

# Summary of Professional Accomplishments

Katarzyna Grzyb



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GDAŃSK 2026

**1 Name**

Katarzyna Grzyb

**2 Diplomas, degrees conferred in specific areas of science or art, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation**

**29 June 2007** Doctor of Biological Sciences (Biology) – University of Gdańsk, Institute of Biology. Dissertation title: *"Identification and Characterization of Creatine Kinase Isoenzymes from Sperm and Skeletal Muscles of Herring (Clupea harengus)"*. Supervisor: Prof. dr hab. Edward Skorkowski

**9 October 2001** Master of Science in Biology (Molecular Biology specialization) – University of Gdańsk, Faculty of Biology, Geography and Oceanology

**3 Information on employment in research institutes or faculties/departments or schools of arts**

**February 2018 – present** Intercollegiate Faculty of Biotechnology UG & MUG, Laboratory of Virus Molecular Biology, Gdańsk

- Assistant Professor – (project-funded position)
- Project Leader – OPUS 14, OPUS 21
- Research area: Development of a vaccine against hepatitis C virus (HCV) and a universal vaccine against SARS-CoV-2.

**February 2013 – December 2016** Intercollegiate Faculty of Biotechnology UG & MUG, Laboratory of Virus Molecular Biology, Gdańsk

- Assistant Professor – (project-funded position)
- Project Leader: NCBiR LIDER and NCN SONATA 3
- Research area: Development of a vaccine against hepatitis C virus (HCV).

**December 2010 – February 2012** – Intercollegiate Faculty of Biotechnology UG & MUG, Laboratory of Virus Molecular Biology, Gdańsk

- Postdoc – (project-funded position) participated in project: *"New Preventative and Therapeutic Hepatitis C Vaccines: From Pre-clinical to Phase 1"*
- Research area: Implementation of novel methodologies for studying the life cycle of hepatitis C virus (HCV).

**March 2009 – September 2010** – CEINGE–Biotecnologie Avanzate, Okairos laboratory (now Reithera), Italy

- Postdoc - 18-month Marie Curie postdoctoral fellowship – participated in project: *"Entry Inhibitors for the Treatment of Hepatitis C Virus Infection"*, EU 6th Framework Programme
- Research area: Antibodies targeting the SR-B1 receptor as a potential immunotherapeutic strategy for the treatment of chronic hepatitis C virus (HCV) infection.

#### 4 Description of the achievements, set out in art. 219 para 1 point 2 of the Act.

##### 4.1 Title of the series of publications constituting the main scientific achievement

Towards an Effective Vaccine Against Hepatitis C Virus: Approaches Based on Recombinant Glycoproteins, Virus-Like Particles, and Minicircle DNA.

##### 4.2 List of publications constituting the main scientific achievement

The main achievements submitted for evaluation are presented in a series of five thematically related publications, which were published after obtaining the doctoral degree and following the completion of an 18-month postdoctoral fellowship in Naples. All articles were published in journals listed in the Journal Citation Reports (JCR). In all publications, I served as the corresponding author, and in one of them additionally as the first author.

##### Publication No. 1

**Grzyb, K\***, Czarnota, A., Brzozowska, A., Cieřlik, A., Rąbalski, Ł., Tyborowska, J., Bieńkowska-Szewczyk, K. (2016). Immunogenicity and functional characterization of *Leishmania*-derived hepatitis C virus envelope glycoprotein complex. *Scientific Reports*, 6, 30627, doi:10.1038/srep30627

*\*corresponding author*

IF<sub>2016</sub> - 4.25; IF<sub>5-year</sub> - 4.3; MEiN<sub>2016</sub> - 40; MEiN<sub>2025</sub> - 140; citation count - 10 (WoS)

*My contribution to this work included: development of the research concept and formulation of research hypotheses, recruitment and training of the project team, scientific supervision of the project team, development of the research methodology, experimental work (implementation of a new Leishmania tarentolae-based protein expression system in*

*the laboratory, expression and characterization of the HCV E1E2 heterodimer, purification of E1E2, preparation of samples for animal immunization, development of the immunization scheme, and analysis of the humoral immune response), obtaining approval from the Local Ethics Committee for animal experiments, data analysis and visualization, interpretation of the results, preparation of the first version of the manuscript, submission of the manuscript to the journal, correspondence with the editor, revision of the manuscript according to reviewers' comments, preparation of responses to reviewers, project administration, and acquisition of research funding through grants in which I served as the principal investigator (LIDER and SONATA 3).*

## Publication No. 2

Czarnota, A., Tyborowska, J., Peszyńska-Sularz, G., Gromadzka, B., Bieńkowska-Szewczyk, K., **Grzyb, K\***. (2016). Immunogenicity of *Leishmania*-derived hepatitis B small surface antigen particles exposing highly conserved E2 epitope of hepatitis C virus. ***Microbial Cell Factories***, 15, 62, doi:10.1186/s12934-016-0460-4

*\*corresponding author*

IF<sub>2016</sub> – 3.68; IF<sub>5-year</sub> – 6.1; MEiN<sub>2016</sub> – 40; MEiN<sub>2025</sub> – 100; citation count – 21 (WoS)

*My contribution to this work included: development of the research concept and formulation of research hypotheses, recruitment and training of the project team, scientific supervision of the project team (including a PhD student for whom I served as an auxiliary supervisor), development of the research methodology, participation in experimental work (analysis of the cellular immune response, ELISpot assay), obtaining approval from the Local Ethics Committee for animal experiments, participation in data analysis and interpretation of the results, contribution to the preparation of the first version of the manuscript, submission of the manuscript to the journal, correspondence with the editor, participation in revising the manuscript according to reviewers' comments and in preparing responses to the reviewers, project administration, and acquisition of research funding through grants in which I served as the principal investigator (LIDER and SONATA 3).*

## Publication No. 3

Czarnota, A., Offersgaard, A., Pihl, A. F., Prentoe, J., Bukh, J., Gottwein, J. M., Bieńkowska-Szewczyk, K., **Grzyb, K\***. (2020). Specific Antibodies Induced by Immunization with Hepatitis B Virus-Like Particles Carrying Hepatitis C Virus Envelope Glycoprotein 2 Epitopes Show Differential Neutralization Efficiency. ***Vaccines***, 8(2), 294. doi:10.3390/vaccines8020294

*\*corresponding author*

IF<sub>2020</sub> – 4.42; IF<sub>5-year</sub> – 3.7; MEiN<sub>2020</sub> – 140; citation count – 20 (WoS)

*My contribution to this work included: participation in the development of the research concept and formulation of research hypotheses, scientific supervision of the project team (including a PhD student for whom I served as an auxiliary supervisor), participation in data analysis and interpretation of the results, establishment of collaboration with researchers from Denmark and correspondence concerning the interpretation of the research results, contribution to the preparation of the first version of the manuscript, submission of the manuscript to the journal, correspondence with the editor and co-authors from Denmark regarding manuscript editing, participation in revising the manuscript according to reviewers' comments and in preparing responses to the reviewers, project administration,*

and acquisition of research funding through a grant in which I served as the principal investigator (OPUS 14).

#### Publication No. 4

Czarnota, A\*, Offersgaard, A., Owsianka, A., Alzua, G. P., Bukh, J., Gottwein, J. M., Patel, A. H., Bieńkowska-Szewczyk, K., **Grzyb, K\***. (2023). The effect of glycan shift on antibodies against Hepatitis C Virus E2 412-425 epitope elicited by chimeric sHBsAg-based virus-like particles. *Microbiology Spectrum*, doi:10.1128/spectrum.02546-22

*\*corresponding author*

IF<sub>2023</sub> – 3.7; IF<sub>5-year</sub> – 4.1; MEiN<sub>2023</sub> – 100; citation count – 5 (WoS)

*My contribution to this work included: participation in the development of the research concept and formulation of research hypotheses, scientific supervision of the project team (including a PhD student for whom I served as an auxiliary supervisor), participation in data analysis and interpretation of the results, establishment of collaboration with researchers from Denmark and the United Kingdom and correspondence regarding the interpretation of the research results, contribution to the preparation of the first version of the manuscript, correspondence with co-authors from Denmark and the United Kingdom related to manuscript editing, participation in revising the manuscript according to reviewers' comments and in preparing responses to the reviewers, project administration, and acquisition of research funding through a grant in which I served as the principal investigator (OPUS 14).*

#### Publication No. 5

Czarnota, A., Raszplewicz, A., Sławińska, A., Bieńkowska-Szewczyk, K., **Grzyb, K\***. (2024). Minicircle-based vaccine induces potent T-cell and antibody responses against hepatitis C virus. *Scientific Reports* 14, 26698, doi:10.1038/s41598-024-78049-3

*\*corresponding author*

IF<sub>2024</sub> – 3.9; IF<sub>5-year</sub> – 4.3; MEiN<sub>2024</sub> – 140; citation count – 4 (WoS)

*My contribution to this work included: participation in the development of the research concept and formulation of research hypotheses, scientific supervision of the project team (including an MSc student for whom I served as a supervisor and a PhD student for whom I served as an auxiliary supervisor), participation in the development of the research methodology, participation in experimental work (analysis of cellular immune response, ELISpot assay), obtaining approval from the Local Ethics Committee for animal experiments, participation in data analysis, visualization and interpretation of the results, contribution to the preparation of the first version of the manuscript, submission of the manuscript to the journal, correspondence with the editor, participation in revising the manuscript according to reviewers' comments and in preparing responses to the reviewers, project administration, and acquisition of research funding through a grant in which I served as the principal investigator (OPUS 14).*

Scientometric data for the five publications constituting the main habilitation achievement:

- Total Impact Factor (IF) according to the list for the year of publication: 19.95

- Total number of MEiN points according to the list for the year of publication: 460
- Total 5-year Impact Factor (2025): 22.5
- Current total number of MEiN points (2025): 620

### 4.3 Scientific goals and results of the main research achievement

#### 4.3.1 Introduction

Hepatitis C virus (HCV) was discovered in the 1980s as the infectious agent responsible for chronic viral hepatitis that was neither hepatitis A virus (HAV) nor hepatitis B virus (HBV). The work of Harvey J. Alter, Michael Houghton, and Charles M. Rice led to the full identification of the virus and confirmation of its etiological role in infection. For this groundbreaking discovery, these scientists were awarded the Nobel Prize in Physiology or Medicine in 2020. Their achievements enabled the development of diagnostic tests for the detection of HCV infection and, consequently, significantly improved the safety of blood transfusions, as transmission of the virus occurs primarily through blood exposure. Moreover, this discovery laid the foundation for the development of highly effective antiviral therapies and initiated global efforts toward vaccine development—an objective that, despite intensive research, has yet to be achieved<sup>1</sup>.

Despite the discovery of HCV and the development of diagnostic methods that have significantly reduced the number of new infections, HCV infection remains a serious global health problem. Currently, approximately 50 million people worldwide are living with HCV infection. The highest prevalence is observed in low- and middle-income countries, where access to antiviral therapies remains limited. In the United States, however, an increase in HCV infections has been observed, particularly since approximately 2010, in association with the escalating opioid epidemic and the increased prevalence of injection drug use<sup>2</sup>. Moreover, the low rate of HCV diagnosis and the large number of undiagnosed individuals, many of whom remain unaware of their infection, continue to pose a major concern. This is mainly due to the absence of noticeable symptoms during the early phase of infection. According to the most recent data, the detection rate of HCV infection in Poland is among the lowest in Europe and is estimated at only about 5%<sup>3</sup>. This is largely due to the lack of screening programs for high-risk groups, which could be incorporated into routine clinical practice. Early diagnosis of HCV infection is crucial, as acute infection progresses to chronic hepatitis C in approximately 75% of patients, potentially leading to severe liver damage, fibrosis, cirrhosis, and hepatocellular carcinoma. Consequently, untreated HCV infection can result in severe morbidity and mortality. Although the introduction of highly effective direct-acting antivirals (DAAs) has revolutionized HCV therapy, approximately one million new infections and 242,000 HCV-related deaths are still reported annually worldwide<sup>3,4</sup>. Recognizing this ongoing burden, the World Health Organization has identified HCV as a priority pathogen requiring vaccine development and has implemented a global elimination strategy aiming to reduce new infections by 90% and HCV-related mortality by 65% by 2030<sup>5</sup>.

For many years, progress in the development of novel antiviral therapies was hindered by the lack of a robust and reliable in vitro model for studying the complete HCV life cycle. The reasons why HCV isolated directly from patient serum cannot be efficiently adapted to cell culture systems remain incompletely understood. Nevertheless, the introduction of surrogate experimental systems, including retroviral pseudoparticles (HCVpp) and subgenomic replicons, enabled detailed investigation of viral entry mechanisms and intracellular replication processes<sup>6,7</sup>. A breakthrough occurred in 2005, when

several research groups independently succeeded in generating infectious HCV particles in the human hepatoma cell line Huh7<sup>8</sup>. This system, termed HCVcc (cell culture–derived HCV), made it possible to perform virus neutralization assays and to analyze individual stages of the viral life cycle in a comprehensive manner. The implementation of these experimental platforms rapidly advanced our understanding of the molecular mechanisms governing virus–host cell interactions. Importantly, this progress directly contributed to the development of highly effective DAAs targeting key viral proteins involved in distinct stages of the replication cycle. Prior to the advent of DAAs, therapeutic options for HCV-infected patients were limited to interferon- and ribavirin-based regimens, which were associated with modest efficacy and a high incidence of adverse effects<sup>9</sup>.

HCV is characterized by a remarkably narrow host range, infecting only humans and chimpanzees. The absence of suitable animal models for HCV infection therefore represents a major obstacle to vaccine development. Although chimpanzees—being the only natural animal host for HCV—played a pivotal role in the discovery and early characterization of the virus in the 1970s and 1980s, their use in biomedical research is now prohibited due to ethical considerations. Currently, *in vivo* studies most commonly rely on chimeric mouse models generated through xenotransplantation of human hepatocytes<sup>10</sup>. While these mice support HCV infection, they lack a functional adaptive immune system, which substantially limits their utility in evaluating vaccine-induced immune responses. Nonetheless, they remain an invaluable tool for testing novel antiviral compounds and therapeutic antibodies. In 2011, an immunocompetent mouse model was proposed in which the expression of human HCV entry receptors enabled infection of murine hepatocytes. However, the relatively low levels of viral replication observed in these transgenic animals significantly constrain their application in large-scale or routine vaccine efficacy studies<sup>11</sup>. More recently, the discovery of rat hepatitis virus (RHV) has provided an additional model for investigating fundamental aspects of viral pathogenesis and host immune responses. However, RHV is genetically distant from HCV, exhibiting limited sequence homology and failing to fully recapitulate the natural course of HCV infection in humans<sup>12</sup>.

The lack of suitable animal models has impeded vaccine development to such an extent that the scientific community has considered the implementation of controlled human infection model (CHIM). The availability of highly effective DAAs has created the theoretical possibility of vaccinating healthy volunteers with an experimental vaccine, followed by controlled exposure to a well-characterized HCV strain in order to assess vaccine efficacy. Despite the potential scientific value of this approach, CHIM studies raise substantial ethical concerns that continue to limit their practical implementation<sup>13,14</sup>.

HCV is further characterized by a high degree of genetic variability, which represents another major challenge for vaccine development. To date, eight major genotypes and more than 100 subtypes have been identified. These genotypes display distinct geographical distributions and differential responses to antiviral therapy, underscoring the necessity of developing a vaccine capable of conferring broad, pan-genotypic protection<sup>15</sup>.

The ultimate objective of an effective vaccine is the induction of sterilizing immunity, ensuring complete protection against infection. In the case of HCV, however, achieving this goal is likely to be particularly challenging. Because the clinical consequences of HCV infection typically manifest only after many years of chronic infection, preventing the progression from acute to chronic disease would already represent a significant clinical benefit. Achieving this objective requires the induction of a robust protective immune response, most likely involving both humoral and cellular arms of adaptive immunity,

capable of eliminating the virus during the acute phase of infection<sup>16</sup>.

The current landscape of HCV vaccine research encompasses a broad and rapidly evolving spectrum of candidates. Traditional strategies have included the use of inactivated or attenuated viruses, which are capable of inducing both robust humoral and cellular immune responses<sup>17,18</sup>. More contemporary approaches comprise subunit vaccines, virus-like particles (VLP), nucleic acid-based vaccines (DNA and RNA), as well as viral vector-based platforms. Among these strategies, recombinant envelope glycoproteins E1 and E2 have attracted particular attention. Due to their surface exposure on the viral particle, they are primary targets of neutralizing antibodies and therefore represent key antigens in rational vaccine design. A vaccine candidate based on the E1E2 heterodimer advanced to phase I clinical trials, highlighting its translational potential<sup>4,19</sup>. However, further clinical development was discontinued, largely due to technical challenges associated with large-scale production and purification of E1E2 from mammalian expression systems<sup>20</sup>. Moreover, a significant limitation of E1E2-based subunit vaccines is their tendency to elicit antibodies with limited breadth of neutralization. Analyses of immune responses induced by genotype 1-derived E1E2 have demonstrated relatively weak neutralizing activity against variants belonging to genotypes 2 and 3, underscoring the challenge of achieving broad cross-genotypic protection<sup>21–23</sup>.

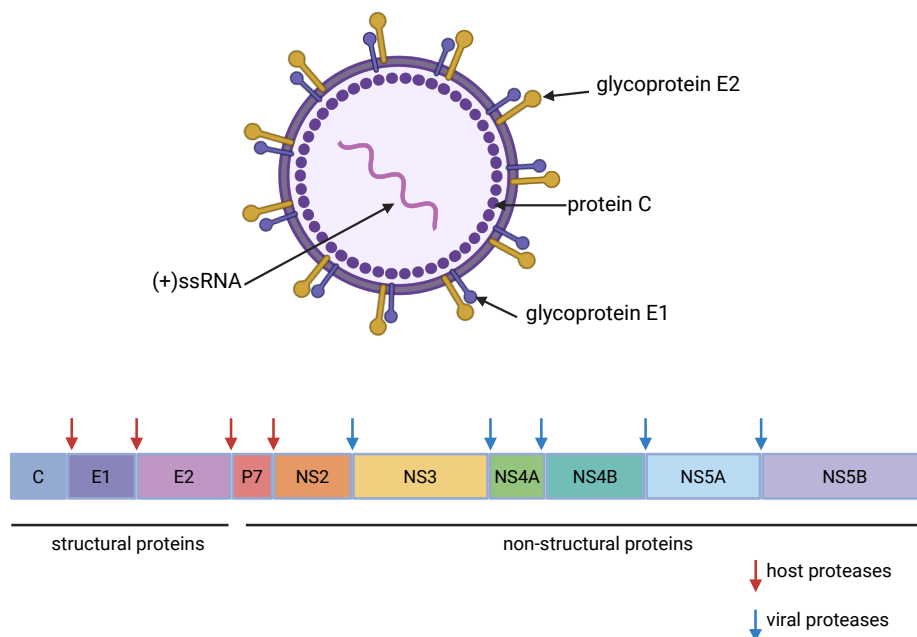


Figure 1: Schematic representation of HCV virion and the viral proteins encoded by its genome. HCV is an enveloped virus belonging to the *Flaviviridae* family. Its single-stranded positive-sense RNA genome encodes a single polyprotein that undergoes proteolytic cleavage mediated by both viral and host proteases. This processing results in the formation of three structural proteins—the core (C) protein and the envelope glycoproteins E1 and E2—as well as seven non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Created in BioRender.com

Given the high mutation rate and pronounced genetic variability of HCV, numerous vaccine development strategies have targeted viral non-structural (NS) proteins, which are relatively conserved and predominantly elicit strong cellular immune response<sup>24,25</sup>.

However, recent phase I and II clinical trials have demonstrated that vaccines based exclusively on NS proteins did not provide protective efficacy<sup>26,27</sup>. These findings highlight the necessity of developing other strategies capable of inducing both a potent cellular immune response and a robust humoral response characterized by broadly neutralizing antibodies. Achieving such a balanced and coordinated immune profile remains a central challenge in HCV vaccine design. The structural organization of HCV virion and the proteins encoded by its genome are presented in Figure 1.

To date, no commercial vaccine against HCV is available. Although direct-acting antiviral (DAA) therapy demonstrates high efficiency against infections caused by nearly all viral genotypes, its widespread implementation in many low- and middle-income countries remains limited, primarily due to the substantial cost of treatment. Furthermore, the emergence of variants harboring resistance-associated substitutions may result in therapeutic failure in a subset of patients<sup>28</sup>. Given the persistently low detection rates of HCV infection, it is widely acknowledged that the development of a prophylactic vaccine would significantly reduce both infection incidence and HCV-related mortality<sup>16</sup>. In this context, following the completion of my 18-month postdoctoral fellowship in Italy and my return to Poland, I directed my research efforts toward the identification and development of novel HCV vaccine candidates. My work focused on two complementary strategies: recombinant E1E2 heterodimer-based vaccines (publication no. 1) and chimeric virus-like particles (VLP) displaying conserved epitopes of HCV E2 glycoprotein (publications no. 2–4). These approaches were primarily designed to elicit a potent humoral immune response characterized by neutralizing antibodies. Recognizing, however, that effective vaccine should engage both arms of adaptive immunity, I subsequently expanded this research framework to incorporate strategies capable of inducing robust cellular immune responses. To achieve this, I designed a DNA-based vaccine platform utilizing minicircle DNA technology, which represents a safer and more efficient alternative to conventional bacterial plasmids previously employed in DNA vaccine development (Publication No. 5).

#### **4.3.2 Development of novel forms of the E1E2 heterodimer in *Leishmania tarantolae* cells**

The HCV envelope glycoproteins E1 and E2 mediate interactions with cellular receptors and play a critical role in viral entry into host cells. Their functional configuration is a highly glycosylated, non-covalent E1E2 heterodimeric complex. Of the two envelope glycoproteins encoded by the HCV genome, E2 constitutes the primary target of virus-neutralizing antibodies. Consequently, numerous research groups have pursued vaccine strategies based solely on recombinant E2. However, accumulating evidence indicates that the full E1E2 heterodimer elicits a more potent and broadly protective immune response, likely due to the presentation of a wider array of conformationally and structurally diverse neutralization-relevant epitopes<sup>29</sup>.

The large-scale production of the E1E2 heterodimer for vaccine applications remains technically challenging. Its intracellular retention in transfected mammalian cells contributes to cytotoxic effects and results in relatively low production yields. In addition, purification of recombinant E1E2 from the complex intracellular environment poses significant technical difficulties<sup>29</sup>. Notably, the E2 glycoprotein contains eleven N-linked glycosylation sites. Extensive studies have demonstrated that N-glycans play a critical role in maintaining the proper conformational structure of the heterodimer and in facilitating viral entry into host cells<sup>30,31</sup>. To date, intensive efforts continue to focus on

strategies that facilitate efficient production and purification of E1E2 while preserving the native antigenic structure of the complex, which is essential for the induction of antibodies with high neutralizing potency.

I initiated this line of research by investigating an alternative expression system for the recombinant E1E2 heterodimer, as production in mammalian cells is both inefficient and costly, despite enabling appropriate post-translational modifications. The unconventional expression platform based on the protozoan *Leishmania tarentolae* emerged as a promising system to produce viral glycoproteins, combining high protein yield with a homogeneous N-glycosylation profile closely resembling that observed in mammalian cells. A distinctive advantage of this system is its ability to generate complex-type N-glycans rather than predominantly high-mannose structures, which are typically produced in yeast- or baculovirus-based expression systems<sup>32</sup>.

Given the absence of prior reports describing the expression of HCV envelope glycoproteins E1 and E2 in *L. tarentolae*, I sought to determine whether this platform could serve as a viable alternative for generating a functional E1E2 heterodimer suitable for vaccine development. This research was supported by a grant awarded under the SONATA 3 program of the National Science Centre. My research strategy therefore encompassed comprehensive biochemical and functional characterization of the expressed proteins. In particular, I evaluated their receptor-binding properties and their ability to induce a specific antiviral immune response. The findings from this study were reported in the first publication included in the series, and the overall experimental design is summarized in Figure 2.

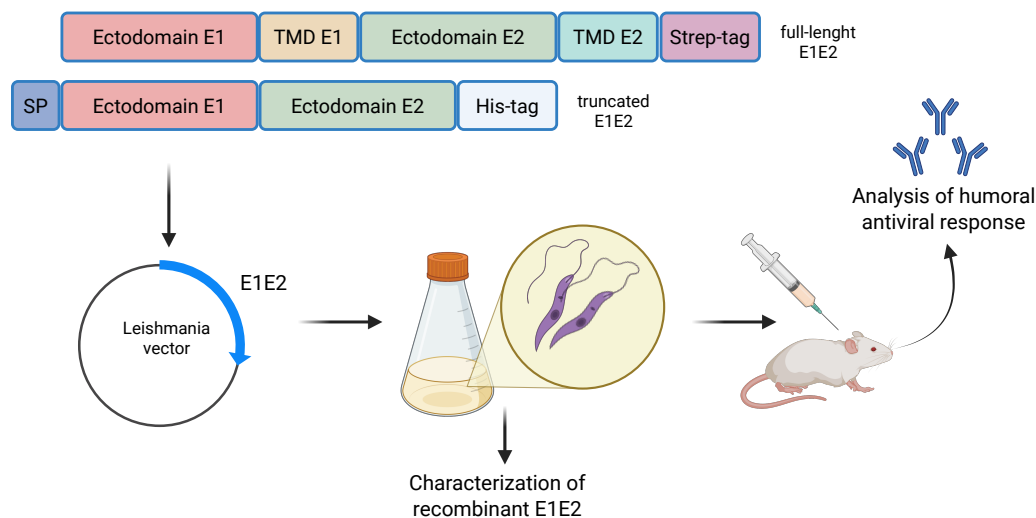


Figure 2: Schematic representation of the construction and production of recombinant E1E2 variants in *Leishmania tarentolae* cells. TMD – transmembrane domain; SP – signal peptide derived from *L. mexicana* facilitating secretion of the protein into the culture medium. Created with BioRender.com based on publication Grzyb *et al.*, 2016.

I initiated the experimental phase by designing two expression constructs: a full-length E1E2 heterodimer and a truncated variant lacking the transmembrane domains (TMDs). Deletion of the TMDs, combined with the incorporation of a signal peptide directing the protein into the secretory pathway, enabled the generation of a soluble,

secreted form of E1E2 and facilitated its release into the culture medium. For protein expression, I employed an inducible vector system that allowed stable integration of the recombinant coding sequence into the *Leishmania* genome and regulated transgene expression under the control of a tetracycline-responsive operator. The E1E2 antigens were designed based on the genotype 1a sequence (strain H77). Subsequently, I performed detailed biochemical and functional characterization of the expressed proteins using immunofluorescence and Western blot analysis with specific anti-E1 and anti-E2 antibodies. Confocal microscopy confirmed expression of both E1E2 variants in *L. tarentolae* cells. In mammalian systems, the full-length E1E2 precursor is cleaved by a host protease into two separate glycoproteins, E1 and E2, which subsequently reassemble into a non-covalent heterodimer within the endoplasmic reticulum. In contrast, I demonstrated that E1E2 produced in *Leishmania* was not efficiently processed by host protease, suggesting the absence or limited activity of the relevant cleavage machinery in this expression system. Despite this difference in proteolytic processing, both E1E2 variants retained functional properties. First, through access to a panel of human monoclonal antibodies (CBH-7, CBH-4G, CBH-4B, HC-1, HC-11), kindly provided by Prof. Steven Fong (Stanford University), I identified multiple conformational epitopes in both variants that are essential for maintaining native protein conformation and are critically involved in viral neutralization. Second, I demonstrated that both forms could bind CD81, the principal entry receptor for HCV on host cells. Subsequently, both E1E2 variants were successfully purified using affinity chromatography. Notably, incorporation of a Strep-tag into the full-length E1E2 construct enabled highly efficient single-step purification directly from the cellular lysate, significantly simplifying the production workflow. The purified E1E2 proteins were then administered to BALB/c mice in combination with the AddaVax adjuvant. The immunization outcomes were highly encouraging, as very high titers of antigen-specific antibodies were detected after the third dose.

Assessment of cross-reactivity by ELISA using E1E2 heterodimers derived from multiple HCV genotypes and subtypes revealed the strongest antibody responses against genotypes 1a, 1b, 5, and 6, whereas the lowest reactivity was observed against genotypes 2a and 3a. These findings are consistent with previously reported data describing the antibody response profile following immunization with genotype 1a-derived E1E2. Earlier studies have demonstrated that immunization with genotype 1a (H77) E1E2 produced in mammalian cells typically elicits antibodies with limited neutralizing breadth against genotypes 2 and 3<sup>21–23,29</sup>. Importantly, our findings were consistent with these observations, confirming reduced reactivity toward genotypes 2a and 3a compared with genotypes 1a, 1b, 5, and 6. However, despite the lower ELISA reactivity against genotype 2a-derived E1E2, sera from both immunized groups were capable of neutralizing genotype 2a HCV in vitro in a cell culture-based assay. Moreover, the high antibody titers induced by both E1E2 variants provide a strong foundation for further vaccine development. These findings suggest that combining E1E2 antigens derived from genotypes 1, 2, and 3 into a single multivalent formulation may enhance the breadth of neutralizing responses and improve cross-genotypic coverage. Indeed, subsequent studies have proposed a pentavalent vaccine candidate composed of E1E2 derived from genotypes 1a, 1b, 2a, 3a, and 6a, which demonstrated superior pan-genotypic antibody responses in murine models compared with monovalent formulations<sup>33</sup>.

In summary, E1E2 produced in protozoan cells represents an attractive and cost-effective alternative for large-scale antigen production, particularly considering the technical challenges and high costs associated with mammalian expression systems. Our

findings, together with evidence from other research groups, support the development of multivalent E1E2-based formulations to broaden cross-neutralization capacity and increase the likelihood of achieving an effective, broadly protective HCV vaccine.

#### 4.3.3 Development of chimeric HBV/HCV Virus-Like Particles displaying conserved E2 epitopes

The extensive genetic diversity of HCV—comprising eight major genotypes and more than 100 subtypes that may differ by up to 30–40% at the nucleotide sequence level—represents one of the principal obstacles to the development of an effective vaccine<sup>15</sup>. Consequently, a rational strategy for achieving broad protective efficacy is to target highly conserved regions within the E2 glycoprotein, thereby increasing the likelihood of inducing cross-genotypic immunity. One such region is epitope I, spanning amino acid residues 412–423 of the E2 glycoprotein. This epitope is recognized by multiple neutralizing antibodies, including the well-characterized monoclonal antibody AP33, which exhibits broad neutralizing activity against viral isolates from diverse genotypes<sup>34,35</sup>. To induce a focused and effective immune response against epitope I, we engineered a strategy in which this sequence was displayed on the small hepatitis B surface antigen (sHBsAg), a protein that spontaneously assembles into virus-like particles (VLP)<sup>36,37</sup>. VLP are recombinant protein that structurally resemble native virions but lack viral genetic material, rendering them safe while retaining high immunogenicity. Their multimeric and repetitive spatial organization enables highly efficient activation of both humoral and cellular immune responses<sup>38</sup>. Importantly, sHBsAg is already widely used as the antigenic component of the licensed hepatitis B virus (HBV) vaccine and is characterized by strong intrinsic immunogenicity. These properties make it an attractive and well-validated carrier platform for the presentation of heterologous epitopes<sup>39</sup>. Funding for the characterization and application of chimeric HBV/HCV VLP displaying epitope I—as a potential bivalent vaccine candidate targeting two major hepatotropic viruses—was awarded to me in 2012 under the LIDER program of the National Centre for Research and Development. The development of these VLP constituted the primary research objective of the doctoral project conducted by Anna Czarnota, for whom I served as an auxiliary supervisor.

In the second publication of the series, we reported for the first time the successful production of chimeric HBV/HCV VLP based on sHBsAg in the *Leishmania tarentolae* expression system. Proper assembly of VLP displaying epitope I on their surface was confirmed by immunogold labelling followed by transmission electron microscopy, employing the epitope I-specific monoclonal antibody AP33 and gold-conjugated secondary antibodies (Figure 3B). The successful implementation of this immunolabeling approach was facilitated by practical workshop in advanced electron microscopy techniques undertaken in Wageningen, the Netherlands, in 2015, which I attended together with the PhD student. Importantly, our studies conducted in a murine model demonstrated that the chimeric VLP were highly immunogenic, eliciting both a cellular immune response directed against the sHBsAg carrier protein and a robust humoral response. The latter was characterized by the production of antibodies recognizing a synthetic peptide corresponding to amino acids 412–425 of the HCV E2 glycoprotein, as well as the yeast-derived sHBsAg protein itself. Demonstrating the preservation of immunogenicity of the sHBsAg carrier was critical for the development of a bivalent prophylactic vaccine targeting both HBV and HCV. These findings clearly indicate that insertion of the HCV epitope I sequence into the immunodominant hydrophilic loop of sHBsAg did not compromise the antibody response directed against HBV.

Recent structural studies of the E1E2 heterodimer have provided critical insights into the mechanisms governing viral interaction with cellular receptors and antibody recognition. These investigations have enabled the identification of several antigenic regions within the E2 glycoprotein, including sites targeted by broadly neutralizing antibodies, which may play a pivotal role in the rational design of next-generation HCV vaccines. As a result of these structural analyses, a so-called “neutralizing face” of the E2 protein has been proposed. This region encompasses amino acid residues 412–446 and 525–535 and includes the CD81 receptor-binding loop, as well as multiple linear and conformational neutralizing epitopes<sup>40–42</sup>.

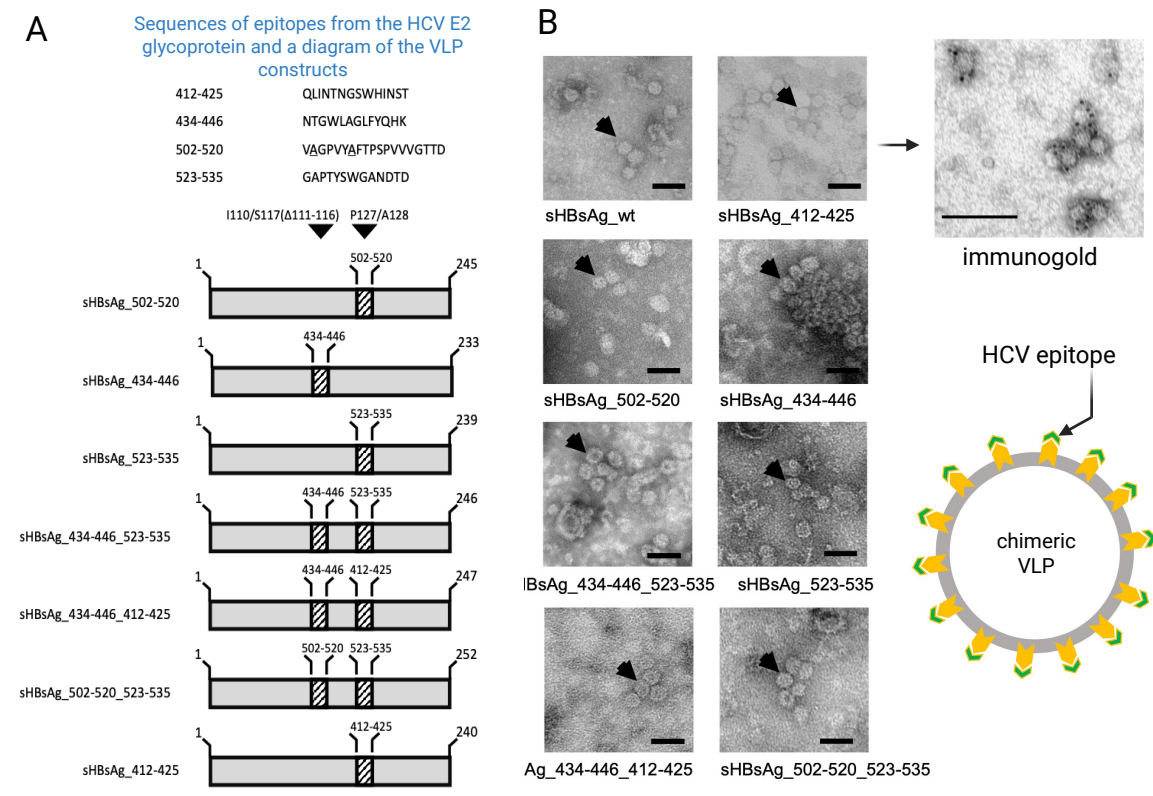


Figure 3: Schematic representation of the construction of chimeric HBV/HCV VLPs. (A) Sequences of epitopes derived from the HCV E2 glycoprotein and a diagram of the constructs illustrating insertion of HCV epitopes into the hydrophilic loop of the small hepatitis B surface antigen (sHBsAg). The insertion sites are indicated by black arrows. (B) Schematic representation of a chimeric VLP displaying an HCV epitope, together with transmission electron microscopy images of the particles. On the right, confirmation of surface exposure of epitope 412–425 on sHBsAg using immunogold labelling with secondary antibodies conjugated to 6 nm gold particles. Based on Czarnota *et al.*, 2016 and 2020.

Given the high immunogenicity of the chimeric HBV/HCV VLP displaying epitope I, we expanded our approach by designing additional VLP constructs presenting other highly conserved epitopes within the E2 glycoprotein. The objective was to develop a vaccine candidate targeting multiple HCV epitopes rather than a single antigenic site. Targeting conserved regions of E2 is currently regarded as one of the most promising strategies for inducing broadly neutralizing antibodies with pan-genotypic activity. Ac-

cordingly, in subsequent studies, in addition to epitope I (412–425), we incorporated regions 434–446, 502–520, and 523–535 into the VLP platform (Figure 3A). These regions are highly conserved within the E2 sequence and play a crucial role in E2 interaction with the CD81 entry receptor on host cells. Consequently, they represent attractive targets for neutralizing antibodies, and induction of immune responses directed against multiple conserved regions constitutes a rational strategy toward achieving broad and potentially universal vaccine protection.

In the third publication of the series, coding sequences corresponding to the selected regions were inserted individually or in defined combinations into the sHBsAg sequence. VLP were produced using the previously established *Leishmania tarentolae* expression system, and correct particle assembly was confirmed by transmission electron microscopy (Figure 3B). To evaluate immunogenicity, eight groups of BALB/c mice were immunized with the respective chimeric VLP. Following completion of the immunization schedule, high titers of epitope-specific antibodies were detected in sera from all experimental groups. These antibodies recognized synthetic peptides corresponding to the E2 epitopes displayed on the surface of the individual VLP constructs. However, distinct differences were observed in (i) serum reactivity against the sHBsAg carrier protein, (ii) binding profiles to E1E2 heterodimers derived from various HCV genotypes under reducing–denaturing versus native conditions, and (iii) neutralization capacity. The principal conclusions from these studies indicate that VLP displaying a single HCV epitope efficiently induced antibodies against the sHBsAg carrier protein, with the exception of constructs presenting epitope 434–446. Insertion of this particular sequence may slightly alter the conformation of the immunodominant hydrophilic loop of sHBsAg, thereby reducing the ability of the corresponding VLP to elicit antibodies against the carrier protein—an effect similarly observed for VLP presenting dual epitopes. Importantly, ELISA analyses demonstrated that antibodies elicited by VLP displaying epitopes 412–425 and 523–535 were capable of binding native E1E2 heterodimers derived from multiple HCV genotypes.

During a scientific conference in Dublin, I initiated a collaboration with Prof. Judith Gottwein from the University of Copenhagen. Prof. Gottwein’s group has access to an extensive panel of HCV genotypes and possesses considerable expertise in performing neutralization assays in the context of HCV vaccine research. This collaboration provided a unique opportunity to comprehensively evaluate the functional properties of antibodies elicited by our VLPs. Within this framework, we assessed the neutralizing capacity of antibodies induced by VLPs displaying epitopes 412–425 and 523–535, as only these constructs had previously demonstrated binding to native E1E2 heterodimers derived from multiple viral genotypes in ELISA assays. Robust neutralizing activity was observed exclusively in sera from animals immunized with VLPs presenting epitope 412–425. The strongest neutralization was detected against viruses representing genotypes 1a, 1b, 4a, and 5a. Antibodies neutralized genotypes 2a and 2b with moderate efficiency, whereas for genotypes 3a and 6a, less than 50% neutralization was achieved even at the highest antibody concentration tested. Notably, the observed differences in neutralization efficiency across genotypes occurred despite the relatively conserved amino acid sequence of the 412–425 region. These findings suggest the involvement of epitope shielding mechanisms, the extent of which may vary among genotypes due to sequence variations outside the 412–425 region that influence the overall conformation of the E1E2 heterodimer. Such structural differences may ultimately affect epitope accessibility to neutralizing antibodies across viral variants. Importantly, these results underscore the intrinsic challenge of designing a universal HCV vaccine antigen. Even when targeting conserved regions of the

E2 glycoprotein, epitope accessibility—and consequently neutralization potency—may differ depending on the viral genotype.

In summary, the data obtained in this study, particularly the functional neutralization analyses, confirm that epitope I constitutes the most promising target for the development of a prophylactic HCV vaccine.

HCV is known to employ multiple mechanisms to evade the host immune response. Owing to these strategies, infection can progress to a chronic state of disease. Viral immune escape mechanisms significantly complicate the design of new vaccine candidates. Moreover, HCV can select variants that are no longer recognized by the host immune system. This phenomenon is largely driven by hypervariable regions within the E2 glycoprotein, often referred to as “immunological decoys,” which constitute major targets for neutralizing antibodies. However, due to their high variability, these regions enable the virus to effectively escape antibody-mediated neutralization<sup>43</sup>. Both viral envelope glycoproteins, E1 and E2, are heavily glycosylated. Numerous studies have demonstrated that N-linked glycosylation of the E1E2 heterodimer contributes to viral evasion of the host humoral immune response through the formation of a so-called “glycan shield,” which sterically hinders the binding of neutralizing antibodies to E1E2<sup>44,45</sup>.

Another important immune escape mechanism involves shifts in N-linked glycosylation sites, referred to as a “glycan shift,” which has been described for epitope I (412–423). Epitope I, a key target of multiple broadly neutralizing antibodies, contains an N-linked glycosylation site at position N417. Substitutions such as N417S or N417T can result in shift of the glycan moiety from N417 to N415. This glycan shift effectively masks epitope I, thereby preventing its recognition by neutralizing antibodies directed against the E2 glycoprotein. Although HCV variants harboring glycan shift mutations (N417S/T) are relatively rare in the general population, their emergence is strongly favored under selective pressure conditions, such as immunotherapy with potent monoclonal antibodies targeting epitope I<sup>42,46</sup>. Therefore, in the context of vaccine strategies directed against this conserved region, it is essential to consider both glycosylation variants and to design antigens capable of overcoming this specific mechanism of viral immune escape.

In our previous studies, we demonstrated that sHBsAg-based virus-like particles displaying epitope I of the HCV E2 glycoprotein are highly immunogenic and capable of inducing antibodies with cross-neutralizing activity against multiple viral genotypes. Building on these findings, in the fourth publication of the series we designed an additional VLP-based antigen specifically intended to address viral escape variants associated with the glycan shift phenomenon. As noted, epitope I contains an N-linked glycosylation site at position N417. Substitution of N417 with serine or threonine (N417S/T) results in relocation of the N-glycan from position N417 to N415, thereby masking the epitope from antibody recognition. To model this escape mechanism, we generated the sHBsAg\_N417S construct by introducing the N417S substitution into the 412–425 epitope sequence displayed on VLPs presenting the wild-type epitope (Figure 4). Virus-like particles containing either the wild-type 412–425 epitope or the N417S variant were produced in the *Leishmania tarentolae* expression system. For their structural and antigenic characterization, we employed the broadly neutralizing monoclonal antibody AP33, which specifically recognizes the wild-type epitope I. Contrary to previous reports indicating a lack of AP33 binding to the E1E2 complex harboring the N417S mutation, we demonstrated that AP33 efficiently binds to both the wild-type (412–425:WT) and the N417S variant when presented on the surface of VLPs. This finding strongly suggests that epitope conformation and accessibility differ depending on the structural context in which

the epitope is displayed—namely, on VLPs versus within the native E1E2 heterodimer. Importantly, this observation was further validated in a mammalian protein expression system (HEK293 FreeStyle), thereby excluding the possibility that the effect resulted from *Leishmania*-specific N-glycosylation patterns.

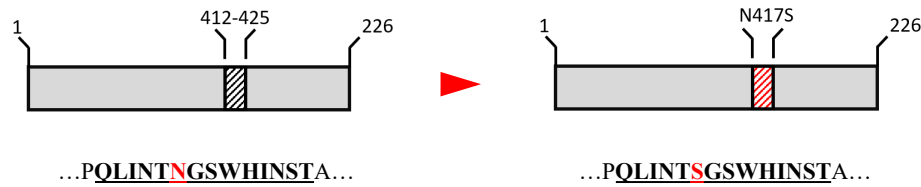


Figure 4: Schematic representation of the generation of the chimeric sHBsAg\_N417S construct by introducing the N417S substitution into the HCV epitope 412–425 sequence. Based on Czarnota *et al.*, 2023.

Immunogenicity studies conducted in the BALB/c mouse model demonstrated that both sHBsAg\_412–425 and sHBsAg\_N417S VLPs were immunogenic and elicited antibodies recognizing peptide sequences encompassing epitope I, regardless of the presence of the N417S mutation. However, pronounced differences were observed in antibody binding to E1E2 glycoproteins and in the neutralization of cell culture–derived HCV (HCVcc) between sera obtained following immunization with sHBsAg\_412–425 and sHBsAg\_N417S VLPs. Sera from mice immunized with sHBsAg\_412–425 efficiently bound native E1E2:WT glycoproteins but showed markedly reduced binding to the N417S variant. In contrast, sera from mice immunized with sHBsAg\_N417S selectively recognized the E1E2 variant carrying the N417S mutation, with minimal reactivity toward the wild-type form. Neutralization assays performed in collaboration with the University of Copenhagen further underscored these differences. Antibodies induced by sHBsAg\_412–425 VLPs—similarly to the monoclonal antibody AP33—effectively neutralized wild-type HCV isolates representing genotypes 1a and 2a but failed to neutralize their corresponding N417S escape variants. Conversely, antibodies elicited by sHBsAg\_N417S VLPs displayed only marginal neutralizing activity, even against viruses harboring the glycan-shift mutation, including the genotype 1a H77 isolate carrying the N417S substitution and the genotype 6a HK6a isolate containing the N417T mutation.

In the present study, we also observed striking similarities in the E1E2 glycoprotein binding profile and HCV neutralization efficacy between sera obtained from mice immunized with sHBsAg\_412–425 and the monoclonal antibody AP33. These findings suggest that immunization with sHBsAg\_412–425 VLPs may induce antibodies closely resembling AP33 in their functional properties. The binding profile of sHBsAg\_412–425 sera was comparable to that of AP33 and demonstrated dependence on key amino acid residues within epitope I, specifically L413, G418, W420, and N415. This comprehensive epitope-mapping analysis was made possible through collaboration with Prof. Arvind Patel (University of Glasgow), who provided expression vectors encoding a complete panel of alanine substitution mutants spanning the full sequence of epitope I within the genotype 1a E1E2 heterodimer.

Although immunization with the N417S variant did not generate an effective neutralizing antibody response against glycan-shift escape mutants, our findings reveal a complex interplay between epitope I–directed antibodies, the glycosylation status of E1E2, and

both the local conformation of the epitope and the global architecture of the heterodimer. Collectively, this study highlights the central importance of epitope I as a vaccine target and represents an important step toward the rational design of immunogens capable of eliciting protective responses against epitope I variants, including glycan-shift-mediated escape mutants.

#### 4.3.4 Development of a DNA vaccine based on Minicircle DNA technology

In our previous studies, conducted together with my research team, I demonstrated that chimeric sHBsAg\_412–425 VLPs can induce antibodies with broad cross-reactivity against multiple HCV genotypes. However, evidence from clinical studies, as well as investigations employing the rat hepatitis virus (RHV) model, indicate that effective control of HCV infection requires the coordinated activation of both humoral and cellular immune responses<sup>47</sup>. Accordingly, the objective of the subsequent project was to develop a vaccine strategy capable of engaging both arms of the adaptive immune system, thereby enhancing protective efficacy against HCV. Funding for this research was secured through the NCN OPUS 14 grant, which focused on evaluating the immunogenicity of conserved epitopes derived from the non-structural (NS) region fused to the previously developed chimeric sHBsAg\_412–425 platform. The initial concept involved designing a single chimeric VLP capable of inducing a comprehensive immune response encompassing both broadly neutralizing antibodies and HCV-specific T-cell responses. Despite substantial optimization efforts, efficient expression of the engineered VLP constructs could not be achieved. Nevertheless, the goal of developing a vaccine capable of inducing both humoral and cellular immune responses against HCV remained central and guided the next stage of the study.

Consequently, in the fifth publication of the series, we introduced a DNA-based vaccine employing minicircle (MC) technology as an alternative antigen delivery platform to conventional plasmid vectors. DNA vaccines have been shown to induce both humoral and cellular immune responses against a variety of pathogens, including HCV<sup>48,49</sup>. Recent studies utilizing DNA constructs encoding either the E1E2 heterodimer or HCV non-structural proteins further underscore the translational potential of this approach<sup>50,51</sup>. Although first-generation DNA vaccines demonstrated limited immunogenicity in humans, technological advancements have substantially improved their efficacy<sup>52</sup>. In particular, minicircle DNA (MC) technology has emerged as a promising next-generation gene delivery platform. Minicircles are small DNA vectors derived from parental plasmids (PPs) that lack bacterial backbone sequences. Removal of bacterial backbone enhances their safety profile and enables higher and more sustained levels of antigen expression. MC vectors can be applied both *in vivo* and *in vitro*, providing prolonged antigen expression while minimizing the risk of undesired immune responses triggered by bacterial sequences present in conventional plasmids used in gene therapy<sup>53,54</sup>. Although MC technology has thus far been explored in a limited number of biomedical applications, it has yielded highly encouraging results, offering innovative solutions in gene therapy, cancer immunotherapy, and vaccine development<sup>55–59</sup>.

During the course of this project, we sought to determine whether the previously developed sHBsAg\_412–425 construct would retain its capacity to induce functional antibodies when delivered via a DNA-based platform, and whether the inclusion of viral proteins derived from the non-structural (NS) region could effectively activate the cellular arm of the adaptive immune response. To address these questions, we designed three minicircle (MC) constructs encoding chimeric HBV/HCV VLPs and two additional

MC constructs encoding HCV non-structural proteins—the viral protease NS3/4A and the RNA-dependent RNA polymerase NS5B. The immunogenicity of these constructs was evaluated in a murine model (Figure 5). The combined immunization regimen, consisting of MCs encoding sHBsAg\_412–425 together with MCs encoding NS proteins, resulted in the induction of a coordinated and comprehensive immune response against HCV. Co-administration of these constructs elicited a strong cellular immune response, particularly directed against NS3, alongside high titers of E2-specific antibodies. Importantly, the induced antibodies recognized native E1E2 heterodimers derived from multiple viral genotypes and displayed an alanine-mutant binding profile closely resembling that of the broadly neutralizing monoclonal antibody AP33, further supporting their functional relevance.

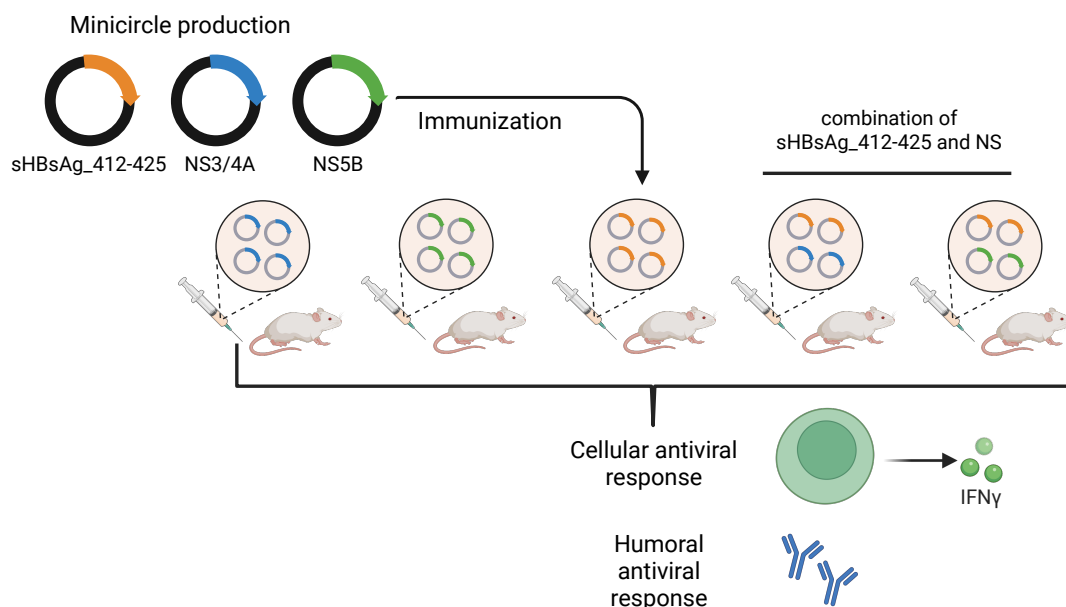


Figure 5: Schematic representation of the immunization strategy using minicircle (MC) DNA constructs encoding chimeric HBV/HCV VLPs (sHBsAg\_412–425), NS3/4A, and NS5B. Based on Czarnota *et al.*, 2024. Created with BioRender.com

In summary, these findings demonstrate that a minicircle DNA-based vaccine encoding both sHBsAg\_412–425 and HCV NS proteins can simultaneously stimulate robust cellular immunity and generate antibodies with functional characteristics comparable to those of the neutralizing antibody AP33. Given the well-recognized challenges associated with designing vaccines capable of effectively engaging both arms of the adaptive immune system, our results highlight the substantial potential of this combined antigen strategy as a pan-genotypic prophylactic vaccine candidate against HCV.

#### 4.4 Summary of the major achievements resulting from the presented series of publications

Based on the presented series of five publications, the following major and original scientific achievements can be identified:

- Pioneering implementation of a recombinant protein expression system based on the protozoan *Leishmania tarentolae* as a viable and cost-effective alternative to

mammalian cells for the efficient production of functionally relevant HCV vaccine antigens capable of inducing strong and specific immune responses.

- Development and functional characterization of novel E1E2 heterodimer variants capable of eliciting HCV-specific antibody responses with cross-genotypic reactivity.
- Design and generation of correctly assembled chimeric HBV/HCV virus-like particles (VLP) displaying conserved epitopes of the HCV E2 glycoprotein, together with comprehensive evaluation of the neutralizing capacity of the induced antibodies in a murine model.
- Identification of the epitope I–displaying VLP (sHBsAg\_412–425) as a leading vaccine candidate, capable of inducing broadly neutralizing antibodies and representing a potential platform for (i) a bivalent vaccine targeting both hepatotropic viruses, HCV and HBV, or (ii) a component enhancing pan-genotypic humoral immunity within multivalent HCV vaccine formulations.
- Investigation of the glycan shift phenomenon, involving relocation of N-linked glycosylation from position N417 to N415 within epitope I of the HCV E2 glycoprotein, and assessment of its implications for antibody recognition and viral immune escape.
- Development of DNA vaccine strategy combining structural and non-structural HCV antigens, based on minicircle DNA (MC) technology, applied for the first time in this context to achieve coordinated stimulation of both humoral and cellular immune responses against HCV.

Despite intensive global research efforts, an effective vaccine against HCV remains unavailable. The identification of novel vaccine candidates therefore continues to represent an urgent and highly relevant scientific challenge. Within the framework of the studies conducted under my leadership, we proposed and systematically developed new concepts for a rationally designed HCV vaccine. Of particular significance is the fact that we were the first to employ a recombinant expression system based on the protozoan *Leishmania tarentolae* to produce both full-length and truncated forms of the E1E2 heterodimer, as well as virus-like particles based on the small hepatitis B surface antigen (sHBsAg). This platform enabled efficient protein production while preserving essential post-translational modifications, particularly N-linked glycosylation, which is critical for maintaining native antigenic structure. Furthermore, the strategy of presenting the conserved epitope I of the HCV E2 glycoprotein (aa412–425) on the surface of sHBsAg-based VLPs resulted in the induction of a strong humoral immune response, including neutralizing antibodies active against the majority of HCV genotypes. Importantly, the binding profile of these antibodies closely resembled that of the well-characterized broadly neutralizing monoclonal antibody AP33, underscoring their functional relevance. We additionally demonstrated that sHBsAg, when used as an epitope carrier within chimeric HBV/HCV particles, retains its intrinsic immunogenicity, eliciting both antigen-specific humoral and cellular immune responses. These findings indicate that chimeric HBV/HCV VLP (sHBsAg\_412–425) may serve as a foundation for the development of a bivalent vaccine targeting both HBV and HCV. At the same time, the genotype-specific differences in neutralization efficiency observed following immunization with sHBsAg\_412–425 VLP—despite the high sequence

conservation of epitope I across genotypes—highlight the structural complexity underlying vaccine design. Variations outside epitope I may influence the overall conformation of the E1E2 heterodimer and consequently modulate epitope accessibility to neutralizing antibodies, emphasizing the challenges inherent in developing a truly universal antigen. Moreover, in response to the occurrence of glycan variants within epitope I, we designed the sHBsAg\_N417S VLP carrying the escape mutation N417S within the 412–425 region of E2. This approach enabled us to investigate the functional consequences of glycan shift from position N417 to N415 and to assess its impact on antibody recognition. Elucidating such viral immune escape mechanisms is essential for the rational design of next-generation vaccines capable of counteracting adaptive viral strategies.

In our most recent work, we were the first to apply minicircle DNA (MC) technology as a platform for the development of a DNA-based HCV vaccine. To achieve coordinated activation of both arms of the adaptive immune system, we designed a vaccine construct encoding both chimeric HBV/HCV VLP (sHBsAg\_412–425) and HCV non-structural (NS) proteins. Combined delivery of these antigens within a single immunization strategy resulted in the induction of a robust cellular immune response directed against NS proteins, alongside a strong humoral response characterized by epitope I-specific antibodies with functional properties closely resembling those of the broadly neutralizing antibody AP33.

Funding for the above research was secured through three competitive grants: NCN SONATA 3, NCN OPUS 14, and NCBiR LIDER. During the implementation of these projects, I systematically expanded my scientific expertise by participating in advanced training courses and specialized workshops. These included an advanced ELISpot course focused on cellular immune response analysis (Hamburg), hands-on workshops in electron microscopy techniques (Wageningen, the Netherlands), and the EURIPRED workshop dedicated to peptide matrix applications in immunology and vaccine research (Berlin). I also completed certified training for researchers involved in the use of animals for scientific and educational purposes.

Several of the developed vaccine candidates have been secured by intellectual property protection—two Polish patents and one European patent have been granted, with financial support from the Technology Transfer Center of the University of Gdańsk. Through the execution of these projects, I made a substantial and lasting contribution to the overall research program of the Department. I significantly expanded the laboratory's methodological capabilities in HCV research by establishing and optimizing recombinant protein production systems, implementing advanced electron microscopy approaches, and developing assays for the comprehensive analysis of humoral and cellular immune responses. All publications included in the presented series involved animal studies; accordingly, I was responsible for the design and planning of animal experiments and for preparing and submitting multiple applications to the Local Ethics Committee. In parallel, I established collaborations with leading HCV vaccine research centres in Copenhagen and Glasgow, which enabled detailed functional characterization of antibodies induced by the chimeric VLPs developed in our laboratory.

The presented work defines new directions in the rational design of HCV vaccines by integrating innovative expression platforms, conserved epitope presentation strategies, and minicircle DNA technology to achieve coordinated humoral and cellular immune responses with pan-genotypic potential.

#### 4.5 Significance of the obtained results for the development of the discipline of Biotechnology

The studies presented in this series make a substantial contribution to the advancement of medical biotechnology, particularly in the field of next-generation viral vaccine design. HCV remains a major global public health concern, affecting nearly 50 million people worldwide. Despite the availability of highly effective direct-acting antivirals, the absence of a preventive vaccine continues to hinder effective control and elimination of infections, resulting in a persistent number of new cases. The development of an HCV vaccine faces numerous challenges, including the extensive genetic diversity of the virus and the lack of appropriate *in vivo* models for evaluating vaccine efficacy. Nevertheless, research in this field continues to progress dynamically. Innovative approaches—including subunit vaccines, virus-like particles (VLPs), viral vectors, and DNA- and RNA-based platforms—have generated promising results<sup>16</sup>.

An effective HCV vaccine must achieve a balanced immune response involving both B- and T-cell-mediated mechanisms. Broadly neutralizing antibodies may prevent infection, whereas virus-specific T-cell responses are essential for viral control and clearance. An optimal vaccine should therefore combine structural and non-structural antigens in order to induce both potent antibody responses and multifunctional T-cell immunity.

Targeting both structural and non-structural HCV proteins—through the diverse approaches applied in our research, ranging from recombinant E1E2 heterodimers to chimeric HBV/HCV VLPs and a DNA platform based on minicircle technology—creates an opportunity to induce durable and pan-genotypic immune responses. Particular attention should be given to the sHBsAg<sub>412–425</sub> construct, which represents a promising candidate for a bivalent vaccine against two hepatotropic viruses or may serve as a component of a vaccine formulation enhancing pan-genotypic humoral immunity against HCV, either in protein-based or DNA-based form. Research focused on conserved epitopes and their optimized presentation directly addresses key challenges in contemporary biotechnology. Achieving universal immunity despite extensive viral variability remains a critical research objective, especially in the broader context of preparedness for future pandemics.

The approaches described above contribute significantly to the development of safe and scalable vaccine technologies and possess a universal character, as they may also be applied to the design of vaccines against other viral pathogens. Owing to these studies, together with ongoing international research efforts, the elimination of HCV through vaccination is becoming an increasingly realistic goal.

#### 4.6 Additional scientific achievements

A candidate for the degree of Doctor Habilitated is expected to demonstrate more than a single scientific achievement; therefore, I would like to present my additional research accomplishments. Below, I present two additional achievements. The first comprises two publications resulting from my postdoctoral fellowship in Italy. The results obtained during this period were published in two highly prestigious journals with high impact factors: *Hepatology* and *Journal of Hepatology*. Both publications are the result of international collaboration among multiple laboratories recognized worldwide for their pivotal contributions to HCV research. Notably, one of the co-authors is Prof. Charles Rice, who was awarded the Nobel Prize in Physiology or Medicine in 2020 for the discovery of HCV.

Meuleman, P., Catanese, M.T., Verhoye, L., Desombere, I., Farhoudi, A., Jones, C.T., Sheahan, T., **Grzyb, K.**, Cortese, R., Rice, C.M., Leroux-Roels, G., Nicosia, A.. (2012). A human monoclonal antibody targeting SR-B1 precludes hepatitis C virus infection and

viral spread *in vitro* and *in vivo*. *Hepatology*; 55, 364-72, doi:10.1002/hep.24692

IF<sub>2012</sub> - 12.0; IF<sub>5-year</sub> - 14.5; MEiN<sub>2012</sub> - 50; MEiN<sub>2025</sub> - 200; citation count - 113 (WoS)

Lacek, K., Vercauteren, K., **Grzyb, K.**, Naddeo, M.R., Verhoye, L., Słowikowski, M., Fafi-Kremer, S., Patel, A.H., Baumert, T.F., Folgori, A., Leroux-Roels, G., Cortese, R., Meuleman, P., Nicosia, A.. (2012). Novel human SR-B1 antibodies prevent infection and dissemination of HCV in vitro and in humanized mice. *Journal of Hepatology* 57, 17-23. doi:10.1016/j.jhep.2012.02.018

IF<sub>2012</sub> - 9.85; IF<sub>5-year</sub> - 28.1; MEiN<sub>2012</sub> - 45; MEiN<sub>2025</sub> - 200; citation count - 67 (WoS)

The focus of my research during my stay in Italy was the characterization of novel human monoclonal antibodies targeting the SR-B1 receptor as potential immunotherapeutic tools, for example in preventing reinfection of transplanted livers in patients with chronic HCV infection. The SR-B1 receptor, similarly to CD81, represents one of the principal cellular receptors responsible for viral entry; therefore, blockade of this receptor may effectively inhibit viral entry into host cells and prevent subsequent replication<sup>60</sup>.

I joined the research group of Prof. Alfredo Nicosia during the characterization of anti-SR-B1 antibodies designated mAb16-71, described in the publication in *Hepatology*. In this study, we demonstrated a strong capacity of the antibodies to block HCV entry in vitro using the Huh7.5 cell line, as well as in a more clinically relevant cellular model employing polarized primary hepatocytes, in which HCV entry factors localize to subcellular compartments similarly to those in native hepatocytes. *In vivo* studies were conducted using a chimeric mouse model permissive to HCV infection following xenotransplantation of human hepatocytes. Administration of the mAb16-71 antibody completely protected animals from HCV infection. In contrast to control animals, mice treated with mAb16-71 exhibited undetectable HCV RNA levels throughout the 12-week observation period. The therapy was well tolerated and did not result in significant alterations in biochemical blood parameters. In summary, the *in vivo* experiments demonstrated that mAb16-71 completely prevented infection of hepatocytes. Importantly, three distinct HCV genotypes (1, 4, and 6) were used in these studies, demonstrating the broad applicability of this potential immunotherapeutic approach across multiple viral genotypes.

Direct spread of HCV from an infected Huh-7.5 cell to adjacent, uninfected cells (the so-called cell-to-cell transmission mechanism) represents an important phenomenon, as it enables efficient viral propagation while avoiding exposure to the host immune system. Within the framework of the above-mentioned publication, we sought to determine whether the mAb16-71 antibody was also capable of blocking this alternative route of viral transmission. My contribution to this study focused primarily on investigating cell-to-cell transmission. Previous studies using Huh-7.5 EGFP-IPS/CD81 target cells, in which CD81 receptor expression was selectively silenced, demonstrated that HCV cell-to-cell spread can occur independently of CD81, a receptor largely responsible for entry of cell-free viral particles. Because Huh-7.5 EGFP-IPS/CD81<sup>-</sup> cells are resistant to infection by cell-free HCV, this cell line was used as target cells in cell-to-cell spread experiments to specifically assess whether mAb16-71 could inhibit this alternative transmission pathway. In these experiments, Huh-7.5 cells infected with HCVcc (genotype 2a) were co-cultured with uninfected EGFP-IPS/CD81<sup>-</sup> cells in the presence of increasing concentrations of

mAb16-71. A dose-dependent reduction in the number of infected EGFP-IPS/CD81<sup>+</sup> cells was observed, whereas no significant changes in HCV transmission were detected in the presence of a control antibody. In summary, these findings demonstrated that mAb16-71 effectively blocks direct cell-to-cell transmission of HCV, a feature of considerable importance for therapeutic strategies aimed at preventing reinfection of newly transplanted livers in chronically infected patients. Moreover, mAb16-71 efficiently inhibits infection by cell-free HCV particles and provides genotype-independent protection against HCV infection *in vivo*.

In parallel, I was involved in the selection and characterization of novel antibodies targeting the SR-B1 receptor. My contribution to the second publication from my post-doctoral fellowship period, published in *Journal of Hepatology*, focused primarily on the experimental work related to the selection and characterization of new monoclonal antibodies designated mAb8, mAb15, and mAb151. Within this study, I performed a series of neutralization assays using the HCVcc system in order to select antibodies with the highest neutralizing potential for subsequent *in vivo* testing. The HCVcc system is an experimental model that enables propagation of HCV *in vitro* in Huh7 cell lines derived from human hepatocellular carcinoma. This system is largely based on the genotype 2a strain (JFH-1), which for the first time allowed efficient replication and production of infectious viral particles in cell culture. The method involves transfection of *in vitro*-transcribed viral RNA into Huh7 cells. The HCVcc platform enables investigation of the complete viral replication cycle, from viral entry and genome replication to the production of new infectious virions. The development of this system represented a breakthrough in HCV research and significantly accelerated progress in antiviral therapy development. Prior to its establishment, studies on HCV were severely limited due to the inefficient replication of patient-derived virus in conventional cell culture systems.

For quantitative assessment of infection, I employed the reporter cell line Huh7-J20, developed in the laboratory of Prof. Arvind Patel, which secretes alkaline phosphatase (SEAP) upon HCV infection. As in the previous publication, the selected antibodies were also evaluated *in vivo* using a chimeric mouse model. In addition to the H77C variant (genotype 1a), infection experiments included the mP05 escape mutant (genotype 1b), isolated from a chronically infected patient following liver transplantation. Beyond the experimental work, my contribution to this publication included antibody selection, data analysis, methodological development, and graphical presentation of experimental results. I also participated in drafting the original manuscript and in revising it in response to reviewers' comments.

In summary, both studies demonstrated that antibodies targeting the SR-B1 receptor may represent a novel therapeutic strategy for preventing HCV infection of newly transplanted livers in patients with chronic disease.

A second line of research in which I was involved, unrelated to HCV, concerned the potential application of platinum nanoparticles in anticancer therapy. These studies were conducted in collaboration with Dr. hab. Jacek Piosik, prof. UG, Head of the Laboratory of Biophysics at the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk. My contribution focused primarily on evaluating the biological effects of platinum nanoparticles complexed with anthracycline drugs—epirubicin or daunorubicin—in human cell lines. The results demonstrated that platinum nanoparticles modulate the activity of these drugs, particularly epirubicin, in a size-dependent manner. Specifically, they enhanced its cytotoxicity in human cancer cells (MelJuSo) while simultaneously exerting a protective effect in non-cancerous cells

(HaCaT). These findings provide an important rationale for the potential use of platinum nanoparticles in optimizing anthracycline-based chemotherapy.

This collaboration yielded valuable scientific data that were published in two journals and established a strong foundation for further joint research initiatives. I believe that this interdisciplinary approach, integrating biophysics with molecular virology, may open new perspectives.

Bełdzińska, P., Zakrzewski, M., **Grzyb, K.**, Hauer, A., Jamrógiewicz, M., Wyrzykowski, D., Bury, K., Gołuński, G., Piosik, J. (2025). Platinum nanoparticles interact with epirubicin in size dependent manner and affect its biological activity. *Eur J Pharm Biopharm.* Nov;216:114866. doi:10.1016/j.ejpb.2025.114866.

IF<sub>2025</sub> – 4.3; IF<sub>5-year</sub> – 4.8; MEiN<sub>2025</sub> – 100; citation count – 0 (WoS)

Zakrzewski, M., Bełdzińska, P., Gajdowska, F., Gołuński, G., Gackowska, K., Strankowska, J., Jamrógiewicz, M., Wyrzykowski, D., **Grzyb, K.**, Gutowska-Owsiak, D., Synak, A., Bojarski, P., Piosik, J. (2025). The effect of platinum nanoparticles on the physicochemical properties of daunorubicin. *RSC Adv.* Sep 25;15(42):35407-35420. doi:10.1039/d5ra02827b.

IF<sub>2025</sub> – 4.6; IF<sub>5-year</sub> – 4.3; MEiN<sub>2025</sub> – 100; citation count – 0 (WoS)

## 5 Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions

My scientific career to date has been characterized by a high degree of mobility and thematic diversity. I began my doctoral studies at the Faculty of Biology, Geography and Oceanology of the University of Gdańsk. I obtained the degree of Doctor of Biological Sciences in 2007, defending my doctoral dissertation with a distinction. During my doctoral studies, I published four original research articles and gained extensive experience in marine biology as well as in techniques for protein isolation, purification, and physicochemical characterization. In recognition of my scientific achievements during this period, I was awarded the Rector's Award of the University of Gdańsk in 2004. My accomplishments were also acknowledged by the Foundation for the Development of the University of Gdańsk, which granted me a scholarship for outstanding young researchers. I have always considered research stays at other institutions to be an essential element of scientific development. Therefore, already during my doctoral studies, I applied for and obtained a three-month Marie Curie fellowship at the Norwegian University of Science and Technology (NTNU), Department of Biotechnology, in Trondheim, Norway. The host laboratory focused primarily on research related to the marine environment and on the characterization of fish muscle proteins and enzymes, particularly from salmon. Analysis of these proteins plays an important role in quality control in the fish processing and seafood industry. This stay in Norway was an extremely valuable experience and closely related to the topic of my doctoral research.

Following the completion of my PhD, I undertook a short research internship at the Laboratory of Virus Molecular Biology at the Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk (MWB UG-GUMed), under the supervision of Prof. Krystyna Bieńkowska-Szewczyk. This experience sparked my interest

in virology and created the opportunity to apply for a Marie Curie fellowship within the 6th Framework Programme of the European Union, enabling me to join the internationally renowned research group led by Prof. Alfredo Nicosia in Naples. During my 18-month postdoctoral fellowship in Italy, I participated in research conducted within the project entitled “*Entry inhibitors for the treatment of hepatitis C virus infection.*” The objective of this project was to develop innovative antiviral therapies targeting the process of HCV entry into host cells, ranging from basic research to potential clinical applications. At that time, the mechanism of HCV entry represented a relatively poorly understood stage of the viral life cycle and constituted a promising target for novel therapeutic strategies. The project aimed, among other objectives, to identify additional cellular receptors and host factors involved in viral entry, as well as to develop and optimize human monoclonal antibodies capable of effectively blocking HCV entry, including their production at a scale suitable for clinical investigation. A detailed description of my work carried out during the postdoctoral fellowship in Naples is provided in subsection 4.6. The company Okairos (now ReiThera), where I carried out my postdoctoral fellowship, specializes in the development of adenoviral vector-based vaccines against infectious diseases, and regular participation in meetings and scientific discussions broadened my understanding of the challenges associated with the development of an HCV vaccine.

My stay in Italy opened numerous opportunities and provided a solid foundation for my further scientific development. After returning to Poland, the expertise I had acquired in virological and immunological techniques, combined with my extensive background in biochemistry, enabled me to successfully apply for both basic and applied research grants. These projects focused on the overexpression and analysis of recombinant HCV envelope proteins as well as on the development of chimeric VLPs for vaccine purposes. Since 2012, I have been affiliated with the Laboratory of Virus Molecular Biology at the Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk (MWB UG-GUMed). Regularly securing competitive research funding ensured continuity of my employment and that of my research team, and most importantly, provided financial support for all studies constituting my main habilitation achievement.

Through funding obtained from the NCN SONATA 3 grant, I was also able to undertake a short research stay in 2013 at the laboratory of Prof. Arvind Patel (MRC–University of Glasgow, Centre for Virus Research). The purpose of this visit was to further develop my expertise in HCV research and to expand the scope of experiments planned within my ongoing projects. This stay resulted in a long-term collaboration, and we subsequently became co-authors of one of the publications included in the habilitation cycle (publication no. 4), published in *Microbiology Spectrum*.

In summary, throughout my scientific career I have engaged in diverse research topics across multiple research institutions, which required adaptation to different experimental models and the continuous acquisition of new skills and methodologies. Following my postdoctoral fellowship, I rapidly demonstrated independence in conducting research, both as a scientist and as the principal investigator of four major research grants. Within these projects, I was responsible not only for experimental work but also for initiating and implementing new research directions, establishing international collaborations, securing additional funding, planning future studies, developing new methodologies, and investing significantly in training and practical workshops for my project team. As a mentor, I have consistently supported—and continue to support—the scientific development of my research team, recognizing that meaningful scientific discoveries are only possible through effective teamwork.

## 6 Presentation of teaching and organizational achievements as well as achievements in popularization of science or art

Despite being employed at the Intercollegiate Faculty of Biotechnology (MWB) in a research position funded through external grants, I have consistently engaged students in the research projects I led, supervising both Master's and Bachelor's theses. I have served as the primary supervisor or direct mentor of seven Master's theses and as the supervisor of four Bachelor's theses. Among my former Master's students are a BioLab scholarship recipient, as well as a laureate of a one-year internship award at the pharmaceutical company Adamed Pharma during the fourteenth edition of the Biotechnology Student Conference.

In addition, I have accumulated substantial teaching experience covering a broad range of courses within the Biotechnology programme. I have conducted proseminars in Molecular Biology Methodology and Genetic Engineering, laboratory classes in Virology, and practical courses in Model Organisms and Their Applications in Science, including modules on *Leishmania tarentolae* and baculoviruses. I have also actively contributed to the organization of Virology laboratory classes and co-authored the corresponding laboratory manual.

I consider the PhD thesis defended with distinction by my doctoral student, Anna Czarnota, for whom I served as co-supervisor, to be a significant teaching achievement. I am currently serving as co-supervisor of a PhD student, Aleksandra Sławińska, whose doctoral project entitled "*A Universal Vaccine Against SARS-CoV-2*" is conducted within the NCN OPUS 21 project, for which I serve as principal investigator.

I am committed to maintaining a high standard of teaching and therefore continue to enhance my pedagogical skills through participation in training programs organized by the Center for Teaching Excellence and Tutoring at the University of Gdańsk, aimed at improving student engagement and instructional effectiveness. Furthermore, I am a member of the Polish and European Societies for Virology and serve as a peer reviewer for manuscripts submitted to internationally recognized journals, including *npj Vaccines*, *Scientific Reports*, *Antimicrobial Agents and Chemotherapy*, *International Journal of Biological Macromolecules*, *Journal of Virological Methods*, *Microbiology Spectrum*, *Pharmacological Reports*, and *Virology Journal*.

As the mother of a nine-year-old child, I am aware of how naturally children are drawn to experimentation and discovery. I have therefore been actively involved in science outreach activities aimed at young audiences. During the "Night of Biologists" event, I conducted workshops for children entitled "*Explosion of Colours*." I have also led workshops on plasmid DNA isolation for high school students as part of the annual "Biotech Week" event organized at the Intercollegiate Faculty of Biotechnology UG-GUMed. Currently, I am involved in organizing a competition for secondary school students under the theme "*Pandemics Through the Ages*."

Below, I present a list of doctoral, Master's, and Bachelor's thesis topics completed under my supervision.

### Co-supervisor of PhD Theses:

- "*Chimeric HBV-HCV virus-like particles as a potential vaccine against hepatitis C virus*" – defended in 2023
- "*A Universal Vaccine Against SARS-CoV-2*" – ongoing

**Supervisor or Direct Mentor of Master's Theses:**

- *"Biochemical characterization of HCV envelope glycoproteins"* – defended in 2014
- *"Analysis of cross-reactivity of sera from mice immunized with HCV E1E2 glycoproteins"* – defended in 2015
- *"Expression of HCV NS3 epitopes fused to the small hepatitis B surface antigen"* – defended in 2019
- *"Characterization of sHBsAg virus-like particles displaying epitopes of the SARS-CoV-2 S protein"* – defended in 2022
- *"Minicircles as a novel vaccine platform against hepatitis C virus"* – defended in 2022
- *"Analysis of humoral immune responses against HCV induced by DNA immunization"* – defended in 2023
- *"Evaluation of neutralizing properties of murine sera following immunization with chimeric HBV/SARS-CoV-2 virus-like particles"* – defended in 2024

**Supervisor of Bachelor's Theses:**

- *"Chimeric virus-like particles as a vaccine candidate against hepatitis C virus"* – defended in 2018
- *"The E1E2 complex as a vaccine against hepatitis C virus"* – defended in 2018
- *"Analysis of cellular immune responses in patients infected with HCV"* – defended in 2020
- *"Recombinant protein- or DNA-based vaccine? A comparison of immune responses directed against HCV"* – defended in 2023

**7 Other relevant information concerning the professional career not included in points 1–6 above**

I am currently the principal investigator of the NCN OPUS 21 grant focused on the development of a universal vaccine against SARS-CoV-2 that would be effective against multiple viral variants. The objective of the project is to identify and expose conserved regions within the spike (S) protein and to propose an effective vaccine capable of eliciting broadly neutralizing antibody responses. Building on our previous research experience, the project aims to evaluate the immunogenic properties of selected conserved regions located within the SARS-CoV-2 S protein, displayed on sHBsAg-based virus-like particles. The research plan also includes assessing the ability of the designed particles to induce immune responses directed against currently circulating variants of SARS-CoV-2. In the context of emerging viral variants, the development of an effective, widely applicable, and universal vaccine remains a highly relevant research priority. I hope that our studies will contribute to a better understanding of immune responses directed against conserved regions within the S protein and provide new insights supporting the development of a universal vaccine targeting both current and future SARS-CoV-2 variants.

The COVID-19 pandemic also motivated us to prepare a review article addressing SARS-CoV-2 variants and the critical role of neutralization assays in COVID-19 research.

The review was published in the high impact journal *npj Vaccines* and unexpectedly received significant international attention within the scientific community.

Chmielewska, A. M., Czarnota, A., Biełkowska-Szewczyk, K., **Grzyb, K\***. (2021). Immune response against SARS-CoV-2 variants: the role of neutralization assays. *npj Vaccines*, 6(1), 142. doi:10.1038/s41541-021-00404-6

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IF<sub>2021</sub> - 9.39; IF<sub>5-year</sub> - 6.7; MEiN<sub>2021</sub> - 100; citation count - 28 (WoS)

Regarding future research on an HCV vaccine, I plan to evaluate the *in vivo* efficacy of the chimeric HBV/HCV VLP-based vaccine using a passive immunization approach in chimeric mice engrafted with human hepatocytes. These mice lack an adaptive immune system; therefore, in the course of the study, it is necessary to administer antibodies derived from immunocompetent animals previously immunized with chimeric HBV/HCV VLPs and subsequently assess their neutralizing capacity following HCV infection. It should be emphasized that, at present, humans remain the only fully immunocompetent model of HCV infection. Consequently, the use of humanized chimeric mice represents the only feasible approach for investigating the *in vivo* impact of antibodies induced by chimeric HBV/HCV VLPs on the dynamics of HCV infection. Due to the technical complexity of generating humanized chimeric mice, studies involving this model are costly and time-consuming, and require the acquisition of additional competitive research funding for their implementation.

I intend to continue my scientific career in the fields of virology and medical biotechnology. My objective is to further develop effective solutions in vaccine design and to advance research on antiviral immune strategies. Achieving the degree of Doctor Habilitated will further strengthen my ability to expand international collaborations and mentor the next generation of researchers.



Signed by / Podpisano przez:

Katarzyna Monika  
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Date / Data: 2026-03-  
24 15:31

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