minimal nutritional requirements, which can be scaled up in bioreactors, reducing the need for land or greenhouses for plant cultivation (Barbosa et al., 2023a).

Microalgae also have a high safety profile, having no toxic endogenous compounds and no risk of animal biological contamination, further reducing associated costs. Production based on chloroplast transformation results in an accumulation of exogenous protein between 0.03-3 mg/L of culture (Rosales-Mendoza et al., 2020) while nuclear transformation allows the production of more complex proteins, but still requires optimization to reach good expression levels. The most popular species is *C. reinhardtii*, for which expression toolboxes have been developed (Legastelois et al., 2017). Nuclear transformation for the expression of antibodies, growth hormones, tumor epitopes, vaccines and peptides with anti-microbial activity has been reported, nevertheless, their expression level remains at low levels when compared with other systems (Geng et al., 2003).

The diversity of microalgae is immense, thus, other microalgal species can be found with higher transgenic expression capacity that can be cultured at higher densities. Recently, the feasibility of using the red microalgal species *Porphyridium purpureum* (*P. purpureum*) for recombinant protein production has been studied. Thus, it has been observed that *P. purpureum* can express recombinant proteins at about 5% of the total cellular protein (5 times more than *C. reinhardtii*) (Hammel, Neupert, et al., 2024).

The transformation of microalgal chloroplasts results in a high level of expression, but does not allow secretion and glycosylation of recombinant proteins. Therefore, different groups have

developed biotechnological methods for nuclear transformation and expression. These methods are based on the optimization of transformation methods, finding new promoters and gene terminators and increasing the genetic tool-box that allow an increased level of expression while also maintaining correct post-translational modifications (Barbosa et al., 2023b). An unknown within this system is the glycosylation profile, which is highly important in terms of immunogenicity. Studies exploring glycosylation in *P. purpureum* are currently few and point to a modified glycosylation profile of the Man 8-9 Xyl 1-2 Me 3 GlcNAc 2 type (Mathieu-Rivet et al., 2020). HCV E2 protein is a great model to study the feasibility of *P. purpureum* as an expression system, as the protein undergoes complex posttranslational modifications such as mammalian specific glycosylation.

Our goal was to assess the feasibility of HCV E2 antigen production in *P. purpureum* by expressing, characterizing and using microalgae E2 produced antigen in immunization studies.

Chapter 2. MATERIALS AND METHODS

The proposed HCV E2 derived antigens were expressed as membrane bound HCV E2 ectodomains chimeras in mammalian cells following a selection process based on expression levels, ER exit, and differential binding capacity to two antibodies recognizing different conformations of the epitope. The selected candidate was further expressed in mammalian suspension cells and purified to a high degree by affinity chromatography coupled with size exclusion chromatography. The purified antigen was further characterized conformationally by antibody binding, CD81 receptor binding, as well as by cysteine bridge analysis through mass spectrometry. Afterwards, it was used in immunization studies, followed by IgG titers quantification and HCVpp neutralizing potency.

In order to test *P. purpureum* suitability as an expression platform, we purified the antigen from lyophilized biomass using affinity chromatography. The purified antigen was further characterized conformationally by binding to specific antibodies and the CD81 receptor and further used in immunization studies. Following immunization, the induced antibody titers as well as the ability of the obtained sera to neutralize HCVpp infection were determined.

Chapter 3. RESULTS

Proposed antigens were obtained by cloning into the pcDNA3.1 vector and selected by a preliminary characterization of the membrane bound form. Thus, by transient transfection in

HEK293T cells, we observed the expression level of the mutants. We noticed that several cysteine substitutions were detrimental for HCV E2 protein ectodomain expression, suggesting defective folding and thus reduced protein stability.

For candidates with a good level of expression compared to the native protein, Endo H digestion was performed to observe their intracellular trafficking and glycan processing. This analysis showed that two out of the 6 selected antigens exhibited a resistant Endo H population, confirming the acquisition of complex glycan structures as a result of native intracellular folding and ER exit of the antigen. One of the two antigens with a good expression level and a native like glycosylation profile, also revealed a better binding of the AP33 antibody recognizing the hairpin conformation. Thus, we selected the E2ΔHVR1-6 antigen for large-scale expression in Expi293 cells and purification by affinity chromatography coupled with size exclusion chromatography.

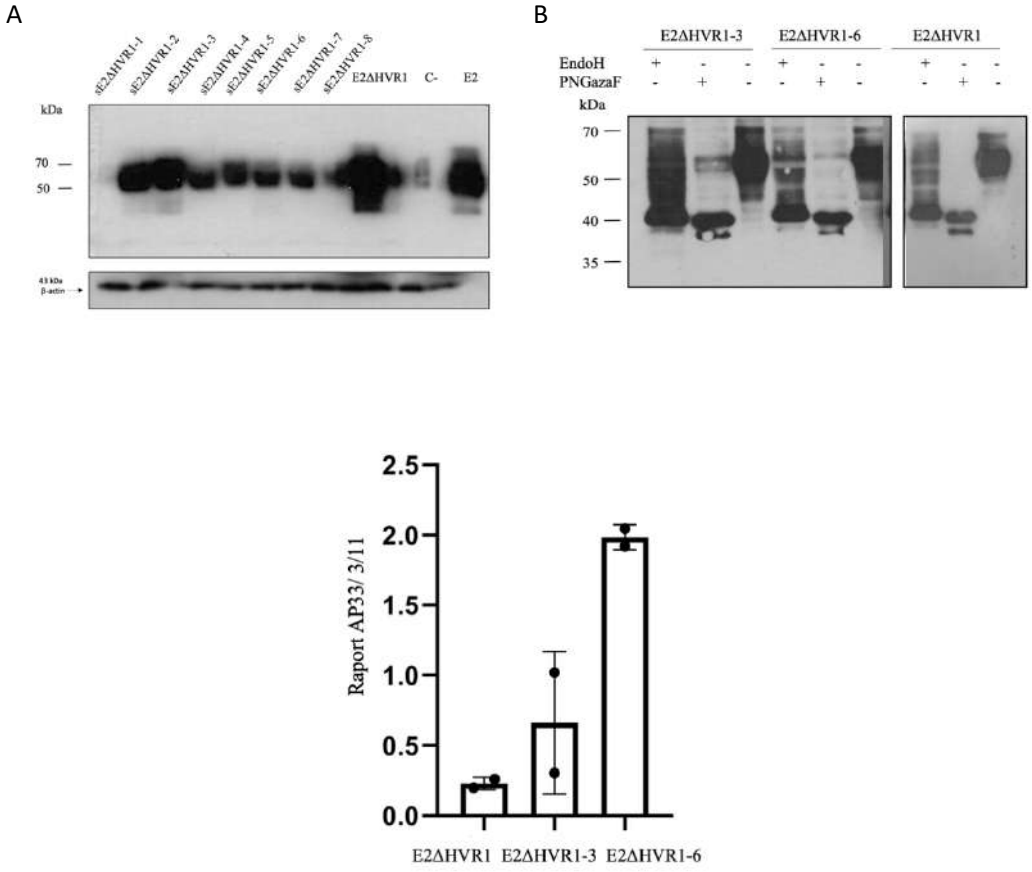


Figure 1. Expression of the novel mutant HCV antigens in mammalian cells (A). HEK293T cells were transfected with pcDNA3.1 plasmids encoding the indicated antigens or empty plasmid as control (C). Cell lysates were analysed by western blot under reducing

conditions (+DTT). Anti-E2 antibody 3/11 was used for detection of E2 proteins. Beta actin was used as a control for the amount of total protein loaded. **Glycosylation of the selected antigens and their intracellular trafficking (B).** HEK293T cells were transfected with pcDNA3.1 plasmids encoding the indicated antigens. Cell lysates were subjected to digestion with either Endo H enzyme, PNGase F or untreated. Detection of glycosylated forms was performed by western blot using anti-E2 3/11 antibodies. **Antigen selection based on differential binding of AP33 antibody by ELISA (C)**. HEK293T cells were transfected with pcDNA3.1 plasmids encoding the indicated antigens. Obtained cell lysates were incubated in 96-well plates coated with GNA in dilutions specific to the level of antigen expression, and detection was performed using 3/11 and AP33, respectively, and HRP-conjugated secondary antibodies. Finally, luminescence was determined and the results are represented as the binding ratio of AP33 to 3/11 for each antigen after normalization to control luciferase units. The graph represents 2 independent experiments performed in technical triplicates with mean and standard deviation presented.

The purified antigen was further used in characterization studies by verifying the level of conformational antibody binding, binding to the extracellular loop of the CD81 receptor, and by characterizing the disulphide bridges formed.

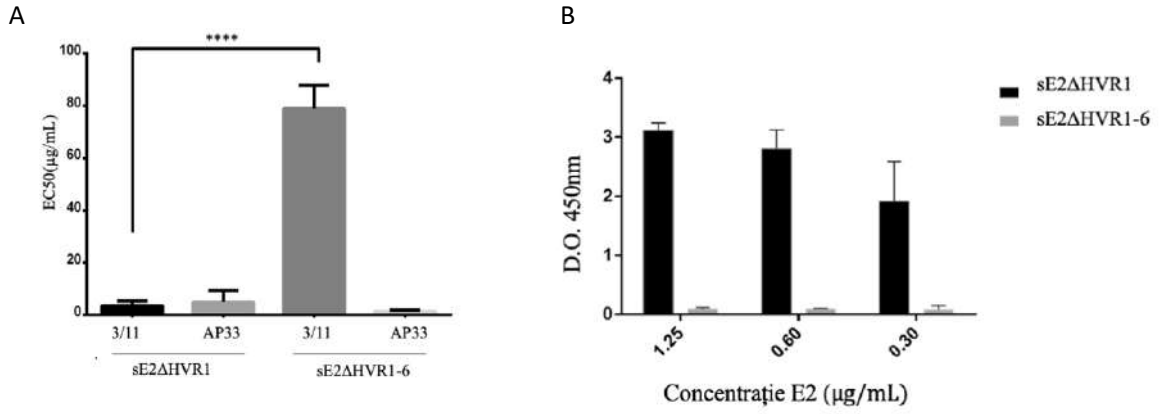


Figure 2. Determination of the level of binding of AP33 and 3/11 antibodies to the novel E2 antigen (A). To determine half of the maximum concentration of an antibody required to bind the antigens, 96-well plates were coated with 100 ng antigen per well. The anti-E2 antibodies tested (3/11 and AP33) were used in 8-fold serial dilutions of 10 (100µg/mL-0.00µg/mL) in duplicate within 3 independent experiments. Anti-mouse (AP33) and anti-rat (3/11) antibodies were used for detection. After calculating the EC50 values from the 3 experiments, they were averaged and the One-way Anova test (Tukey's multiple comparisons

test) ****P < 0.0001 was applied. **Recognition of E2 protein by the extracellular loop of the human CD81 receptor (B)**. 96-well plates were coated with 200 ng CD81-LEL-Fc per well for successive incubation with the indicated concentrations of sE2ΔHVR1 and sE2ΔHVR1-6. Detection was performed by incubation with horseradish peroxidase-conjugated anti-human-Fc antibody. The experiment was performed in duplicate twice.

By testing antibody binding capacity in the context of a purified protein of known concentration, we can directly observe the effect of antibody binding through a binding curve at different dilution points. Additionally, this experiment tracks the amount of antibody required to reach half the maximum concentration that can bind the antigen.

Thus, we can observe that for sE2ΔHVR1-6 the amount of 3/11 antibody required to reach the EC50 is about 80-fold higher than for sE2ΔHVR1 protein. We also observe a decrease in the required concentration of AP33 used to reach EC50, suggesting a direct conformational change in the epitope that significantly prevents the binding of 3/11 while maintaining the binding level for AP33.

Further, we wanted to see whether sE2ΔHVR1-6 can still interact with CD81-LEL. It is the extracellular loop of the CD81 receptor that participates in the interaction with the N-terminal region of E2, an interaction facilitated by the open-type conformational state of the 412-423 epitope. Through this binding experiment we observed that the interaction with sE2ΔHVR1 is dose-dependent, while for sE2 ΔHVR1-6 the interaction is completely blocked, regardless of the antigen concentration used. This result indicates that from a functional point of view, the proposed antigen no longer exhibits the conformational dynamics that allow for receptor binding.

For a better understanding of the structure of the proposed antigen, we performed mass spectrometry analysis to characterize the cysteine residues pairing in disulphide bridges. Thus, we used reduced (with reducing agent) or unreduced (without reducing agent) E2 samples that were alkylated and digested with chymotrypsin. The modification of cysteine residues involved in the formation of a bridge occurs only in the reduced form of the sample, as they are not accessible to alkylation under non-reducing conditions.

Thus, in the samples analysed, we identified two bridges frequently described in the literature for different genotypes, C564+C569 and C581+C585 (de la Peña et al., 2022; Krey et al., 2010; Metcalf et al., 2023), suggesting these are conserved. In order to compare the overall structure of the mutant antigen with that of the wild-type protein, we also analysed the latter. Thus, in addition to the two identified common bridges, we also identified the formation of a common bridge between C494 and C503, not found in the literature. C429 appears to form

heterogeneous bridges, being found both occupied and unoccupied in the wild-type protein. However, in the proposed antigen, it appears to form a different bridge with a newly introduced cysteine residue. Nevertheless, the data show a correct global folding of the antigen and attest at least partially to the formation of a bridge in the frontal domain of the protein.

The antigen was expressed on a large scale, purified and characterized. By size exclusion chromatography, no aggregate protein complexes were observed compared to the wild-type protein, which reinforces the hypothesis of correct folding. Antibody binding analysis shows that the antibody binding the open conformation of the epitope, 3/11, binds the sE2ΔHVR1-6 antigen with approximately 80% less capacity than the wild-type protein, and 10% more for the AP33 antibody, the one recognizing the hairpin conformation. This result indicates a stabilization of the 412-423 epitope with a good exposure.

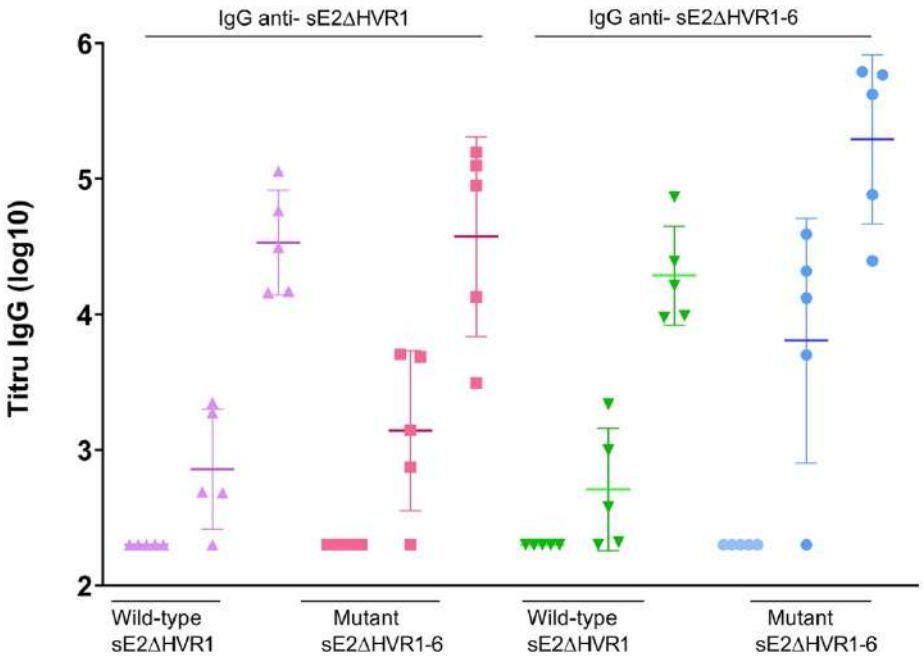
The characterization, however, reveals an inability of our vaccine antigen to bind to the receptor protein CD81. This could be explained by the reduced exposure of the tryptophan 420 residue which is essential in the binding of the E2 protein to CD81. However, binding data to AP33 contradict this hypothesis, as the same tryptophan residue is also required in antibody binding. If we exclude the hypothesis that this interaction is blocked by the substitution of an essential contact point, we can reason that the binding is indeed affected by a conformational shift. The MS data obtained on the formation of disulphide bridges in the novel antigen indicate a conservation of bridges already identified in the literature (Khan et al., 2014b; Krey et al., 2010; Metcalf et al., 2023) and reveal a possible heterogeneity of the bridges formed by the newly introduced cysteine residues. Thus, the induced conformational change is local to the epitope of interest, and this conformation most likely is not the one recognized by the cellular receptor. In the absence of CD81, the E2 ectodomain exhibits an internal CD81-binding loop hidden behind the 422-isoleucine residue or the 412-423 epitope on the front layer. When receptor binding occurs, E2 most likely undergoes two conformational changes, with the front layer interacting with CD81 by folding the 412-423 epitope around the CD81 E2 interaction site, while the CD81-binding loop moves away from the protein ectodomain. Thus, the two hydrophobic residues at the tip of the loop (tyrosine residues 529 and tryptophan residues 531) can bind to the host cell membrane (Kumar et al., 2023, Bankwitz et al., 2010).

Recent studies show that in the absence of CD81 or in low pH conditions, the 422 isoleucine maintains the CD81 binding loop close to the ectodomain. (Kumar et al., 2023). It is also known that removal of HVR1 causes increased binding to cellular receptors. Thus, E2 is thought to first interact with SRB1 through the HVR1 region, making the 412-423 epitope accessible for CD81 interaction, this interaction in turn inducing conformational modifications of the binding

loop (520-539) that allow for receptor binding (Bankwitz et al., 2010). A recent crystallography study, suggests that the protein may be in a closed conformational state, where HVR1 covers the AR4A antibody epitope (which recognizes the N-terminal region of E2) and prevents binding to CD81 in a temperature-dependent manner (Augestad et al., 2020). Theoretically, an immunogen without HVR1, stabilized in a pre-binding conformation could induce antibodies of a lower diversity and better quality. These antibodies could bind to the viral envelope while it is still present in the conformation preceding binding to the CD81 receptor, thus blocking further conformational changes of the envelope and blocking virus-receptor interaction.

The two purified and characterized antigens were subsequently used in immunogenicity *"in vivo"* studies. Through these studies we were able to determine the immunogenicity of the mutant antigen compared to the wild-type protein. To gain more insight into the heterogeneity of the immune response, we performed two completely independent immunization studies using both Balb/C and CD1 mice. Balb/C represents an inbred strain of mice with low genetic variability and an immunologic response biased towards Th2-type activation. CD1 mice, in contrast, exhibit a balanced Th1-Th2 response, as well as high intrinsic genetic variability as outbred mice. The two studies were performed with different administration routes and variable antigen doses. For Balb/C mice we administered a 50 μg prime followed by 2x20 μg boosts peritoneally while for CD1 mice we used a 10 μg prime followed by 2x5 μg boosts administered intramuscularly.

A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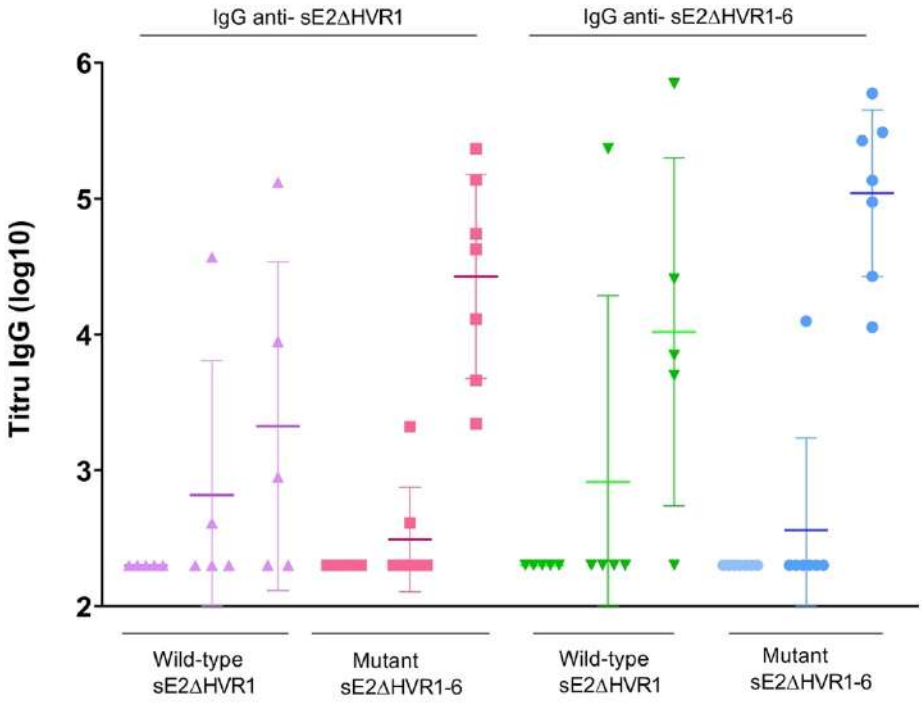
IgG anti- sE2ΔHVR1-6
Titru IgG (log10)
6
5
4
3
2
Wild-type sE2ΔHVR1
Mutant sE2ΔHVR1-6
Wild-type sE2ΔHVR1
Mutant sE2ΔHVR1-6

B


IgG anti- sE2ΔHVR1
IgG anti- sE2ΔHVR1-6
Titru IgG (log10)
6
5
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3
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Wild-type sE2ΔHVR1
Mutant sE2ΔHVR1-6
Wild-type sE2ΔHVR1
Mutant sE2ΔHVR1-6

Figure 3. Analysis of the total IgG humoral immune response induced by immunization of Balb/C mice with sE2 antigens produced in mammalian cells (A). Groups of 5 mice per batch were immunized at 14-day intervals with 3 doses of adjuvant sE2ΔHVR1 or sE2ΔHVR1-6 protein or Al(OH)3 adjuvant alone. The final sE2ΔHVR1 or sE2ΔHVR1-6 antibody titer at 0-, 28- and 42-days post-immunization was determined by ELISA and calculated using a 4-parameter logistic regression curve fitted to a mixture of immune serum (n=5). **Analysis of the total humoral IgG immune response induced by immunization of CD1 mice with sE2 antigens produced in mammalian cells (B).** Groups of 5 mice per batch were immunized at 14-day intervals with 3 doses of adjuvanted sE2ΔHVR1 or sE2 ΔHVR1-6 protein or AddaVax adjuvant alone. The final sE2ΔHVR1 or sE2ΔHVR1-6 antibody titer at 0-, 28- and 42-days post-immunization was determined by ELISA and calculated using a 4-parameter logistic regression curve fitted to a mixture of immune serum (n=5).

Thus, by ELISA experiments, total IgG, IgG1 and IgG2a antibody titers were determined for the two immunization studies, against the wild-type protein as well as against the mutant. For the Balb/C mice we observe that the total IgG antibody level is similar for the anti-wild-type antigenic response, but following the anti-mutant antibody level, we observe an increase in titer levels for the serum from the autologous antigen immunization both by comparison with the serum from the wild-type antigen immunization and when comparing the titer value for the same serum recognizing the two different proteins. For CD1 mice (Figure 4, B) we note an expected greater variability of the immune response. Surprisingly, even when administering a lower antigen dose, sE2 ΔHVR1-6 is able to induce high antibody titers against both antigens suggesting an improved immunogenicity.

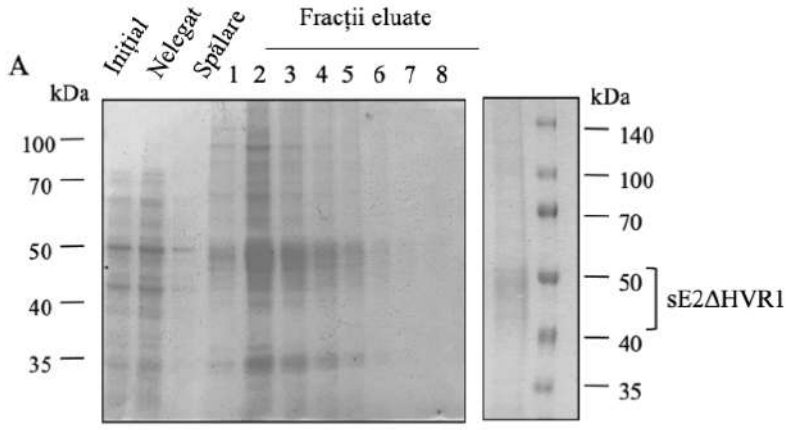
Inhibition of 3/11 antibody binding and increased binding of AP33 antibody by the proposed E2 antigen suggest a correct epitope exposure. The glycosylation profile determined by Endo H digestion indicates the ER exit of the membrane bound ectodomain, suggesting a native folding. Moreover, the aggregation and protein oligomerization profiles combined with native cysteine bridges identification support a native global folding of the antigen. Independent immunization studies have shown that the antigen is immunogenically superior to the wild-type variant, with serum harvested from immunization with the candidate antigen better recognizing the wild-type protein.

We may conclude that the strategy of stabilization of -flexible highly conserved regions of HCV E2 targeted by nAbs is a promising one and that sE2ΔHVR1-6 indeed presents a

conformation capable of directing the immune response towards a cross-neutralizing response. At the time of writing this PhD thesis, we have no information on the cross-neutralizing capacity of the obtained sera. But from preliminary data we observe a better neutralization of the autologous genotype following immunization with the proposed antigen. Further on in the project we will test the neutralizing capacity of the sera in the context of neutralization-resistant isolates, as well as their potency in the context of other genotypes.

Next, we wanted to test the feasibility of *P. purpureum* microalgae in the context of vaccine development. Following expression of the antigen in microalgae, it was extracted and purified from the microalgae by sonication and affinity chromatography and used in characterization studies. To verify the conformation of the purified antigen we performed ELISA binding experiments to conformational antibodies as well as to the CD81 receptor. Thus, we verified protein folding by binding to two conformational anti-E2 antibodies, AP33 (recognizing the frontal domain), H53 (recognizing the distal domain together with the frontal domain) and to the CD81 receptor (recognizing the frontal domain). For all three interactions we obtained a dose-dependent level of binding in ELISA experiments, thus confirming specific antibody-antigen and receptor-antigen recognition and a native global folding.

A



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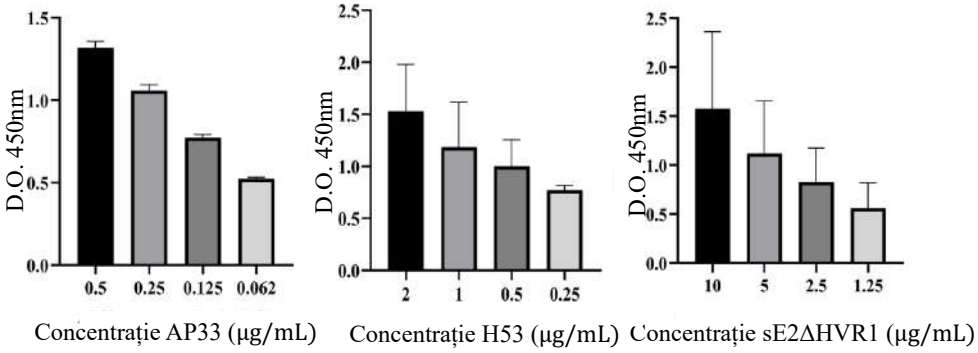
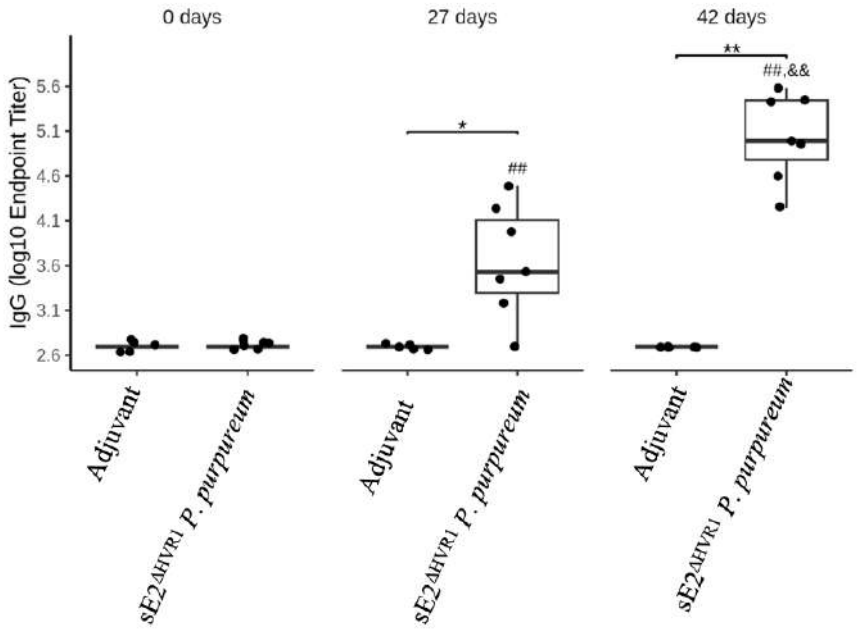


Figure 4. sE2ΔHVR1 antigen purification from the soluble fraction of red microalgae lysate (A). The soluble extract of the lysate obtained was successively added to a NiNTA resin-packed gravity column. The fractions eluted by stepwise gradient (20-450mM) imidazole were confirmed by SDS-PAGE with Coomassie Blue staining (A) The fractions were pooled, dialyzed in PBS and concentrated using a 30 kDa size-limiting Amicon (A-right) Figure taken from and adapted after (Hammel, Cucos, et al., 2024). **Conformational characterization of sE2ΔHVR1 antigen purified from red microalgae (B).** 96-well Nunc MaxiSorp Nunc MaxiSorp plates were incubated with purified antigen (left, middle) or CD81 receptor (right) overnight. The next day the wells were incubated with serial dilutions of antibody (AP33/H53) or antigen dilutions (for CD81 binding). Reading was performed using horseradish peroxidase-conjugated secondary antibodies. Experiments were performed twice in technical duplicate. Figure adapted from (Hammel, Cucos, et al., 2024).

With available purified antigen as well as in the form of biomass, we decided to investigate the humoral immune response by intramuscular administration (with microalgal purified protein) and under oral booster conditions after administration of an intramuscular prime with E2 from mammalian cells (with orally administered microalgal biomass; Prime + Boost+). For each immunization study, an adjuvant batch was added, and for the oral booster study, an intramuscularly administered batch of antigen produced in mammalian cells at the maximum dose used (as a positive control for the immune response) were added as controls; sE2 ΔHVR1Expi293), a wild-type microalgae feeding booster (as a negative control for response to the booster; Prime + Boost -) and a wild-type microalgae immunization (as an additional negative control for response to the oral booster; WT P. purpureum). Serum was collected before each dose and at 42-days after immunization and tested for anti-E2 response by ELISA.

A significant immune response was induced by the oral administration of antigen-expressing biomass compared to wild-type biomass. Quantitatively, however, the titer obtained by oral administration is much lower than that obtained by immunization with antigen derived from mammalian cells, which is to be expected given the lower total amount of antigen administered. The intramuscular administration of purified microalgal antigen showed a greater increase in the anti-E2 immune response than oral administration, but less than that of mammalian cells obtained antigen, most likely due to the lower amount of immunogen administered. The secretion of IgG indicates an efficient activation of the immune system, a correct presentation of the antigen inducing a complex response that will further be tested for neutralizing activity.

A



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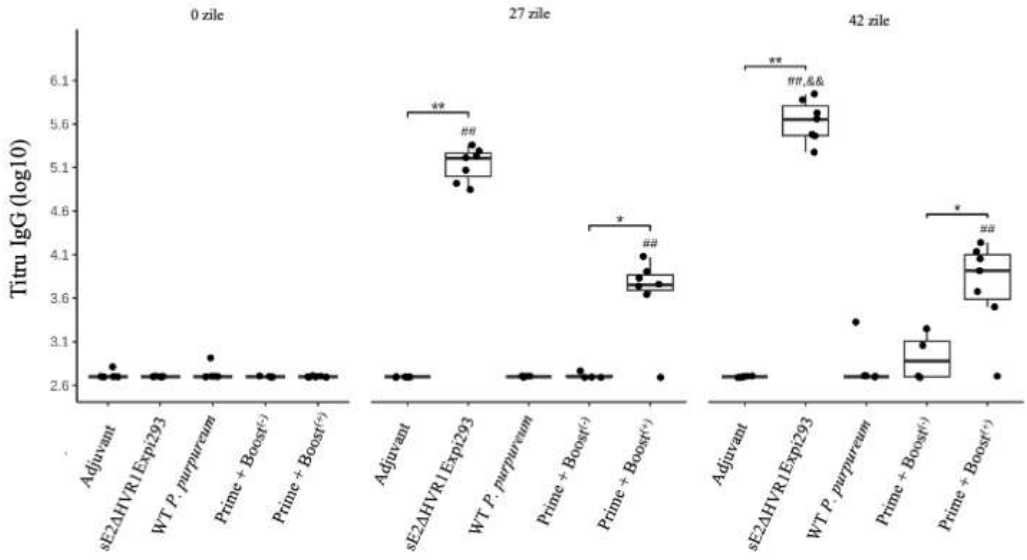

Figure 5. Analysis of the total IgG humoral immune response induced by immunization of Balb/C mice with sE2 antigens produced in microalgae. Groups of 5 or 7 mice per batch were immunized at 14-day intervals with 3 doses of adjuvanted sE2ΔHVR1 protein produced in microalgae by intramuscular administration (sE2ΔHVR1 P. purpureum) or adjuvant only **(A),** or by administration of an initial intramuscular dose followed by oral administration of E2 non-expressing (Prime + Boost-) or E2 expressing (Prime + Boost+) algal pellet, or by administration of E2 produced in mammalian cells (sE2 ΔHVR1 Expi293) or by oral

administration of E2 non-expressing algal pellet only (WT P. purpureum) **(B)**. The final sE2 ΔHVR1 antibody titer at 0-, 27- and 42-days post-immunization was determined by ELISA and calculated using a 4-parameter logistic regression curve fitted to a mixture of immune serum (n=5). Comparisons made between groups at the same time point (*P < 0.05; **P < 0.01), at days 27 and 42 compared to day 0 (#P < 0.05; ##P < 0.01) and at 42 days compared to 27 days (&&P < 0.01) are shown. Figure taken from and adapted after (Hammel, Cucos, et al., 2024).

The immunization-derived sera were tested for neutralizing activity against infection with HCV pseudoparticles displaying complete E1E2 envelope proteins on their surface, belonging to the isolate used as an immunogen (H77). As a positive control for neutralization of infection we used the anti-E2 antibody AP33 in each experiment, which has a high and specific neutralizing potency. As a positive control for immunization, we used the serum from the immunization with the antigen produced in Expi293, which has a capacity to inhibit infection of approximately 60%. To follow the non-specific neutralizing activity of the immune sera, we used as a negative control the serum from the batch of mice immunized with adjuvant alone, harvested at the same time as the immune sera, for which we observed a non-specific inhibition of about 10%. As predicted, immune sera from immunization with purified microalgal antigen administered parenterally showed a neutralizing activity of 30-40%, significantly higher than adjuvant, but also significantly lower than that of serum from immunization with mammalian cells derived antigen. However, the higher neutralization of Expi293 expressed antigen is explained by the fact that this antigen was purer (95% vs 65%) and was used in much higher amounts (25 μg vs 10 μg per dose) to ensure a positive control of the immunization study, i.e., the maximum possible response. Therefore, comparisons between the two antigens are not useful in this study.

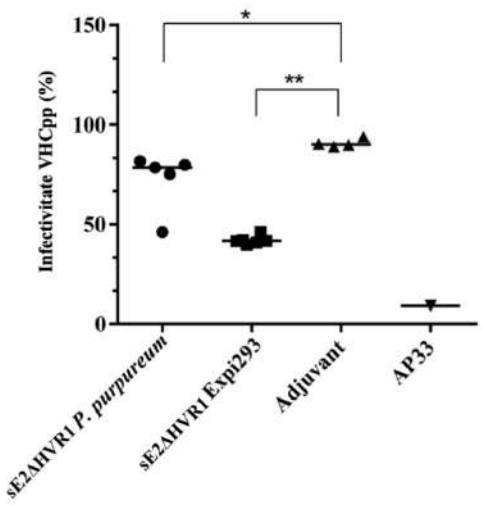

Figure 6. Analysis of the neutralizing ability of the immune serum obtained following immunization with sE2 antigens produced in microalgae. Huh7 cells were infected with viral pseudoparticles produced in HEK293T cells. HCVpp infection was performed in the presence of serum from individual mice immunized with adjuvant, sE2 from microalgae (sE2ΔHVR1 P. purpureum), sE2 from mammalian cells (sE2ΔHVR1 Expi293) or in the presence of a strong neutralizing antibody as a neutralization control (AP33). Cells were harvested 72 hours post-infection and used to quantify luciferase levels using the Luciferase Assay System kit (Promega). Data are presented as percentage of HCVpp infectivity in the presence of post-immune serum out of the percentage of infectivity of infected but untreated cells. Mean percentage infectivity obtained in two independent experiments performed in triplicate are represented. Statistical analysis was performed using the Kruskal-Wallis test (*P < 0.05; ****P < 0.0001). Figure taken from and adapted after (Hammel, Cucos, et al., 2024).

CONCLUSIONS AND PERSPECTIVES

The current study reports the development and characterization of a novel HCV E2 antigen based on the conformational stabilization of a highly conserved epitope. The new vaccine candidate exhibited an antigenic profile more favourable for AP33 recognition, a broadly neutralizing antibody. Moreover, the proposed antigen induces a more potent immunological response than the wild-type protein, generating a quantitatively higher titer in the two independent immunization studies.

However, the quality of these antibodies is essential for a protective immune response. By stabilizing the conformational dynamics of the protein front layer, we expect to obtain an immune response capable of neutralizing most genotypes as well as neutralization-resistant

isolates. Neutralization experiments will therefore define the aim of this study, confirming the proposed antigen as a potential vaccine.

Of equal importance, however, are structural studies that are currently lacking, to confirm the cysteine bridge formation. Further, the epitope flexibility would be evaluated by HDX-MS under various temperature and pH conditions. Refining and improving the immunogen can also be achieved by coupling to the E1 envelope protein which indeed provides a complete and faithful immune response against the viral envelope. At the time the study was initiated, there was not yet a soluble form of the heterodimer accompanied by structural studies revealing the correct heterodimer folding. Recently, however, studies and antigenic models have emerged that allow the integration of the novel antigen proposed in the current study into the E1E2 heterodimeric form. Therefore, we further propose to study this antigen in the full heterodimer form following a similar workflow.

In this study we demonstrated for the first time the feasibility of using microalgae as an expression platform for vaccine production. The results obtained demonstrate an expression level superior to any other photosynthesizing system previously used for viral antigen expression. The antigen obtained is correctly folded, and at least partially correctly glycosylated, demonstrating the ability of *Porphiridium purpureum* to express complex proteins that retain post-translational modifications specific to mammalian cells. The antigen obtained was shown to be immunogenic both purified and administered as an intramuscular injection and when administered as a biomass by feeding. Its oral immunogenicity can be improved in the future by the use of mucosal immunity specific adjuvants as well as by adjusting the antigen dose and administration schedule. Moreover, the resulting antibodies are neutralizing. By using a modified and stabilized antigen such as the one proposed in the first part of the study, we can also improve the quality of the immune response.

Preliminary data also show secretion of the antigen produced in microalgae into the extracellular environment. This could facilitate a more efficient production with even higher yields of complex antigens. The use of a soluble antigen would also decrease the costs associated with microalgal lysis and improve the level of protein purification, leading to a much approachable workflow. In the future, we also want to investigate the secreted antigen forms and their immunogenic potential.

Given the favourable properties of the new antigen, as well as the development of a new expression system, another next step would be to establish advanced preclinical studies in humanized liver chimeric mice or transgenic mice which permit viral entry (Dorner et al., 2013). Furthermore, the characterization of the B-cell repertoire activated by our antigens

would be the final proof of our aim to induce the expansion of a targeted subset of the B cell germ lines. These studies may also lead to the identification of new antibodies that we can produce in the institute and use in a variety of contexts, from the development of new diagnostic methods to reagents used in the development of HCV vaccines.

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1. Hammel, A.*, Cucos, L. M.*, Caras, I., Ionescu, I., Tucureanu, C., Tofan, V., Costache, A., Onu, A., Hoepfner, L., Hippler, M., Neupert, J., Popescu, C. I., Stavaru, C., Branza-Nichita, N., & Bock, R. (2024). The red alga Porphyridium red alga as a host for molecular farming: Efficient production of immunologically active hepatitis C virus glycoprotein. Proceedings of the National Academy of Sciences of the United States of America, 121(24), e2400145121. https://doi.org/10.1073/pnas.2400145121 IF 12.779, AIS 4.48, Q1

2. Pantazica, A. M.*, Cucos, L. M.*, Stavaru, C., Clarke, J. L., & Branza-Nichita, N. (2021). Challenges and Prospects of Plant-Derived Oral Vaccines against Hepatitis B and C Viruses. Plants (Basel, Switzerland), 10(10), 2037. https://doi.org/10.3390/plants10102037 IF 4.658, AIS 0.654, Q1

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* co-first author, equal contribution

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