


# The Identification of the Hepatitis C Virus

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## History of blood-borne viral hepatitis

After the discovery of the hepatitis B virus (HBV) by Dr. Baruch Blumberg and colleagues in the late 1960s (1–3), serological tests were developed to detect this virus in blood donors which led to the realization in 1974 that the majority of post-transfusion hepatitis cases were not due to blood transmission of this virus (4). With the discovery of the hepatitis A virus (HAV) in 1973 by Drs. Steve Feinstone, Bob Purcell and colleagues (5) and the introduction of tests to detect this virus, it was found in 1975 that the vast majority of non-HBV blood-borne hepatitis was not due to HAV (6). Originally perceived as of minor importance, the work of Dr. Harvey Alter and others in the field went on to show the important role of this disease in developing chronic liver disease (7). Termed blood-borne Non-A, Non-B hepatitis (NANBH), this triggered a long search spanning 15 years to identify the causative agent or agents.

The initial breakthrough in the discovery of HBV was the identification of an unusual antigen observed in the blood of Australian aborigines, later shown to be the major envelope glycoprotein of HBV (1–3). However, no such specific antigen was identified for the NANBH agent(s) prior to the discovery of HCV in 1989 (8). The visualization of

27nM diameter particles in the stools of infected animals and humans proved decisive in the discovery of HAV (5) but no such virus particle could be associated with NANBH (8). The critical discovery of HIV was enabled by its lytic propagation *in vitro* in peripheral blood mononuclear cells (PBMNCs) derived from AIDS patients by Drs. Françoise Barré-Sinoussi and Luc Montagnier (9) but the difficult *in vitro* propagation of HCV did not occur until 2005 by Dr. Takaji Wakita and colleagues (10) and to this day, it still remains difficult to grow efficiently in cell cultures. So, methods used to identify other hepatotrophic viruses and HIV proved unable to identify HCV.

## The long molecular search for HCV (1982–1989)

My own quest to identify the causative agent(s) of NANBH began in 1982 with the belief that emerging molecular biological methodologies could be capable of success. Initially, Dr. Tatsuo Miyamura from the Japanese NIH visited and worked in my laboratory at the Chiron Corporation for one year bringing human liver samples derived from autopsied materials. However, the quality of the mRNA was poor resulting from degradation of the tissue after death. We then realized that to obtain intact mRNA, we would have to work with materials from the living chimpanzee model which had been shown to be a reliable model for NANBH by a few laboratories around the world (11–14). This animal model had proven to be of great value in HBV research. Consequently, we initiated a collaboration with Dr. Daniel Bradley of the US Centers for Disease Control (CDC) who was a leader in the NANBH chimpanzee model. Fig. 1 shows typical results from his laboratory in which acute NANBH could be passaged in the common chimpanzee (*pan troglodytes*) from a human factor 8 preparation contaminated with the NANBH agent(s).

From 1983 onwards, Dr. Bradley provided me with a continuous supply of blood and liver biopsy samples from many of his NANBH-infected and control, uninfected chimpanzees and for several years thereafter, one major theme of my laboratory was to identify rare NANBH-specific poly-A+ mRNAs derived from the etiological agent(s) of NANBH via interrogation of very large bacterial cDNA libraries derived from NANBH-infected chimpanzee liver samples. We used highly radioactive hybridization cDNA probes derived either from NANBH-infected liver poly-A+ mRNA (the + probe) or from control, uninfected livers (the – probe) to identify many genes apparently upregulated in NANBH-infected livers using duplicate blotted filters of the cDNA libraries (Fig. 2).

We now know that many genes within the liver are modulated (up- or down-regulated) by HCV infection, including those involved in the innate and adaptive host immune responses (15,16). Despite obtaining many upregulated NANBH-specific genes, none could be ascribed to being

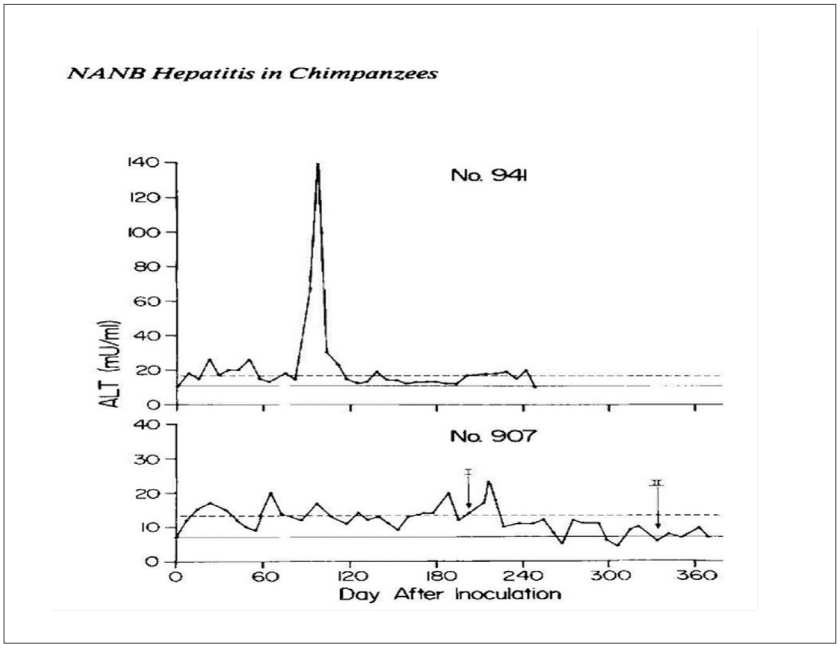


*Figure 1.* Alanine aminotransferase (ALT) activity (reflective of liver damage) in serial serum specimens from chimpanzees with non-A, non-B hepatitis. Chimpanzee no. 941 was inoculated with plasma II from chimpanzee no. 771 (122).

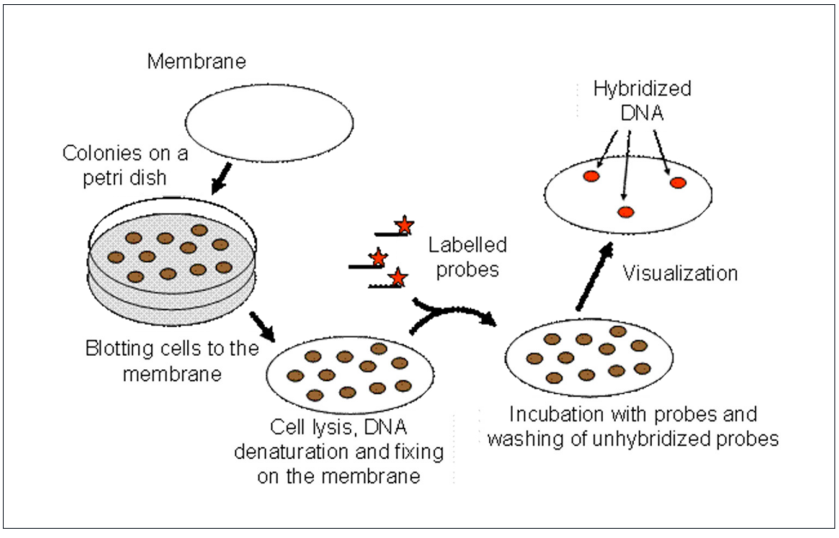


*Figure 2.* Method of identifying genes modulated by NANBH infection using radiolabeled cDNA hybridization probes derived from poly-A+ mRNA derived from NANBH-infected livers (the + probes) and control, uninfected livers (the – probes). In this way, genes up- and down-regulated by NANBH infection were identified.

derived from a NANBH etiological agent since they all hybridized to the chimpanzee and human genome and therefore were not extra-chromosomal, a criterion used for designating a possible pathogen derivation. In an attempt to increase our probability of success, we requested to Dr. Bradley that he try to produce chimpanzee liver and blood samples of similar infectivity titers to those reported by other NANBH research groups around the world (around 1 million chimpanzee infectious doses per ml of human plasma (17) or chimpanzee (18) plasma and similar infectious titers per gram of NANBH liver tissue from the same animals. We co-funded work in Dr. Bradley's laboratory to attain these goals in 1985 via his production of large plasma pools and liver samples from his chimpanzees #910 & #771 (19,20; Fig. 3). Unfortunately, interrogating cDNA libraries from these sources of tissue from 1985–1987 still failed to identify any clones derived from an etiological NANBH agent(s).

In 1979, Dr. Yohko Shimizu and colleagues at the US NIH demonstrated that one NANBH agent, termed the tubule-forming agent (tfa), elicited double layered membranes and membranous tubules within the endoplasmic reticulum of hepatocytes in infected chimpanzees (21). Dr. Bradley pointed out that such cytoplasmic membranous changes were reminiscent of changes within cell cultures infected by RNA viruses such as the flaviviruses and togaviruses, from which he suggested that the tfa may be related to such viruses (22). He also showed that the infectivity of the tfa was destroyed by treatment with organic solvents and that it could

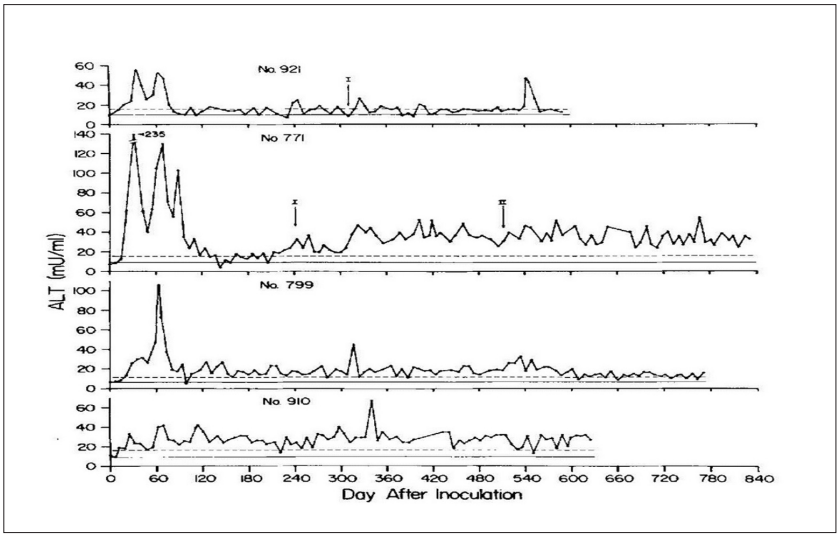


*Figure 3.* Alanine aminotransferase (ALT) activity in serial serum specimens from chimpanzees with non-A, non-B hepatitis. A plasma pool made from #910 was shown to have an infectivity titer of ~ 1 million chimp infectious doses per ml (19,20).

pass through a filter with pore sizes of 80nM, leading him to suggest that the tfa was either a small lipid-enveloped virus similar to flaviviruses and togaviruses or that it was HBV-like (a 40nM enveloped DNA virus), or that it was a novel type of virus (19,22,23).

This led my laboratory to interrogate his chimpanzee samples with hybridization probes derived from the HBV DNA genome as well as from various flaviviral and togaviral RNA genomes. There were papers published around this time suggesting that one NANBH agent could be HBV-like (24). However, we could not detect any hybridization signals between these known viral genomes and the NANBH chimpanzee samples. We also interrogated (again unsuccessfully) the chimpanzee NANBH samples using picornaviral and coxsackie viral genomes, since Dr. Bradley had observed 27nM viral particles resembling these non-enveloped viruses within some chimpanzee hepatocytes that received inocula previously-treated with organic solvents (19,22,23). None of this time-consuming work resulted in the identification of a specific molecular probe or handle for the NANBH agents.

In another attempt to identify a virus related to the NANBH agent(s) my laboratory, working with that of Dr. John Gerin's laboratory at the US NIH, identified the genome of the hepatitis D virus (HDV). Originally discovered in the form of an unusual antigen (the "delta antigen") by Dr. Mario Rizzetto within hepatocyte nuclei in some HBV patients experiencing severe hepatitis (25), its infectivity for non-human primates was shown to be associated with a RNA molecule that required HBV for infectivity and transmission (26). We showed that this RNA molecule was indeed the HDV genome being a covalently-closed-circular, single-stranded RNA whose complementary anti-genomic RNA encoded the delta antigen (27). Highly reminiscent of infectious plant pathogens like viroids and virusoids, the HDV genome exhibited strong intramolecular base-pairing under physiological conditions to form a double-stranded RNA rod-like structure that could only be visualized in the electron microscope under strongly denaturing conditions (27,28; Fig. 4).

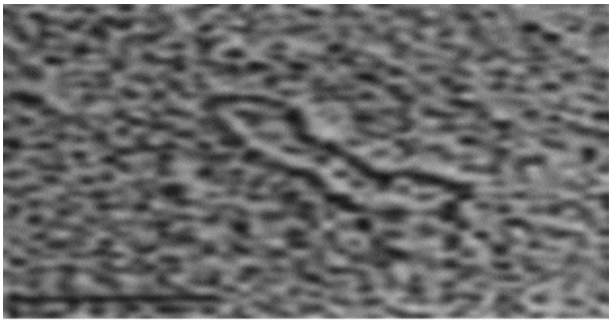

*Figure 4.* The covalently-closed-circular, single-stranded RNA genome of the HDV pathogen as observed in the EM under strongly denaturing conditions (27,28).

Replicated by host RNA polymerase II in a rolling circle mechanism, the HDV RNA genome, like the similar plant pathogens, was shown by Dr. Taylor's laboratory to possess an innate ribozymic activity, self-cleaving itself from a larger precursor RNA and then able to self-ligate (29,30). This HDV ribozyme has proven to be a useful tool in general molecular biological research and the availability of recombinant delta antigen and its specific antibodies led to improved diagnosis of HBV carriers superinfected with HDV. Therapeutic strategies have also been facilitated by further knowledge of its replication strategy (31).

Dr. Robert Purcell's laboratory at the US NIH was the first to propose that HDV may be related to HCV based on the similarity in tubular membranous structures observed within the hepatocytes of chimpanzees infected by either virus (32). However, Dr. Amy Weiner in my laboratory could not detect any cross-hybridization between the HDV genome and nucleic extracts derived from Dr. Bradley's NANBH-infected chimpanzee samples, thus ruling out the possibility of there being any close genetic relationship between the two agents (ref. 33; Fig. 5).

A possible relationship between the blood-borne NANBH agent and retroviruses was indicated by a report of reverse transcriptase activity in NANBH sera samples (34) and by the propagation of foamy-like viruses in cell cultures infected with NANBH samples (35), but despite intense efforts, we were unable to confirm these findings.

A new exciting era of NANBH research was heralded by Dr. Shimizu at the NIH who cloned B cells derived from NANBH individuals and showed that their secreted antibodies could bind specifically to thin liver sections derived from NANBH-infected livers but not to control, uninfected livers (36). Generally termed as the "Shimizu antibodies", this discovery spawned great activity in the field because hitherto, no NANBH-specific antibodies or antisera had been confirmed using similar histochemical binding assays.

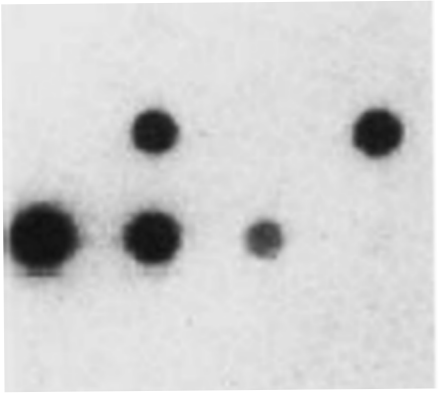

*Figure 5.* Dot blot hybridization of radio-labeled HDV cDNA to various standards and NANBH nucleic acids extracted from chimpanzee #910 plasma pool (top far right dot shows no detectable hybridization signal to NANBH-derived nucleic acids (33).

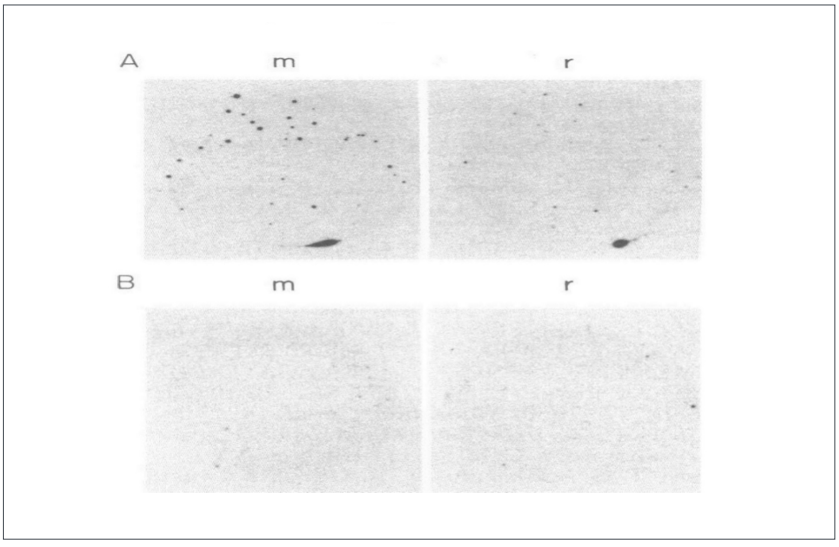


*Figure 6.* Detection of amylase genes using a recombinant immuno-screening method employing bacteriophage λgt11 lysogens and an amylase IgG probe (37).

In an effort to confirm the reactive antigen specific to the Shimizu antigens (hopefully encoded by the NANBH etiological agent), we employed the cDNA immuno-screening method devised by Drs. Young and Davis (37). Developing a highly efficient λgt11 bacteriophage cloning and expression vector, they demonstrated the ability to identify various genes by screening resulting proteomic cDNA bacterial libraries with specific monoclonal antibodies or polyclonal antisera of known high affinity and titer (Fig. 6).

Unfortunately, this approach was not successful when we screened cDNA libraries derived from NANBH-infected chimpanzee livers with the Shimizu antibodies. Indeed, it was well known at that time that immuno-screening cDNA libraries did not always meet with success. Earlier in our work, I had considered a similar immuno-screening approach using sera from NANBH patients and chimpanzees as a presumptive source of antibodies to the NANBH agent, but had deemed it too risky because no such antibodies had been demonstrated despite intensive efforts by the field. Also, the NANBH agent(s) was known to be highly persistent in most patients suggestive of a weak, inadequate humoral immune response, as known to be the case in cases of persistent HBV infection following exposure of immune-competent adults to the virus. However, in the context of our unsuccessful work on Shimizu antibodies (which Dr. Shimizu later showed, by purifying and sequencing the target protein, were targeting host proteins not encoded by HCV), my next-door laboratory neighbor Dr. George Kuo (then working on non-HCV projects)

strongly encouraged me to gamble on screening cloned NANBH libraries with NANBH patient sera. I found his argument to be persuasive that the lack of demonstration of NANBH-specific antibodies to date could possibly be due more to limiting concentrations of NANBH antigen within the thin liver sections used in histochemical screening, rather than the true absence of circulating NANBH antibodies. Dr. Bradley was also of this opinion and was providing chimpanzee samples to another group pursuing this same approach (although this group subsequently terminated this approach due to lack of success and its perceived risk).

Initially, Dr. Qui-Lim Choo in my laboratory screened many of my NANBH-infected chimpanzee liver cDNA libraries derived from poly-A+ mRNA with sera derived from NANBH-infected individuals as a presumptive source of specific NANBH agent antibodies. Despite using many different chimpanzee and human sera samples derived from the minority of individuals that appeared to have spontaneously eradicated their acute NANBH infections based on normalization of serum ALT levels and who we therefore hoped had a strong protective immune response to the etiological agent, we could not identify any cDNA clones that were extra-chromosomal in origin and hence deemed unlikely to be derived from the causative NANBH genome. Based on the possibility that the NANBH agent could be togavirus-like which do not have a poly-A+ tail (or produced poly-A+ mRNAs during replication), I then turned to making λgt11 proteomic libraries derived from the NANBH-infected chimpanzee plasma pool from animal #910. In order to do this, this plasma pool was ultra-centrifuged such that the smallest virus known to man would be pelleted. Initially, RNA extracted from this pellet was then reverse-transcribed using random primers. The resulting cDNA was cloned into λgt11 and screened by Dr Choo using sera from apparently "convalescent" NANBH-infected chimpanzees and patients. Yet again, no molecular clones derived from the NANBH agent(s) genome could be identified. By now, after several years of trying unsuccessfully to track down the causative NANBH agent(s) using numerous different approaches, it felt like I was groping around in the dark looking for the proverbial "needle in a haystack".

As indicated earlier, it was well appreciated by molecular biologists at that time that cDNA immuno-screening often failed to identify genes of interest even when well-characterized antisera or monoclonal antibodies of high affinity were available. Therefore, wanting to reassure myself that this method really could be successful at identifying an infectious agent *de novo*, I used our previously characterized infectious HDV plasma extracts and cloned it into λgt11 prior to screening with HDV-infected patient sera that we already knew contained high titer antibodies to the delta antigen. Numerous HDV clones were successfully obtained in this

way giving us confidence that this method could possibly work for identifying the NANBH genome(s). However, the infectivity titer of HDV plasma was known to be many orders of magnitude higher than the best NANBH chimpanzee plasma pool and so I knew that successful detection of NANBH clones was still very risky and uncertain (indeed, of at least 6 plasma-derived libraries that I made during this period without the assistance of PCR amplification technology , which had not yet emerged, only one was to prove successful). In addition, the worrying possibility of a weak humoral immune response to NANBH infection still existed.

I then made a second attempt to identify molecular HCV clones derived from the infectious chimpanzee #910 plasma pool, this time implementing two significant changes. The first involved cloning random-primed, reverse-transcribed cDNA from RNA extracted from the ultra-centrifuged plasma pellet, as well as cloning the DNA existing within this pellet. This was because we did not know if the NANBH agent(s) was a RNA or DNA virus. The second change was to screen this bacterial proteomic library with serum from patients diagnosed with chronic, persistent NANBH infection in the hope that perhaps these chronic patients might have higher titers and affinities of NANBH agent-specific antibodies as compared with apparent "convalescent" individuals experiencing acute, resolving NANBH infection (we later showed this indeed to be the case after we successfully identified HCV and developed anti-HCV antibody tests). Dr. Choo and myself selected a chronic NANBH patient with unusually high ALT levels that we hoped was indicative of a strong immune response to the NANBH agent(s). This resulted in the identification of several clones (Fig. 7).

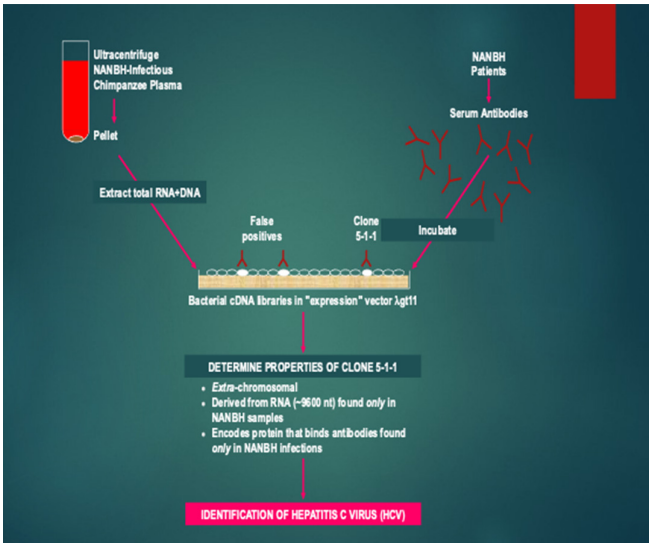


*Figure 7.* Identification of clone 5-1-1.

In addition to identifying positive clones encoding MS2 bacteriophage RNA (a carrier that was essential for me to use before the emergence of the powerful PCR nucleic acid amplification technique), Dr. Choo found clone 5-1-1, a small clone containing only ~ 100bps, but one that we could later prove was derived from an etiological agent of NANBH. We showed that clone 81 (overlapping with 5-1-1) was derived from a large RNA of around 10,000 ribonucleotides in length that was found only in NANBH-infected samples, not control uninfected samples, that was single-stranded and positive-stranded with respect to encoding the 5-1-1/81 antigen (Fig. 8).

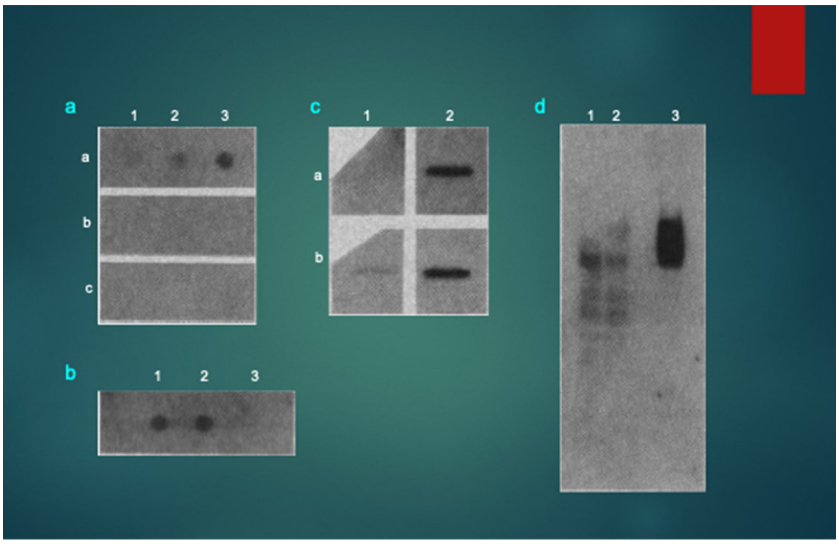


*Figure 8.* (38). Hybridization of clone 81 cDNA to RNA. (A) Spot hybridization of 2, 4, or 12 ug of total liver RNA extracted from either chronic NANBH-infected chimp 910 (al to a3) or from two control, uninfected animals (bl to b3 and cl to c3) with $^{32}$P-labeled nick-translated clone 81 cDNA. (B) Spot hybridization of nucleic acid extracted from viral plasma pellets before (spot 1) or after treatment with either excess deoxyribonuclease 1 (spot 2) or ribonuclease A (spot 3). Hybridization probe as in (A). (C) Each strand of clone 81 cDNA was subcloned into phage M13mp18 and then labeled by incubating with Klenow Escherichia coli DNA polymerase 1 in the presence of hybridization probe primer and [a-$^{32}$P]dCTP. Each probe was then hybridized to slot blots containing either identical portions of viral RNA derived from infectious plasma (al and bl) or 2 pg of purified clone 81 double-stranded cDNA (a2 and b2). (D) Northern blot analysis of 30 pg of total RNA (track 1), 30 pg of unbound RNA (track 2), and 20 ug of bound RNA (track 3) after chromatography on oligo(dT)-cellulose. RNA was derived from the liver of infectious chimpanzee # 910. The top of the smear in track 3 corresponds to around 10,000 ribonucleotides in length. $^{32}$P-labeled nick-translated clone 81 cDNA was used as the hybridization probe.

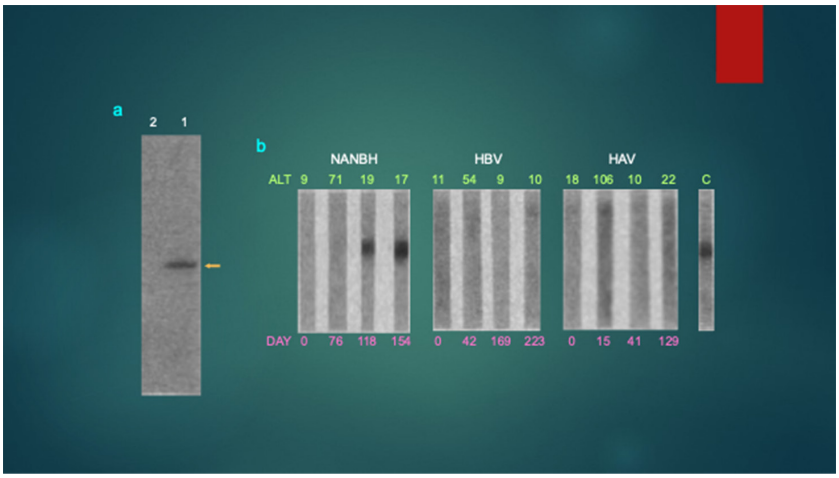


*Figure 9.* (38). Western blot assay for anti-5-1-1 antibodies. (A) Incubation of the chronic NANBH patient serum used to isolate done 5-1-1 with blots of total bacterial lysates expressing either a fusion of the 5-1-1 antigen with superoxide dismutase (SOD) (lane 1) or control SOD (lane 2). (B) Sequential serum samples from experimentally infected chimpanzees were reacted with identical strips cut from a preparative blot of total lysate containing the 5-1-1/SOD fusion protein. Day 0 represents the day of virus inoculation. Infections were monitored by serum ALT concentrations (international units per liter). Control strip C was incubated with the same patient serum used in (A).

Furthermore, using a Western blot analysis, only chimpanzees (4/4) infected with NANBH seroconverted to anti-5-1-1 antibodies unlike animals infected with HAV or HBV (0/7; representative examples shown in Fig. 9).

Furthermore, we showed that 7 of 11 USA NANBH patients had circulating antibodies to the 5-1-1 antigen but these were absent in control, uninfected individuals. Moreover, as we obtained more and more overlapping cDNA clones from the same cDNA library, we began to see distant but significant primary sequence identity with flaviviruses like Dengue virus. These data convinced us that we had indeed identified a major etiological agent of NANBH, which led to my first public disclosure of the discovery of HCV in the spring of 1988 at the University of California in San Francisco, work subsequently published in 1989 (38).

In the next stage of our work, Dr. Kuo developed a high throughput radioimmunoassay to detect antibodies targeting the antigen C100-3, which was encoded by a few adjacent clones overlapping with 5-1-1, which we used to assay a pedigreed panel of known infectious and known non-infectious NANBH samples from Dr. Harvey Alter. This blinded study revealed that our assay did indeed have specificity for NANBH and furthermore, demonstrated that such antibodies were present in infectious NANBH samples meaning that we could therefore use this assay to

*Figure 10.* The core HCV discovery team (40). Drs. Michael Houghton (upper left), Qui-Lim Choo (upper right), George Kuo (lower left), Daniel Bradley (lower right).

detect infectious blood donors and patients (39). In addition, using a highly pedigreed panel of 10 post-transfusion NANBH patients of Dr. Harvey Alter at the USA NIH, we showed that all 10 were positive for HCV antibodies and that there was at least one HCV-positive blood donor involved in 9 of these 10 cases (39). After assaying other NANBH patient cohorts from other collaborators from the USA, Italy and Japan, it became clear that the majority of NANBH samples were positive for HCV antibodies (39). This constituted confirmation that we (Fig. 10) had identified the major cause of blood-borne NANBH.

## Ramifications of the molecular isolation of HCV

### 1. Diagnosis

Our first priority then was to protect the global blood supply by developing blood screening diagnostics to detect circulating HCV antibodies. Using the protein encoded by clone 5-1-1 and two adjacent clones, we were able to produce the C100-3 antigen in recombinant yeast which was used to detect circulating HCV antibodies in the first commercial EIA assays (initially produced by Ortho Diagnostics and shortly afterwards by Abbott Laboratories). This was shown to detect the majority of infectious blood donors and chronically-infected patients (39,41).

Meanwhile, we and many other laboratories around the world began to characterize the viral proteins encoded by the HCV RNA genome (42–47) from which it became clear that the virus encoded a large polyprotein precursor that was cleaved co- and post-translationally by a variety of cellular and viral proteases into structural virion proteins and a large number of proteins required for viral replication (Fig. 11).

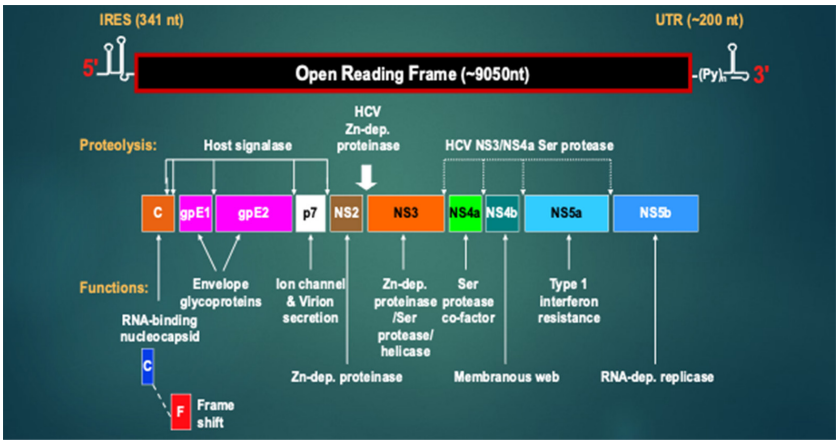


*Figure 11.* The HCV polyprotein precursor and its cleavage products (42–47).

By producing recombinant proteins from the various protein domains encoded by HCV, we were able to detect and map additional B cell epitopes, both linear and discontinuous epitopes conserved among the different strains of HCV, that we incorporated into a series of EIA tests of ever-increasing sensitivity (48,49). This led to commercial EIA tests detecting circulating HCV antibodies with >95% sensitivity as well as confirmatory tests (50). Dr. Weiner then developed the first PCR-based test to detect circulating HCV genomic RNA including in some individuals lacking HCV antibodies (51). Shortly afterwards, another laboratory colleague, Dr. Jang Han, showed that the 5'untranslated region (UTR) of the HCV genome was highly conserved among the many different genotypes and strains of HCV and was even highly conserved with the animal pestiviral 5'UTRs (52). It should be noted that although the genomic RNA of the HCV strain that we isolated (HCV1) does contain a short poly-A region, this is in the gene encoding the nucleocapsid C gene (43) and is not at the 3' terminus of the HCV genome ( which can be a product of spurious PCR reactions; 52).

The highly conserved 5'UTR allowed very sensitive nucleic acid tests to be developed first by PCR methods (53) and later by transcription-mediated amplification (TMA) methods (54).

Over the course of a few years after the identification of the HCV RNA genome, the co-introduction of these HCV antibody and HCV genomic nucleic acid detection tests effectively arrested the global transmission of HCV from blood donors as well as allowing the diagnosis of all HCV patients. Such tools also became invaluable to monitor patient responses

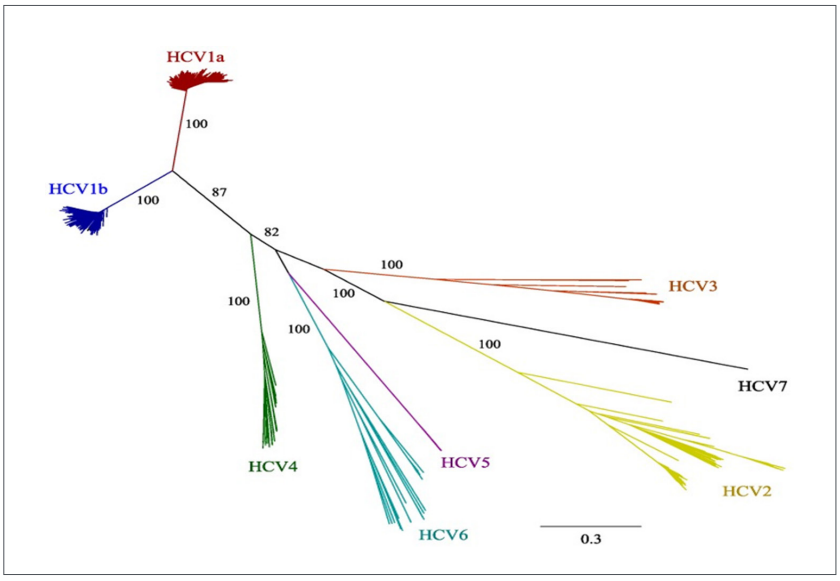


*Figure 12.* Phylogenetic tree of full-length HCV genomic RNA sequences (116).

to interferon and ribavirin therapy (55,56) and much later, to specific direct-acting-antivirals (DAAs; 57).

By this time, it became evident that HCV was a very heterogeneous virus that is now known to comprise at least 7 major genotypes around the world with each genotype comprising many related sub-types (Fig. 12; refs 58–60,114,116).

The first variant subtype identified was from the laboratory of Dr. Tatsuo Miyamura at the Japanese NIH who identified what is now known to be the most common global subtype (1b; 58). Subtype 1a is the most common in North America, meaning that the major genotype 1 (comprising subtypes 1a & 1b) is the most common around the world (61–63,114,116). Median inter-genotype diversity was calculated to be 32.39% at the nucleotide level and 25.02% at the amino acid level (116). The lowest nucleotide diversity is observed between HCV major genotypes 1 and 4 (29.03%) and the highest between HCV genotypes 2 and 3 (35.46%; 116).

## 2. Direct-acting-antiviral (DAA) development

Up until 2014, standard-of-care treatment of NANBH patients comprised type 1 interferon combined with the guanosine analogue, ribavirin (55,56). Requiring at least 6 months of treatment, it was associated with significant toxicity and resulted in virus eradication in only 40–50% of patients. The isolation and characterization of the HCV genome and its

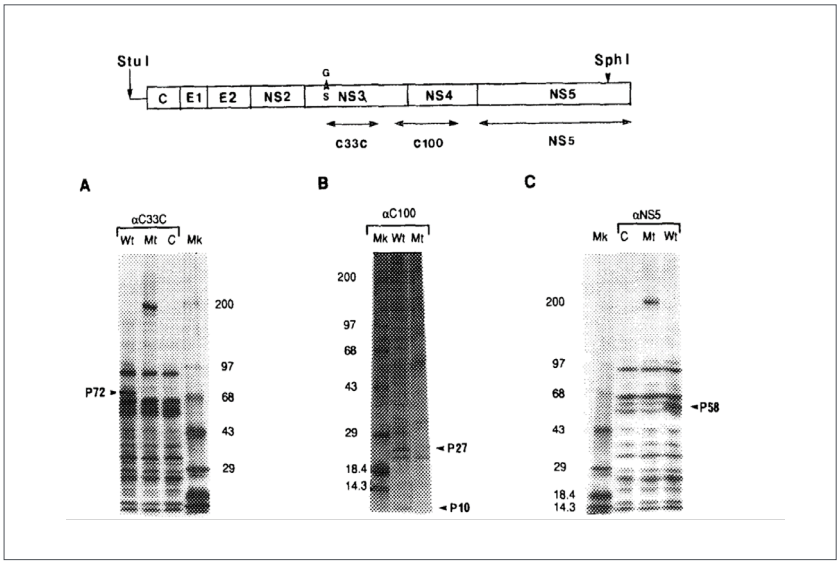


*Figure 13.* The HCV NS3-encoded serine protease activity. The polyprotein precursor fails to be properly cleaved in active site mutations (mt) versus wild-type enzyme (wt; 64).

encoded enzymes and proteins in 1989 enabled the HCV field, comprising numerous academic and corporate laboratories around the world, to pursue the development of direct-acting-antivirals (DAAs) to treat HCV patients. My laboratory (64) and those of Dr. Charles Rice (65) and Dr. Kunitada Shimotohno (66) first showed the presence of a serine chymotrypsin-like protease within the HCV NS3 protein (Fig. 13).

Later shown to complex with the NS4a co-factor (67,68), this activity was targeted by many groups and led to the approval of first generation antiviral DAAs, boceprevir and telaprevir, in 2011. While potent and of great value to patients, these drugs were associated with significant toxicity that led to the subsequent approval of more potent and better tolerated 2nd (Simeprevir and Asunaprevir) and 3rd generation drugs (Paritaprevir, Grazoprevir, Glecaprevir and Voxilaprevir; 69).

The HCV NS5b protein encodes the viral RNA-dependent RNA polymerase for which the nucleoside inhibitor prodrug, Sofosbuvir (Sovaldi), was approved for human use in 2013 (70). Non-nucleoside inhibitors of this enzyme have also been developed (Dasabuvir;71). All of this drug development work was greatly facilitated by the development of HCV cell culture systems, from the laboratories of Dr. Ralf Bartenschlager and Dr. Charles Rice, able to replicate the HCV genome (72,73). Although not capable of producing progeny virus, these systems served as valuable tools to screen for inhibitors of the HCV replication cycle and allowed the

field to generate a huge amount of valuable information regarding HCV replication mechanisms. Furthermore, these replicon systems enabled the development of unconventional drugs targeting the NS5a protein. By screening for antiviral drugs and then selecting for drug-resistant mutations, the extremely potent Daclatasvir targeting the NS5a dimer was first developed by a group at Bristol-Myers-Squibb (74) followed by many active chemical derivatives from other pharmaceutical companies (Ledipasvir, Velpatasvir, Ombitasvir, Elbasvir). These drugs represent the most potent antivirals ever developed against any virus and they bind to a pocket formed by the NS5a dimer (Fig. 14; 75,76).

HCV drug development was also greatly facilitated by the ability to test efficacy in a mouse model developed by Drs. David Mercer, Lorne Tyrrell, Norman Kneteman and colleagues, able to support robust HCV replication in transplanted human hepatocytes (117).

The HCV DAAs are used together in various combinations to effectively shut down viral replication, resulting in eradication of HCV in the large majority of patients, usually within 2–3 months of oral administration. The first drug combination to accomplish this historic landmark was Harvoni (Ledipasvir + Sofosbuvir) from Dr. John McHutchison and colleagues at Gilead Sciences, which was approved for human use in 2014 followed later by Epclusa (Sofosbuvir + Velpatasvir) in 2016. The latter cocktail is very effective against all HCV genotypes. HCV represents the first chronically-infecting virus of man that can be eradicated using antivirals. This has led the World Health Organization (WHO) to issue the objective of eliminating HCV infection as a major public health threat by

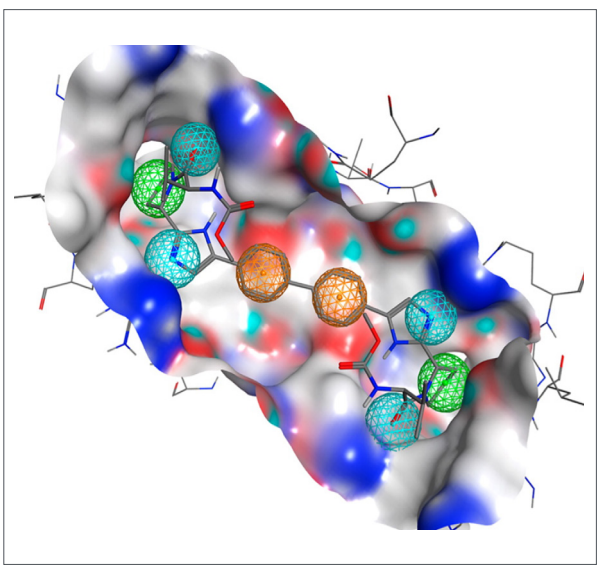

*Figure 14.* Essential pharmacophoric features for potent directly acting NS5A inhibitors (76). These features are a central biaryl core (orange spheres), two H-bond acceptor sites (cyan spheres), and two hydrophobic sites (green spheres). The pharmacophoric sites are mapped over Daclatasvir (green sticks).

2030 via a "test and treat" policy and by developing a much-needed vaccine. Notably, Egypt and other countries have already made substantial progress towards curing their HCV-infected peoples with DAAs (77).

### 3. Towards vaccines against HCV

Unfortunately, vaccine development for HCV has been slow due to many factors. Five years after we identified the HCV genome, Drs. Choo, Kuo, Ralston, Spaete and myself with other colleagues published strong evidence for prophylactic vaccine efficacy in the reliable chimpanzee model (78–80). Using adjuvanted recombinant envelope glycoproteins E1 & E2 (in the form of a native heterodimer, 79) derived from transfected mammalian cells, we could show that of 7 animals immunized 3 times over the course of 6–7 months, 5 were sterilized against homologous virus challenge while the other 2, although becoming acutely infected, eradicated the virus within a few months. In contrast, 4/4 control unimmunized animals became chronically infected after challenge (80).

Since HCV-associated disease manifests itself only in the chronic phase of infection, usually after many years, this data was most encouraging for the feasibility of developing an effective HCV vaccine. Unfortunately, the chimpanzee is an endangered species and very costly to use, such that private and public funding to extend this work took many years to become available. Perhaps vaccine over-pessimism from the field in general also contributed to this budget constraint despite the pandemic nature of HCV infection. Eventually, we showed that vaccinated chimpanzees challenged with a heterologous viral strain also significantly reduced the incidence of chronic infection (Table 1; 81,82). To date, our E1E2 vaccine remains the only HCV vaccine candidate shown to be efficacious at reducing chronic HCV infection in animal models.

After many years when the ability to produce infectious HCV pseudo-

| Viral challenge | Group | Total | Acute infections | Chronic infection (%) | |
|---|---|---|---|---|---|
| Homolog | gpE1/gpE2 | 12 | 7 | 2(17) | P=0.03 |
| HCV-1 | Unimmunized | 10 | 10 | 7(70) | |
| Heterolog | gpE1/gpE2 | 19 | 19 | 3(16) | P=0.02 |
| H77 | Unimmunized | 14 | 14 | 8(57) | |
| Total | gpE1/gpE2 | 31 | 26 | 5(16) | P=<0.001 |
| | Unimmunized | 24 | 24 | 15(63) | |

*Table 1.* Summary of outcome of chimpanzee challenge studies using animals vaccinated with adjuvanted recombinant gpE1/gpE2 vaccine or unimmunized control animals and then challenged with either homologous (HCV1) or heterologous (HCVH77) subtype 1a viruses that predominate in North America (ref. 82).

particles in the laboratories of Drs. Jane A, McKeating and François-Loïc Cosset (83,84), then infectious virus itself by Dr. Wakita and collaborators (85) finally became possible, we were able to show that adjuvanted recombinant E1E2 envelope glycoproteins derived from a single HCV 1a strain elicited broad cross-neutralizing antibodies in vaccinated mice (86), guinea pigs (86), chimpanzees (87) and then human volunteers (Fig. 15; 88,89).

The use of chimeric HCV pseudoparticles (pp) and cell-cultured (cc) viruses containing E1E2 derived from multiple genotypes has greatly facilitated our knowledge of cross-neutralizing antibody responses (90,115). However, antibody-mediated neutralization of infectivity is weaker against genotypes 2 and 3 relative to 1,4,5 and 6 (87–89) and we have demonstrated different neutralization potencies even within the same genotype and subtype (121), suggesting that an optimum vaccine may need to comprise E1E2 derived from multiple genotypes, as is common for vaccines against other viruses (and bacteria).

Both neutralizing antibodies and HCV-specific cellular immune responses correlate with protection against the development of chronic HCV infection (91–99) and we showed that in addition to cross-neutralizing antibodies, the HCV E1E2 vaccine also elicits strong T cell lymphoproliferative responses in vaccinated humans (89,123). Furthermore, in the last 20 years, various groups have isolated cross-neutralizing HCV monoclonal antibodies targeting multiple, discrete epitopes within E1 and E2 (100–112), some of which have been demonstrated to be protective in HCV animal models (102,107,109,110) and to synergize with each other (108). Using monoclonal antibody competition assays, we showed that human volunteers vaccinated with recombinant E1E2 elicited antibodies to many of these discrete cross-neutralizing epitopes (113).

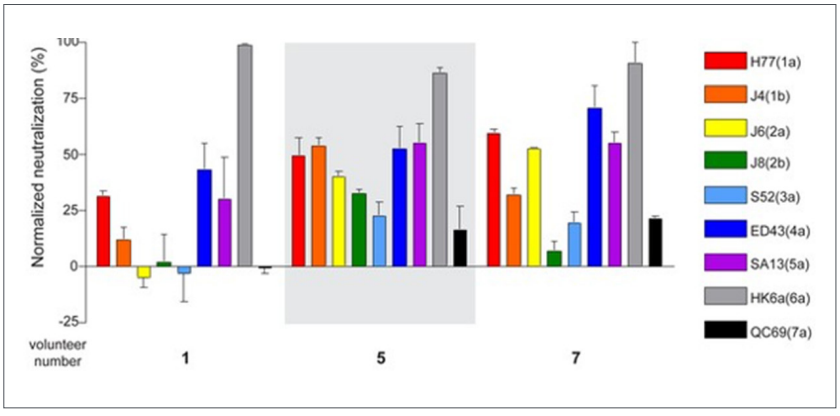


*Figure 15.* Human volunteers #s 1, 5 & 7 were vaccinated with adjuvanted gpE1/gpE2 and their antisera (diluted 1:50) assayed for the ability to neutralize different world-wide strains of HCVcc (89).

Along with the knowledge that acutely-infected chimpanzees (118,120) and people-who-inject-drugs (PWIDs; 119) who can eradicate HCV infection without therapy are then largely immune to developing chronic, persistent infection following re-exposure to homologous or heterologous HCV, these accumulated vaccine and immune correlates data do indicate the feasibility of developing a HCV vaccine. Further clinical studies of our HCV vaccine, designed to elicit broad cross-neutralizing HCV antibodies and broad, HCV-specific cross-reactive CD4+ & CD8+ T cell responses, are planned over the next few years with the objective of making it available first to high-risk PWIDs around the mid-2020s.

Together with the "test and treat" policy, successful vaccine development could mark the end of HCV being a major public health issue by 2030, if not before. The tremendously rapid development of SARS-2-Cov-2 vaccines using lipid/RNA and adenoviral vector technologies (124) are now also being applied to HCV vaccinology, giving the HCV field even more hope of delivering a global HCV vaccine within the 3rd decade of this millennium.

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