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An Assessment of the Use of Chimpanzees in Hepatitis C Research Past, Present and Future: 1. Validity of the Chimpanzee Model

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Summary — The USA is the only significant user of chimpanzees in biomedical research in the world, since many countries have banned or limited the practice due to substantial ethical, economic and scientific concerns. Advocates of chimpanzee use cite hepatitis C research as a major reason for its necessity and continuation, in spite of supporting evidence that is scant and often anecdotal. This paper examines the scientific and ethical issues surrounding chimpanzee hepatitis C research, and concludes that claims of the necessity of chimpanzees in historical and future hepatitis C research are exaggerated and unjustifiable, respectively. The chimpanzee model has several major scientific, ethical, economic and practical caveats. It has made a relatively negligible contribution to knowledge of, and tangible progress against, the hepatitis C virus compared to non-chimpanzee research, and must be considered scientifically redundant, given the array of alternative methods of inquiry now available. The continuation of chimpanzee use in hepatitis C research adversely affects scientific progress, as well as chimpanzees and humans in need of treatment. Unfounded claims of its necessity should not discourage changes in public policy regarding the use of chimpanzees in US laboratories.

Key words: *chimpanzee, hepatitis C, hepatitis C virus, hepatocellular carcinoma, Pan troglodytes.*

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Introduction

The use of chimpanzees in biomedical research remains at the centre of debate, both within the scientific community and among the general public. The suffering experienced by the chimpanzees involved (1–5), combined with growing evidence of the lack of human relevance of the data produced (6–10), provide compelling ethical and scientific arguments that have contributed to worldwide legislative decisions to ban, or at least significantly restrict, experimentation on chimpanzees and other great apes (1). These concerns are reflected in the United States, both in public opinion (11), and in legislative actions, such as the *CHIMP (Chimpanzee Health Improvement, Maintenance and Protection) Act* in 2000, the Burr Amendment of 2007 (12), and the 2009 reintroduction of the *Great Ape Protection Act (GAPA)*; 13) — legislation that seeks to prohibit the conducting of invasive research on all great apes.

In spite of the above considerations, invasive experimentation on chimpanzees continues to be performed in the USA. As the only significant user of these animals in such research, approximately 1,000 individuals were held in US laboratories as of late 2009 (14). It has been claimed, by some advocates of their use, that chimpanzees are a crucial tool in the fight against serious human dis-

eases such as AIDS, cancer and hepatitis (15), constituting a scientific necessity that ‘trumps’ ethical and practical concerns. While robust scientific evidence has been published to rebut these claims with respect to AIDS (6) and cancer (16), as well as the general utility of chimpanzee research with regard to human medical benefit (7, 8), little attention has been devoted specifically to the question of chimpanzee research into hepatitis C.

Science and medicine are naturally compelled to address hepatitis C, a disease that currently affects up to 200 million people and leads to billions of dollars in healthcare costs. Annually, there are around four million new infections and tens of thousands of deaths, as the disease often progresses to liver cancer, treatable only in approximately half of the cases. There is currently no vaccine available against the disease. As a result, hepatitis C research has been an area of intense investigation for many years. In common with other viral infections, a huge effort has been made to further understand the virus, the immune responses to it, the ensuing disease and pathology, and the roles of host and viral factors therein. Extensive work has also been undertaken toward the development of vaccines and antiviral therapies. This effort has comprised clinical, epidemiological, *in vivo*, *ex vivo*, *in vitro* and *in silico* approaches, but has also extensively utilised chim-

panzees as the only organism other than humans that can be reliably infected with hepatitis C virus (HCV). What remains unclear, however, is the role that chimpanzees could, or should, play in the discovery and development of these urgently needed therapies and vaccines. This can only be established by a thorough evaluation of the research requirements that only chimpanzee use can meet, and which cannot be met by any alternative approaches.

This report aims to elucidate and critically evaluate the current and future 'need' for chimpanzee experimentation in this field. An indication of the prior value of chimpanzee data must be obtained, by evaluating their contributions to tangible progress and assessing to what degree the data produced are predictive of, and relevant to, human hepatitis C infection. Further, a full appraisal of the scientific methodologies currently available to HCV research must be conducted, to identify precisely which questions can be answered, what data can be obtained, and where any gaps in knowledge might exist (this is addressed in detail in the companion article, which will appear in the next issue of *ATLA* [17]).

While retrospective analyses can indicate the value, predictive nature and human relevance of the chimpanzee model, such conclusions may or may not be pertinent to that model's value in future research, as new clinical and *in vitro* research methods may have superseded it. If non-chimpanzee approaches are able to provide all the necessary information to enable the development and testing of HCV therapies, then there is no need to use chimpanzees. If there is no current scientific requirement for chimpanzees, their value as a model is academic, and their use can no longer be justified. Importantly, a negative outcome for the chimpanzee model (e.g. revealing it not to be robust and predictive for humans) would invalidate it, and would preclude its further use in any case. However, if there might be data that only chimpanzees can provide, there must be a critical and ethical appraisal of such necessity with regard to the aim of realising HCV therapies. Would the perceived benefit of additional and specific data, only available through chimpanzees, positively impact HCV research as a whole to a significant enough degree to warrant the ethical and financial cost of those experiments? For these reasons, a retrospective analysis of the validity of chimpanzee use in hepatitis C research is a crucial part of this process.

Hepatitis C: The Virus, Disease and Treatment

A human hepatitis-like illness that did not conform to type-A or type-B hepatitis diagnoses was

identified in the 1960s, based on studies of humans who had received blood transfusions (18). However, the causative agent of "non-A, non-B hepatitis" (NANBH; 19) was not identified until 1989 (20), when tests on human serum samples from blood donors and recipients finally confirmed that the agent was HCV (21, 22).

HCV infection now poses a serious and growing problem worldwide, with significant human and financial impacts. Approximately 170–180 million people are infected, representing some 3% of the world's population, and the number of infected people is growing at a rate of three to four million per year (23–25). In the USA alone, it is estimated that four million people are infected, with up to 10,000 deaths and 40,000 new infections per year, while in Europe, five million are HCV-positive (24). HCV infection is the leading cause of liver transplantation in developed countries (26, 27), with an enormous burden in direct healthcare costs that exceeded one billion US dollars more than a decade ago (24). Projections indicate that HCV-related chronic liver disease will affect four times as many people in 2015 than it did in 1990 (24).

The virus chiefly infects hepatocytes, though peripheral blood mononuclear cells are also infected (28–30). Acute infection is very often asymptomatic (31), and a significant number of infected individuals eliminate the virus without treatment. Estimates of the proportion of infected individuals who spontaneously resolve their infections, vary from 15% to 50%, though an average of 25–30% seems to be a reasonable consensus (32, 33).

Chronic infection, however, often leads to liver fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC; 34). While approximately 75% of infections become chronic, almost all of these — and 60–70% of *all* HCV infections — progress to liver disease. Between 5% and 20% of infected people (and around one-third of those chronically infected) eventually develop liver cirrhosis, and many individuals go on to develop HCC (35). More than half of all HCC cases, and two-thirds of all liver transplants, are directly due to HCV infection (36). HCC is one of the most prevalent and deadly cancers in the world (37), and up to 5% of HCV-infected people will eventually die from this form of cancer (33).

In spite of intense research since the discovery of HCV in 1989 (20), an effective prophylactic human vaccine remains elusive. The difficulty in developing successful HCV vaccines and antiviral therapies is largely due to the significant genetic heterogeneity of the virus, which is a consequence of the high activity of the error-prone and non-proofreading viral polymerase. This leads to the presence of viral quasispecies in infected people, confounding the generation of effective neutralising antibodies and other immune responses, thus

instigating immune escape and the tendency for infection to become chronic (38).

Therapeutic options currently amount to just one approved intervention, which entails treatment for 12–72 weeks with a combination of non-specific antivirals in the form of pegylated interferon-alpha (PEG-IFN- α) and ribavirin (39–41). While the mechanism of action of this treatment is not well understood, IFN-mediated host-directed immune modulation and antiviral effects, augmented by ribavirin (42), typically induce a sustained viral response (SVR) in between 40% and 60% of patients (43–47), which results in HCV being undetectable six months after the end of treatment (45, 46, 48). The benefits of this treatment, for those who respond to it, are considerable: the virus is eliminated from the patient's liver and blood cells and remains undetectable, in almost all instances, for many years (48, 49); it ameliorates fibrosis and cirrhosis; and it helps prevent progression to HCC (24, 50). However, there are many caveats. The response to treatment is highly genotype-specific (51), ranging from 60–90% for HCV genotypes 2 and 3, but it can be as low as 30–70% for genotype 1, the type of HCV most prevalent in the USA (33, 52). On average, this means that almost half of infected individuals fail to respond to therapy — and this proportion is increasing (24, 42). Further, treatment is poorly tolerated and causes significant adverse reactions, including anaemia, depression, fever and fatigue, with the result that many patients discontinue the medication (41, 42, 53–56). A standard course of treatment costs over \$20,000 (57), which prevents many people from being treated (58). It is clear that superior therapies and a vaccine are imperative for the effective management of hepatitis C.

How Chimpanzees are Used

Chimpanzees have been used in hepatitis C/NANB research for three decades (Figure 1). Despite an initial steep rise in their use from 1979 through to the mid-1980s, the trend since then has been downwards, with the current relative research interest (2009) approaching one-third of its peak value in 1985 and a historical low. This considerable reduction in chimpanzee use is encouraging, ethically, as it indicates a lack of need for chimpanzees — arguably because of lack of utility and/or diminishing scientific need due to replacement technologies. Figure 2 illustrates this point further, outlining the contrasting rise in hepatitis C research, over the same period, which does not involve the use of chimpanzees or any other non-human species (see companion article [17]). No matter how low the numbers of chimpanzees currently used in HCV research, the consequences for

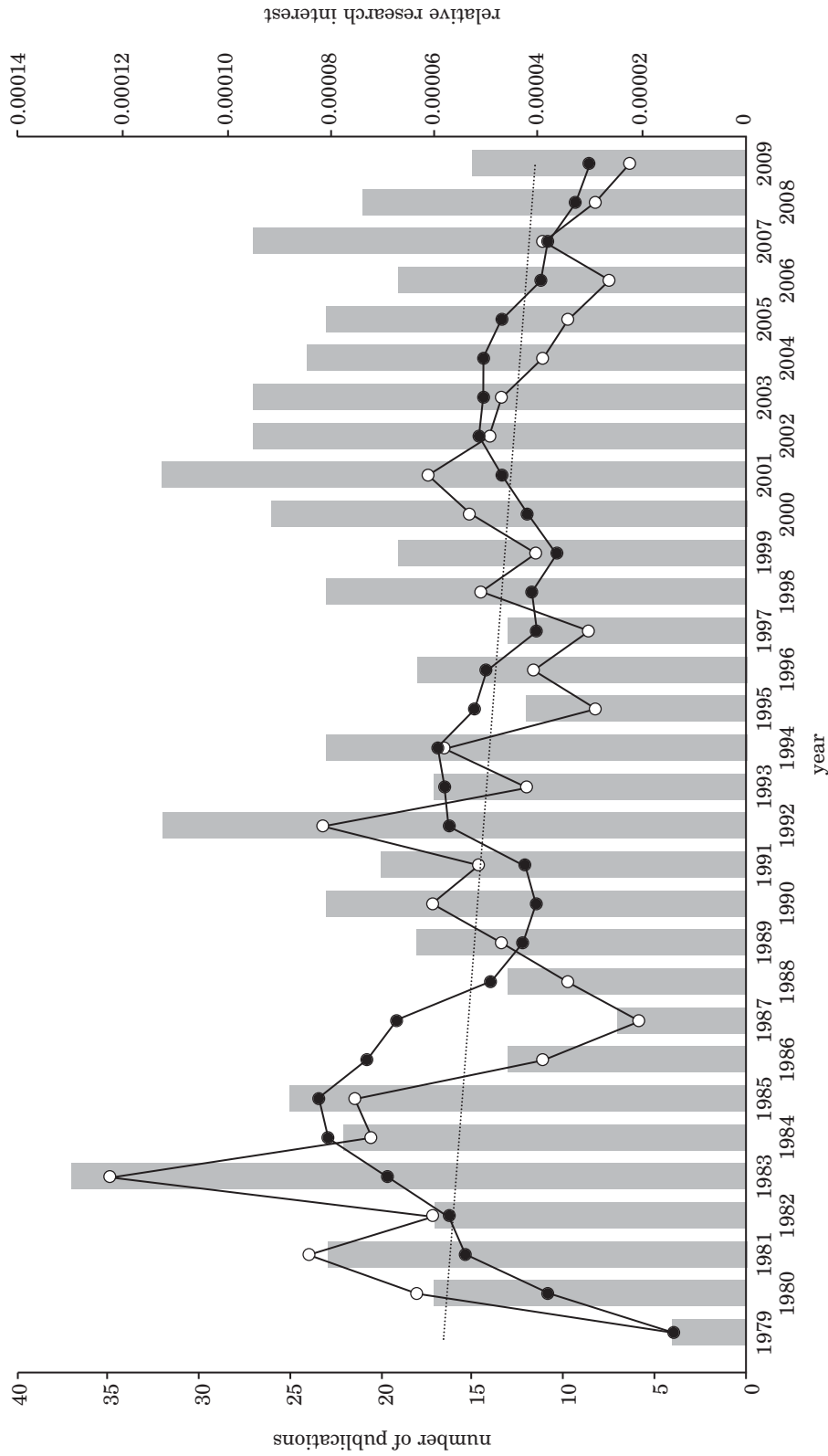
those individuals in active protocols are severe and pose grave ethical concerns.

It is estimated that approximately 500 chimpanzees have been used in HCV-related investigations from 1998 to 2007 (59). These chimpanzees were subjected to invasive and stressful procedures (59), including frequent blood sampling, repeated liver biopsies, intravenous or intrahepatic inoculation of HCV, and injections/infusions of potential vaccines. The details of the procedures performed on chimpanzees are almost invariably scant. No elaboration of blood sampling procedures is given — these can be weekly or, in the case of programmes of six months or longer, bi-weekly or monthly (e.g. 60–62). Blood draws can be achieved by training the chimpanzees to present their arms for phlebotomy, or can require a 'knockdown' in which chimpanzees are immobilised by being shot with dart guns loaded with anaesthetic (63). Knockdowns are common, and are required for the other procedures mentioned above, though they are not explicitly cited in the literature. Given their traumatic nature, it is important to note the degree of knockdowns. It is not uncommon for chimpanzees to be surrounded by laboratory staff during the procedure, which induces severe stress. Fear of the gun causes chimpanzees to attempt to avoid it, resulting in wayward darts hitting sensitive areas, such as the eyes and genitals, or even puncturing their lungs (64). Generally, little explanation is provided regarding the frequency and general procedure for biopsies and inoculations. Liver biopsies, initially, may be performed up to three times per week, with a chimpanzee typically undergoing dozens of these procedures in any particular investigation (e.g. 65–69). Such biopsies may be done percutaneously by using a needle, or as part of open surgery to obtain more substantial wedge biopsies (70). Either way, postoperative pain is common and severe (71). Procedures for inoculation with HCV and/or testing potential vaccines are also invasive: infection can take up to ten challenges (72, 73), and may involve intrahepatic inoculation (74) entailing open surgery (75); testing of vaccines involves multiple procedures, with up to 11 immunisations being administered over a 48-week period (e.g. 76–78). Altogether, it has been estimated that a chimpanzee may be subjected to around 30 knockdowns in any one investigation, and that each knockdown can require the use of up to five darts (64). Because chimpanzees are used repeatedly in different research programmes, some individuals have been subjected to more than 300 knockdowns and over 130 liver biopsies (64).

Validity of the Chimpanzee Model

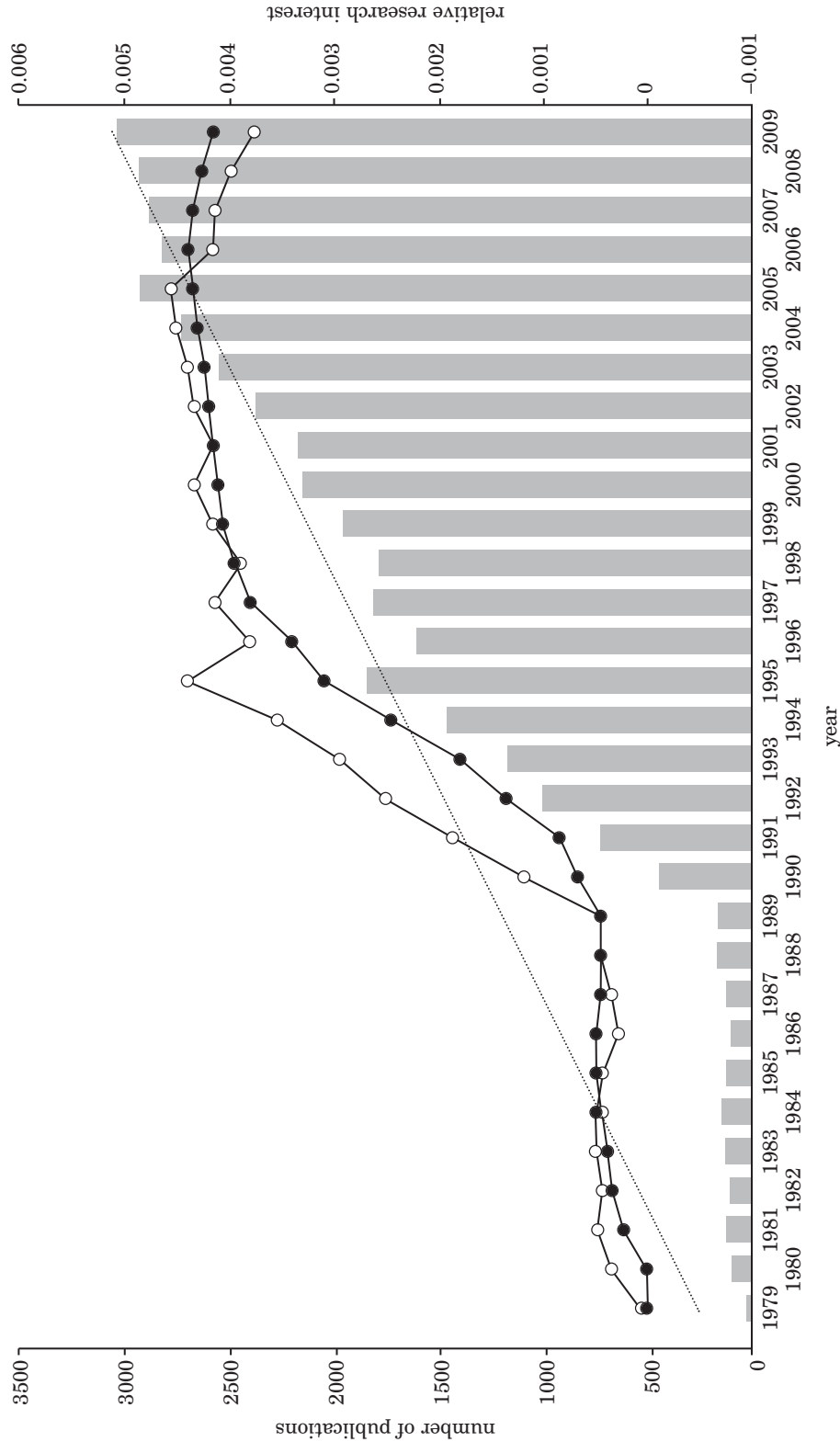
Against this ethically problematic background, the scientific worth of chimpanzee experiments in

Figure 1: Thirty-years of papers associated with chimpanzees and hepatitis C virus (HCV) research



Relative research interest provides a better view of research activity and importance than an absolute number of papers (solid bars), by relating publication statistics to the overall number of scientific publications indexed by PubMed. It is calculated as: weighted publications/year (subject specific)/total weighted publications/year (in PubMed), where weighted publications/year = number of publications/year multiplied by relevance factors (as defined by PubMed). The smoothed relative research interest is the relative research interest smoothed by a sliding window of five years. It is calculated as: mean $(R[y-4], R[y-3], R[y-2], R[y-1], R[y])$, where y = year, R = absolute relative research interest for year y . All data shown were derived from the 'Statistics' feature provided by the GoPubMed literature search engine. The absolute relative research interest is indicated by the black data points; the smoothed relative research interest is indicated by the white data points; and the dotted line indicates the trend. The smoothed relative research interest of chimpanzee HCV papers decreased from approximately 0.00008 in 1985 to 0.00003 in 2009; a decline of over 60%.

Figure 2: Thirty-years of papers associated with non-animal methods of hepatitis C virus (HCV) research



Relative research interest provides a better view of research activity and importance than an absolute number of papers (solid bars), by relating publication statistics to the overall number of scientific publications indexed by PubMed. It is calculated as: weighted publications/year (subject specific) / total weighted publications/year (in PubMed), where weighted publications/year = number of publications/year multiplied by relevance factors (as defined by PubMed). The smoothed relative research interest is the relative research interest smoothed by a sliding window of five years. It is calculated as: mean (R[y-4], R[y-3], R[y-2], R[y-1], R[y]), where y = year, R = absolute relative research interest for year y. All data shown were derived from the 'Statistics' feature provided by the GoPubMed literature search engine. The absolute relative research interest is indicated by the white data points; the smoothed relative research interest is indicated by the black data points; and the dotted line indicates the trend. The relative research interest of HCV papers based on non-animal research methods increased approximately 80-fold over the three decades from 1979-2009, inclusive, in marked contrast to the decline in chimpanzee HCV research of over 60%.

HCV research must be measured. It is not possible to assess their scientific value, past or present, based solely on statements and opinions in the literature. For example, it is often claimed that chimpanzee data have contributed significantly and crucially to our knowledge of the virus and of the disease. According to some reports, this animal model was required for demonstrating that a transmissible, filterable agent with a lipid-containing envelope was the cause of NANBH (79–83). In the actual discovery of HCV, these animals were said to have provided a means to amplify the causative agent of NANBH in order to acquire sufficient amounts of virus for cloning and identification (20, 52, 83). It was also claimed that chimpanzees were essential in the development of HCV infectious clones (84–87), and in demonstrating that HCV infection does not elicit protective immunity (88–90).

The chimpanzee “has been considered the primary choice for studying the relationship between the virus and host anti-viral immune responses, as well as for evaluating immunopathogenesis and the efficacy of prophylactic vaccination” (91), and as “a useful model for HCV infections” by virtue of the genetic similarity of humans and chimpanzees (90). Through the past decade, it has been claimed that chimpanzees are:

- the only way that vaccine immunogenicity and efficacy can be tested (86);
- the only means of analysing the early events of HCV infection due to its asymptomatic nature in humans, and of determining whether antibodies raised against HCV have neutralising activity (90);
- the only means of monitoring the entire course of infection because of ethical issues surrounding the frequent sampling of liver tissue in infected humans (86); and
- the only approach to studying infections with specific and well-characterised inocula (87).

Speculative claims of future crucial involvement in HCV research are also frequent. It is alleged, for example, that chimpanzees will be important in the definition of correlates of protection, to determine the duration and extent of cross-genotype vaccine protection, to understand mechanisms of chronicity, and to derive optimal vaccine formulations (52).

It is widely implied that the chimpanzee model has been extensively used, simply because the chimpanzee is the only species, other than man, which can be infected with HCV (e.g. 32, 52, 92, 93). This suggests that the researchers’ hands were forced to some degree, as no other *in vivo*

model was available, and is borne out by a consideration of the acknowledged significant differences between the course of HCV infection and pathology in humans and chimpanzees. It is widely appreciated that “experimental infection in primates is in many respects poorly representative of human infection” (92), and that “the chimpanzee model of HCV infection may not recapitulate all aspects of the virus–host interaction of the infection in humans” (94). Though species-specific estimates vary (90), in general, there is a much lower rate of chronic infection in chimpanzees, due to greater viral clearance (95–97). Persistent infection has been estimated at approximately 85% for humans, but it might be as low as 30–40% for chimpanzees (87). Immunologically, it has been demonstrated that the production of antibodies to the HCV envelope proteins is much less robust in chimpanzees, possibly due to reduced viral replication during acute infection (97, 98), and it seems that HCV envelope proteins mutate to a lesser degree in infected chimpanzees compared with humans (99). Chimpanzees infected with HCV do not progress to having liver fibrosis and cirrhosis in the way that humans do, and have much milder symptoms (90). Furthermore, HCV-induced hepatocellular carcinoma is very rare in chimpanzees (87), and, unlike in humans, there is a lack of mother-to-infant transmission of HCV (100).

In light of these differences and the other serious issues outlined above, all claims of the indispensability of the use of chimpanzees in HCV research must be critically examined. It is of no consequence with regard to the validity and necessity of chimpanzee HCV research, if the involvement of chimpanzees in a particular line of investigation was of secondary importance. Any involvement must be shown to be absolutely crucial. The data must be relevant to, and predictive of, human biology; there must be no alternative way in which these data could have been obtained; and the data must have led to tangible medical progress. If the results from chimpanzee experiments fail to meet any of these criteria, then it must be concluded that their use was unnecessary and unethical.

HCV Research: Contributions of Chimpanzee, Clinical and *In Vitro* Methods

Early observations of NANBH and the discovery of HCV

Human-based research features heavily in accounts of the discovery of hepatitis C (e.g. 83, 101), and of the discovery and early characterisation of its causative agent. The acknowledged

human-based contributions include: demonstrating that NANBH was the salient complication of transfusion therapy; defining NANBH's natural history; identifying surrogate markers of the disease, such as alanine aminotransferase; and lowering the incidence of transfusion-associated NANBH, even prior to the identification of the virus itself (102).

However, such clinical research appears alongside assertions of the vital nature of contemporary chimpanzee involvement, of which an examination is presented here. NANBH was first described in 1975, when newly developed serological tests based on clinical research demonstrated that most cases of transfusion-associated hepatitis were not caused by the hepatitis A or B viruses (HAV and HBV; 19, 103, 104). Human studies also suggested that a new infectious agent was responsible for the disease that led to liver damage and even cirrhosis (105), and was not only manifest following approximately 10% of blood transfusions, but also spontaneously in the population (106–108). Around half of NANBH patients went on to suffer from chronic disease (109), and some developed hepatocellular carcinoma (110).

The results from chimpanzee studies definitively demonstrated the transmissibility of NANBH (79, 80). However, this had been previously suggested by the aforementioned human studies. Further, one of the serum samples from a NANBH patient, which was used to 'infect' a chimpanzee in these studies, was already known to have transmitted the disease to a nurse following an accidental needle-stick injury. Two other sera which were used originated from blood donors whose blood had been implicated in cases of post-transfusion hepatitis in human recipients (80). As the authors of the chimpanzee study noted, "We have shown that non-A, non-B hepatitis is transmissible to young chimpanzees by sera from human beings whose blood had transmitted the disease to other human beings" (80). They also reported that a number of previous attempts to transmit NANBH from humans to chimpanzees had failed.

Attempts to isolate and identify the NANBH virus then floundered for more than a decade — in large part because potent molecular biological methods, such as the polymerase chain reaction (PCR; 111), were not available. The major breakthroughs were made in the mid-to-late 1980s, following the painstaking screening of cDNA expression libraries derived from NANBH virus-infected chimpanzee liver samples (101). The protocol involved the speculative 'fishing' of NANB-genome specific clones out of these libraries by using sera from infected humans and chimpanzees as 'bait' — the hypothesis being that antibodies in these sera, specific to the NANB infectious agent (generated by the host immune system in response to the infection), would bind to

the NANB polypeptides present in the expression library, thus identifying immuno-positive polypeptides for selection and further characterisation. Many millions of clones were screened, initially, with no success. Finally, a positive NANBH clone was isolated via a screening process that utilised a cDNA library from a single chimpanzee with a markedly high plasma titre of the NANBH infectious agent, which was screened with serum from a human chronic NANBH patient with very high serum alanine aminotransferase levels (indicating severe liver damage; 20).

Subsequent comparative experiments, involving the use of sera from infected and uninfected humans, verified the NANBH-specificity of this clone, and revealed the viral genome to be RNA, not DNA, which facilitated the identification of other viral clones (101). Further work with human sera led to the development of an immunoassay that served as a blood-screening test for what was now known as hepatitis C virus (22, 112), which prevented many HCV infections and, ultimately, HCV-related deaths via the transfusion of contaminated blood.

Therefore, it is evident that the crucial steps in the early characterisation of the disease and the infectious agent were based on human investigations, and that chimpanzee involvement was not integral or essential. Given the lack, at that time, of many of the molecular techniques that now exist, when attempts were made to positively identify HCV, chimpanzees were undoubtedly useful in the generation of serum samples with high titres of the infectious agent, the use of which greatly increased the chances of success in discovering HCV-specific clones during the laborious screening process. More virions provided a greater proportion of HCV-specific clones, which in turn facilitated their isolation. Yet, *in retrospect*, it is perhaps likely that the use of uncharacterised (i.e. low/'standard' titre samples) in contrast to these high-titre sera would not have precluded, or even compromised, the identification of HCV-specific clones. Though the infectivity of human HCV sera is low in chimpanzees, which suggests low titres of virus and reduced levels of HCV genomic material for cloning, it is now known that the poor infectivity of such sera is on account of the genomic RNA being immune complexed (113, 114). Uncharacterised human sera would probably have been equally useful for cDNA library construction, and to the eventual identification of HCV clones and the virus itself.

Infectious molecular clones of HCV

The development of infectious molecular clones of HCV is one of the more salient examples put forward by advocates of the importance of chim-

panzee research. For example, it is claimed that the chimpanzee provided the only means of assessing the infectivity of HCV clones, and that subsequent experiments with these infectious clones led to several valuable insights into the natural history of HCV infection and host immune responses (86).

HCV clones are produced by reverse-transcriptase PCR (RT-PCR) of HCV genomic RNA present in infectious serum samples. The resultant cDNA clones can then be inserted into expression vectors, which, when specifically linearised by a restriction endonuclease, allow the generation of run-off transcripts producing RNA molecules with precise 3' termini, representing HCV genomic RNAs (115). Because these RNA molecules are positive (i.e. genomic) sense, they can be used *in vitro* or *in vivo* directly, as mRNA templates for the translation of the HCV viral proteins, thus initiating the viral life cycle (116).

Initially, the reality was not as straightforward as the theory. In some of the first reports of successful infectious clones in chimpanzees, investigators referred to previous experiments in which dozens of pre-screened clones (in terms of restriction analysis, polyprotein processing and viral-polymerase production) failed to demonstrate infectivity when intrahepatically injected into chimpanzees (84, 85). This was thought to be due to the absence from the clones of important nucleotides from the 3'-untranslated region (3'-UTR) of the HCV genome, as well as mutations incorporated into the clones due to errors during the RT-PCR process (84, 85). To remedy this, lessons were learned from similar attempts at infectious clone production with other related viruses (e.g. 117), in which *consensus* sequences of the specific viral isolates being used were determined. This overcame problems caused by inherent error-prone replication of the viral genome during cloning and as part of the viral life cycle, which introduces mutations into progeny genomes, thereby rendering them defective (118–120). These introduced mutations are not prevalent in the viral population, and are therefore identifiable in relation to the consensus sequence of that population. By aligning clones of an HCV genotype 1a isolate H77, obtained from the serum of an infected patient with a high viral titre, and then establishing a consensus sequence, HCV clones were finally produced that were shown to be infectious when inoculated into the livers of chimpanzees (84, 85).

It is claimed that the investigators in these first successful studies tested the infectivity of their clones via the inoculation of chimpanzees, as opposed to the infection of cell lines, "because it was unclear whether such a genome would replicate in cell culture" (118), and due to the "lack of reliable *in vitro* propagation systems of HCV" (85). This assertion has subsequently been compounded

by affirmations that the very development of these clones was *dependent* on chimpanzees (86, 87). It can be argued that only the first of these three statements is valid, and that the last statement is false. It was certainly unclear whether any cloned genome would replicate in cell culture. Yet, given the considerations above, it must be concluded that this uncertainty was no greater than the uncertainty that any clone would replicate in chimpanzees. Both approaches had encountered numerous failures up to this point. It is also likely that *in vitro* approaches were being overly criticised. A recent review cites several successful cell culture approaches, contemporaneous with the first reports of successful chimpanzee inoculation (121). For example, a persistently HCV-infected Huh7 human hepatoma cell line had been created by transfection with putative full-length HCV-1 transcripts, which demonstrated HCV replication and the production of biologically-active progeny virus (122). In addition, a HepG2 human hepatoma cell line transfected with near-full-length HCV RNA formed the basis of a stable and reproducible system exhibiting robust HCV replication and the production of infectious viral progeny (123). The investigators opined that these systems were valid for the study of key aspects of the HCV life cycle, such as viral replication, persistence and pathogenicity, and to test anti-viral agents.

A study of the literature offers several explanations as to why *in vitro* approaches like these might have been prematurely and incorrectly discounted in favour of *in vivo* methods. The ability of cell lines to provide a means of assessing infectivity of clones, and of investigating the viral life cycle, could have been generally underestimated. In common with early *in vivo* attempts, the viral clones were missing important sequences from the 3'-UTRs (124–126) and, prior to the use of consensus clones, they were carrying many deleterious mutations (84, 85, 118). But, perhaps more importantly, it has been claimed that *in vitro* methods posed too many practical problems regarding the measurement of HCV replication, due to difficulties in differentiating between the high input-level of template RNA required to efficiently transfect the cells, and the sparse amounts of product RNA because of the low efficiency of *in vitro* HCV replication (127). In fact, not only did *in vitro* systems exist with respectable levels of HCV replication (such as those cited previously), but also a number of sensitive, reliable and routine methods were available to specifically detect and quantify HCV replicative activity. To illustrate this point, HCV replication can be detected and measured via:

- RT-PCR and/or ribonuclease protection assay of negative strand RNA intermediates, which are only produced during viral replication;

- the incorporation of radioactive nucleosides, such as ^3H -uridine, into *de novo* RNA molecules;
- the *in situ* RT-PCR of HCV RNA present in long-term-cultured cells;
- the infection of freshly cultured, non-infected cells with culture media from transfected cell lines, indicating the production of infectious HCV;
- the immunostaining of viral proteins; and
- the visualisation of the virus, or virus-like particles, via electron microscopy (122, 123).

Further, recent examinations of 14 HCV clones and their *in vitro* and *in vivo* properties (118, 127) list parallel references for the *in vitro* and *in vivo* studies of each HCV clone. Of the 14 clones, six are referenced in chimpanzee studies prior to cell culture experiments (as might be expected in light of the assumed importance of the chimpanzees and the assumed unsuitability of cell cultures). Yet, four clones are referenced in *in vitro* studies prior to the cognate chimpanzee citation. The remaining four clones were either referenced in one system, but not in the other, or the *in vitro* and *in vivo* approaches were reported concurrently. In addition, an extract from one of the first papers reporting a successful infectious clone in chimpanzees may be instructive with regard to the choice of chimpanzees over cell cultures for the study of infectious clones. Reasons of practicality, given that a chimpanzee protocol was already in use, seem clear: “We previously established an *in vivo* transfection system for RNA transcripts of infectious clones of hepatitis A virus in tamarins, as well as in chimpanzees. Therefore, the infectivity of RNA transcripts of full-length HCV clones was tested by injecting transcription mixtures into the liver of chimpanzees” (85).

It can be argued that it was not scientifically justifiable or necessary to use chimpanzees to test the infectivity of HCV molecular clones. Nevertheless, chimpanzees continued to be used to investigate the infectivity and characteristics of a number of subsequent HCV clones (128, 129), including consensus clones of other genotypes such as 1b (strain J4; 62) and 2a (strain J6; 73). These clones were used in reverse genetic analyses of the HCV genome, to establish the functions of its various regions and encoded enzymes, yielding valuable data (75, 130). Crucially, however, similar experiments and findings have been obtained *in vitro*. For instance, the regulated and non-regulated expression of HCV clones (subgenomic and full-length) in various cell lines (121) has allowed the examination of: subcellular localisation of HCV

proteins and assembly of virus-like particles (131–134); functions of wild-type and mutant HCV proteins, such as the NS5b polymerase (131); the effects of HCV proteins on host-cell growth and gene expression (135, 136); the functional roles of HCV UTR stem-loops (137); the antiviral effects and modes of action of IFN and ribavirin (138, 139); the role of CD26 in HCV infection (140); the effect of HCV proteins on IFN-induced intracellular signalling (141); the determinants of membrane association of the viral polymerase (142); and various properties and characteristics of the HCV NS3-NS4A complex (143).

It is fortunate that *in vitro* methods were delivering comprehensive and useful data and showing great promise for future utility. An alternative to the chimpanzee model has always been asserted as an imperative, due to widely-recognised caveats, including “limitations in the variable course of HCV infection in chimpanzees” (121), their “endangered status” (121), and the “expense associated with their use” (87). It was auspicious that *in vitro* approaches to HCV research were continuing to evolve with the revolutionary development of subgenomic and consequently full-length replicons.

Acute versus chronic hepatitis: Factors determining viral clearance and progression

A significant amount of chimpanzee HCV research has focused on analysing the early events following infection, as it is likely that these events critically influence the outcome with regard to viral clearance or persistence (65). It is claimed that the controlled infection of chimpanzees provides the only means of investigating the early events in HCV infection, since natural infection in the human population is only symptomatic (and therefore detectable and amenable to research) when infection becomes chronic (87, 90). Further, it is suggested that, because chimpanzees clear the virus at a greater rate than infected humans do, the former represent an ‘attractive’ model to investigate clearance, since the same factors *may* lead to clearance in humans (65). While chimpanzees have been used to obtain relevant data, such as demonstrating that animals who resolved acute HCV infection had stronger cytotoxic T-lymphocyte (CTL) responses than those which went on to develop chronic hepatitis (144), equivalent and extremely informative data have emerged from prior and contemporary human studies of the roles of cellular and humoral immunity during acute HCV infection and viral clearance. For instance, the critical role of cellular immunity was indicated by observations that HCV-negative humans, especially those who had had prior virus exposure, exhibited cellular immune responses to it

(145–147). In addition, examinations of people exposed to HCV-contaminated blood products revealed the persistence of cellular immune responses decades after recovery, with a concomitant disappearance of anti-HCV antibodies (148), and drew attention to the importance of particular human leukocyte antigen (HLA) alleles in viral clearance (149). Clinical observations indicated that CD8⁺ CTLs, known to recognise both conserved and variable regions of HCV proteins in the context of several different HLA molecules, had been detected in the peripheral blood and liver in humans (150, 151). Human studies had also indicated vigorous T-cell responses in acutely infected patients, in whom viral clearance was associated with a strong CD4⁺ helper T-cell response (152–154).

The accidental exposure of healthcare workers by needle-stick injury, provides a powerful means of studying virological and immunological events following from a human perspective, during the incubation phase of acute HCV infection. One such study led to the following information: “the vigour and quality of the antiviral T-cell response determines the outcome of acute HCV infection; that the ability of HCV to outpace the T-cell response may contribute to its tendency to persist; that the onset of hepatitis coincides with the onset of the CD8⁺ T-cell response; that disease pathogenesis and viral clearance are mediated by different CD8⁺ T-cell populations that control HCV by both cytolytic and noncytolytic mechanisms; and that there are different pathways to viral persistence in asymptomatic and symptomatic acute HCV infection” (155). Further, the comparative study of chronic and long-term recovered HCV patients revealed that HCV-specific CD8⁺ cells in chronically-infected patients had impaired proliferative and effector functions, which possibly contributed to viral persistence (156). Chimpanzees have also been extensively used in attempts to elucidate the characteristics of protective immunity to HCV reinfection, following a prior and resolved infection (157, 158). The results have been conflicting: some chimpanzees were not protected against challenge with a quasispecies, even of the same viral strain (88, 89), while others exhibited rapid viral clearance after rechallenge with both homologous and heterologous HCV (159), a protection that was even extended to HCV of other genotypes (160). Most chimpanzee studies similarly indicate that, following a prior infection, rapid control of HCV rechallenge occurs. A more recent investigation suggested that this was limited to homologous virus genotypes — heterologous HCV challenge resulted in viral persistence (60). Notably, the mediation of viral clearance via cellular immunity, alluded to earlier, does not appear to be augmented by humoral immunity. In chimpanzees that resolved infection or resisted reinfection, neu-

tralising antibodies were not present at significant levels (157). However, this finding was not groundbreaking, as this had been previously demonstrated in a clinical study on humans with acute HCV infection (161). Similarities between human and chimpanzee immune responses disappear in chronic infection — while neutralising antibodies are not found in chronically-infected chimpanzees, humans with chronic infection have high levels of neutralising antibodies and the virus acquires neutralisation-escape mutations (162).

The examination of chimpanzee blood and liver samples, by using microarray technology, has been used to investigate changes in gene expression during acute infection and the virus clearance process. These studies revealed, for example, a biphasic pattern of viral clearance which involved different mechanisms fundamental to the cessation of viral replication to clear viraemia, and that was followed by the elimination of infected hepatocytes (clearance from the liver) (65, 87). This had been previously deduced, via clinical research and mathematical-modelling, with experimental results from HCV-positive patients undergoing IFN- α -based therapy (163, 164).

Interferons

Microarray-based studies with chimpanzees have shown that a significant number of genes, the expression of which is affected during both acute-resolving and chronic HCV infection, are IFN-stimulated genes (ISGs), which further implicates the role of type-I IFNs in the control of HCV infection (69, 165, 166). Similar conclusions have also stemmed from human and *in vitro* studies. For example, clinical investigations have demonstrated the critical role of IFN- α in HCV clearance (44, 45, 167), and *in vitro* infection studies have shown that IFN- α inhibits HCV replication in primary human hepatocytes (168) and in lymphocytic cell lines (169). Mathematical modelling based on clinical studies indicated that IFN- α blocked the production of HCV virion (163), and *in vitro* investigations with subgenomic replicons showed that they, too, are sensitive to IFN- α (170, 171), allowing the detailed *in vitro* study of HCV replication and investigation of IFN activity and its effects on ISGs (172–174).

The analysis of differential gene expression, via microarray technology and RT-PCR, of human liver and blood samples from HCV-infected and uninfected individuals, has confirmed the intimate involvement of interferons in HCV infection control. It also identified specific genes with antiviral activity, along with many other molecular pathways involved in the anti-HCV immune response (175–177), as well as revealing signature genetic differences in patients who do not respond to IFN

therapy (178, 179). One investigation on such patients, that involved the use of chimpanzees, reported similar results to previous chimpanzee experiments in terms of the ISGs induced (180), but the results were also similar to those from clinical and *in vitro* experiments (181, 182). This situation not only gives validity to these non-chimpanzee approaches, but it raises questions as to why chimpanzees were being used in the investigation at all, especially as human liver-biopsy specimens were used alongside the chimpanzees.

Development of Antivirals and Vaccines

Basic immunity research to inform vaccine development

Fundamental to the intelligent and rational development of HCV vaccines is the determination of the exact types of immunity that are effective against the virus during acute infection, enabling the most salient immunogens and immune responses to be targeted. It has been argued that chimpanzees are uniquely suitable for this purpose because they permit frequent biological sampling during the acute phase of the disease (90), but there are human-specific alternative approaches. Acute infection, even during the very early phase, can be, and has been, extensively studied in humans, and is exemplified by studies of patients exposed to contaminated blood products and the victims of accidental needle-stick injuries, etc. (see above, e.g. 155). Elegant prospective studies of acute HCV infection have been conducted, and these can be achieved, for example, by the routine screening for the virus in new admissions to a young offenders institution (183). This study identified asymptomatic HCV-positive individuals, whose outcomes and courses of infection with regard to humoral and cellular immune responses were monitored. The results revealed that both types of immune response to HCV were weak, or even absent, in patients who exhibited spontaneous viral clearance. The sampling of liver tissue, while invasive and potentially painful, is also not the preserve of chimpanzee research. Many HCV investigations have entailed the use of human liver biopsies, and have resulted in important discoveries. For example, human liver biopsies have been involved in the elucidation of the molecular pathways implicated in HCV core protein-mediated angiogenesis (184). The role of microRNAs (miRNAs) in HCV infection and IFN therapy (185) were similarly investigated by using human liver tissue. Human liver biopsies were also useful for identifying differential gene expression in patients who do, and do not, respond to antiviral

therapy (178), and to uncover IFN-specific genes in responders that effect antiviral responses, such as the viperin protein (175). Human liver tissue samples might also be helpful for the study of disease progression: gene expression patterns that correlate with progression to fibrosis can be identified (186–192), and disease progression, in patients with persistently normal alanine transaminase (ALT) levels, can be assessed (193, 194).

It is therefore evident that immunity to HCV during acute infection, and productive research into the development of HCV vaccines, can be investigated extensively, even in liver biopsy samples, without the use of chimpanzees. Indeed, it was often acknowledged that the lack of robust tissue culture systems had hampered the identification of potential antigens with key determinants of neutralisation, and the development of effective HCV vaccines (e.g. 195, 196). Further, the *in vitro* investigative approaches discussed above have been developed since the various claims of chimpanzee indispensability in this area were made. These *in vitro* studies have positively impacted vaccine development in many ways, including: the facilitation of viral neutralisation studies to identify neutralising antibodies and relevant epitopes, etc.; the discovery of factors, cofactors and receptors involved in the infectious process; and the identification of host genes that play a role in the infection. Given that these methods can be used to study HCV infection from its inception, they also circumvent the need to take regular liver tissue biopsies from newly infected chimpanzees. Not only can these studies be achieved without recourse to chimpanzee use, but also a move away from the chimpanzee model toward alternatives offers many advantages. It is quicker, easier, more humane, and more human-relevant to utilise *in vitro* methods. The use of these methods overcomes acknowledged and intractable problems inherent to chimpanzee use, such as the restricted number of experiments that can practically be performed, and the limited inference that can be made with statistical significance from chimpanzee data, given the low sample sizes involved (90), which typically number between two and four animals (86).

Vaccine testing

The development of an effective HCV vaccine undoubtedly remains a major challenge. The immunological correlates of protection are still to be fully determined, and even were they to be established any time soon, serious challenges would remain. For example, multiple HCV genotypes and circulating quasispecies in infected individuals require any vaccine to elicit broad immunological responses, both in terms of cross-neutralising antibodies to inhibit viral spread, and

an efficient cellular immune response to clear infected host-cells. Further, a therapeutic vaccine must contend with the T-cell failure that is associated with persistent HCV infection (197).

Thus far, many vaccines of varying types have been created and tested, with more in the pipeline — “an exponential growth” in the preclinical testing of HCV vaccines has occurred over the past three to four years (198). Although there is no comprehensive and publicly available database of HCV vaccines, such as the ones that exist for HIV (e.g. the International AIDS Vaccine Initiative [IAVI] database [<http://www.iavireport.org/trials-db/Pages/default.aspx>] and the Nonhuman Primate HIV/SIV Vaccine Trials [NHPVT] database [<http://www.hiv.lanl.gov/content/vaccine/home.html>]), some recent reviews have summarised the vaccine development attempts made to date (e.g. 52, 58, 197). The strategies have included the use of peptide and recombinant envelope glycoprotein vaccines, DNA vaccines, virus-like particles, and various live viral vectors, such as vaccinia and adenoviruses. Despite this exponential growth in preclinical HCV-vaccine testing, Lang and Weiner (58) and Stoll-Keller *et al.* (197) cite a total of only nine different prophylactic vaccines that have been tested in chimpanzees (58, 197). The outcomes of trials of these vaccines, when tested in chimpanzees, are summarised in Table 1. Much of the cited preclinical investigation of prophylactic vaccines involves the use of mice or even baboons, and not chimpanzees (52). Of 11 candidate vaccines cited by Houghton and Abrignani (52), three referred to chimpanzee preclinical data (199, 200, and unpublished data), while seven referenced mouse data (201–207) and one referenced baboon data (201). A more recent report of chimpanzee vaccine tests referenced almost 30 immunogenicity studies of candidate HCV vaccines (208). In addition to those already cited above, 17 were mouse studies (202, 209–224), two used rats (225, 226), one used pigs (as well as mice; 221), and just four involved chimpanzees (77, 200, 227, 228).

The development of therapeutic vaccines has also received much attention. While Choo *et al.* (199) used chimpanzees to test specifically the *prophylactic* potential of an envelope-protein vaccine with adjuvant, this vaccine was also tested for therapeutic potential in human Phase I clinical trials (52) from 2003–2005 (ClinicalTrials.gov identifier NCT00500747) — no clinical follow-up is as yet apparent. In 2005, chimpanzees were cited in two therapeutic vaccine trials either “in progress”, via personal communication, or as “unpublished data” (52), involving adenovirus/DNA prime–boost and adjuvanted HCV polyprotein vaccines, respectively. Other cited preclinical therapeutic vaccine trials involved not chimpanzees, but rhesus macaques (229) and mice (230).

Otherwise, the reviews have focused on human trials of vaccines examining therapeutic efficacy (58, 197). These include: clinical reports of humoral and cellular immune responses following a recombinant E1 envelope protein vaccination (‘InnoVac-C’; 231, 232); and inoculation with the multi-peptide ‘IC-41’ vaccine (233–235), and ‘personalised peptide’ vaccination (236), both of which decreased viral RNA in only a small proportion of the patients; and Phase I/II clinical trials of the DNA-based non-structural protein 3 (NS3) vaccine, ‘ChronVac-C’, the vaccine based on heat-killed recombinant yeast expressing NS3-Core fusion protein, ‘GI-5005’, and the recombinant vaccine based on modified vaccinia virus Ankara (MVA) expressing HCV non-structural proteins (ClinicalTrials.gov, 198, 237).

A major objective of therapeutic vaccine development is to induce and augment innate immune responses, such as the production of IFNs and the activity of natural killer (NK) cells, both of which are specifically down-regulated by HCV during infection. *In vitro* research has informed this area significantly. For example, the HCV NS3/4A protease is known to disrupt host signalling-pathways that induce IFN- β , among other antiviral host-defence genes such as IFN regulatory factor 3 (IRF-3) and NF- κ B, via the retinoic acid-inducible gene I (RIG-I; 238) and the Toll-like receptor 3 adaptor protein (TRIF; 239). Inhibitors of HCV NS3/4A can therefore restore these host defence mechanisms (240). In addition, NK cells were shown to be inhibited by HCV binding — specifically, engagement of the viral E2 protein with the CD81 receptor on the NK cells blocks NK cell activation and proliferation, as well as cytokine production and cytotoxic granule release (241), and NK cell-directed IFN- γ production (242).

With regard to all of the above chimpanzee data, it is difficult to interpret the results to infer significant relevance for the efficacy of any future human vaccine. The data are highly variable, which might be expected due to natural biological variability between individual chimpanzees. However, more importantly, it is acknowledged that the innate high rate of resolution of HCV infection in chimpanzees (compared to humans) poses a problem — “Given that many chimpanzees spontaneously resolve acute hepatitis C, definite conclusions await human studies” (77, 197). Further, optimism at many of the ostensibly encouraging results must be tempered by the fact that “protection against chronic infection following challenge with a heterologous strain was limited” (197). With regard to DNA vaccines, it has been conceded that “DNA based immunisation results obtained in one animal species cannot be extrapolated to other species, and this is especially relevant for HCV” (243). In 2008, Youn *et al.* reported that, of the vaccines tested in chimpanzees to date (208), all except one have failed to prevent chronic infection completely (196).

Table 1: Hepatitis C vaccines tested in chimpanzees

Vaccine type	Vaccine details	Trial outcome (immune response/protection)	Refs
Recombinant envelope glycoprotein	No adjuvants	Humoral and cellular responses did not prevent infection, but delayed viraemia in two chimpanzees	78
Recombinant envelope glycoprotein	With adjuvants	Strong humoral response provided five chimpanzees with protection from rechallenge with homologous HCV. Two chimpanzees remained unprotected. Pooled results: 62% of unvaccinated chimpanzees infected on rechallenge; 17% of vaccinated chimpanzees infected upon homologous HCV rechallenge; and 11% of vaccinated chimpanzees infected upon heterologous HCV rechallenge	199
DNA	Encoding E2 envelope glycoprotein	Humoral and cellular responses in two chimpanzees. Both individuals were infected upon homologous rechallenge, but the infections resolved	291
VLPs	Core and envelope proteins	Strong humoral and cellular responses in four chimpanzees, similar to previous tests in mice and baboons. Animals were infected upon rechallenge, though viraemia was controlled	196
Recombinant vaccinia virus	Encoding several HCV proteins	HCV infection cleared after homologous rechallenge in four chimpanzees	208
DNA prime/protein boost	Multi-component	One chimpanzee resolved infection. Another individual fell ill, but infection was controlled after rechallenge with heterologous HCV	200
DNA prime/adenoviral boost	–	One chimpanzee showed sterilising immunity and one individual resolved infection. Four became persistently infected	228
DNA prime/modified vaccinia Ankara (MVA) boost	–	Humoral and cellular responses. Control of viral load upon homologous rechallenge, but three of four chimpanzees became chronically infected	227
Adenoviral prime/DNA boost	–	Strong cellular response. Four of five chimpanzees resolved infection when challenged with heterologous HCV	77
DNA	Non-structural (NS3-NS5B)	Vigorous cellular response comparable to five humans who had spontaneously cleared acute HCV infection	330

HVC = hepatitis C virus; VLPs = virus-like particles.

In contrast to the confounding nature of the chimpanzee data exemplified here, clinical and *in vitro* data have precipitated significant progress in vaccine development (23, 197). To illustrate, longitudinal studies of two cohorts of acutely-infected patients involving HCV pseudoparticles (HCVpp) have confirmed a correlation between the rapid or delayed induction of high-titre neutralising antibodies, and viral clearance or chronic infection, respectively (244, 245). Further *in vitro* and clinical investigations have shown that these antibodies are not responsible for the control of HCV infection, with the mutation and evolution of the HCV envelope pro-

teins outpacing the neutralising antibody response (162). Studies utilising HCVpp and cell culture-derived HCV (HCVcc) showed that neutralising antibodies target HCV entry events post-binding, via a specific epitope in the HCV E1 envelope glycoprotein, and the CD81 and Scavenger Receptor B1 (SR-B1) host receptors (246).

Development and testing of antiviral agents

The currently available therapy for HCV — PEG-IFN/ribavirin — is effective in only around half of

patients, is genotype-specific, is highly toxic, and is expensive (prohibitively so, in many instances). The urgent need for new antivirals is clear, and *in vitro* research has greatly contributed to this area of investigation. The molecular characterisation of the HCV genome and proteome permitted the precise definition of targets for antiviral agents and the structure-based rational design of HCV enzyme inhibitors (83, 247). The use of HCV replicons, cell culture and other associated methods of investigating the HCV life cycle *in vitro* (172, 248) were breakthroughs described as “revolutionary” (198), as they enabled the design, development, screening and testing of specific anti-HCV antivirals known as ‘specifically-targeted antiviral therapies for hepatitis C’ (STAT-Cs). Due to their specificity, most, if not all, of these agents should be more tolerable and should possess greater and more robust anti-HCV activities than the current standard IFN/ribavirin treatment (198). As a result of *in vitro* methods, numerous STAT-Cs have been and are currently being tested, and many more are in the course of development — directly targeting, for example, viral entry, translation, and assembly, but also working indirectly via modulation of the immune response, etc.

Up-to-date and comprehensive summaries of these therapies are available, which detail numerous and varied agents (e.g. 50, 198, 249), not all of which can be, or need to be, extensively described here. With regard to assessing the necessity of chimpanzee use and the impact of alternative methods on the development of HCV antivirals, however, a number of examples are informative:

- *Entry inhibitors*: Civacir (human HCV antibody-enriched immune globulin) and HCV-AB68 (human monoclonal anti-E2 antibody) have been clinically evaluated in liver transplant recipients, though both exhibited little or no suppression of HCV RNA levels (250, 251). Civacir had previously been shown to neutralise infectious inoculates, and prevent or delay infection, in chimpanzees (252, 253).
- *Translation inhibitors*: Several have been tested, including the antisense oligonucleotides ISIS14803 and AVI-4065, for which a clinical trial was terminated due to lack of antiviral activity or elevated plasma ALT (254). Other options considered include ribozymes that target and cleave the HCV internal ribosome entry site (IRES) region, such as Heptazyme, which showed efficacy in human trials, but its use was terminated due to animal toxicity (255), and IRES inhibitors such as VGX-410C (Mifepristone), which was not efficacious in clinical trials (256).
- *Assembly inhibitors*: An HCV-assembly inhibitor that operates via alteration of envelope-protein glycosylation, Celgosivir (MX-3253), is currently in Phase II clinical trials (257).
- *Viral-polymerase inhibitors*: These are another important class of antiviral agents, and comprise nucleoside, non-nucleoside and pyrophosphate mimics, and other classes of agents (258). Non-nucleoside inhibitors have been tested *in vitro* by using the replicon system, and in biochemical assays by using purified viral polymerase (259, 260), with positive results. The benzothiadiazine, A-837093, for example, exhibited potency and specificity *in vitro*, and reduced 1a-genotype and 1b-genotype viral load in chimpanzees, although resistance quickly developed and rebound occurred (261). The nucleoside analogue, MK-0608, was efficacious *in vitro*, and showed favourable pharmacokinetics in rats, dogs and rhesus macaques. It was subsequently tested in chimpanzees, where it reduced viral load significantly, though rebound occurred after the cessation of dosing (94). Valopicitabine (NM283; Idenix) exhibited efficacy in clinical trials, but was associated with severe gastrointestinal adverse reactions and antagonistic drug–drug interactions that caused its development to be suspended (262, 263). The inhibitors, R1626 and R7128, have shown encouraging human efficacy, especially in combination with PEG-IFN/ribavirin (262), though the development of R1626 was terminated at the end of 2008, due to adverse events and limited sustained efficacy (198, 264); R7128 remains in clinical trials (198). IDX184 is being evaluated in clinical trials, after exhibiting synergistic antiviral effects with PEG-IFN/ribavirin, as well as efficacy in chimpanzees (198). The polymerase inhibitors, VCH916, RO5024048, ABT-333 and A-831, are in Phase II clinical trials (237).
- *Protease inhibitors*: These are a major focus of STAT-C development, given that the HCV life cycle crucially involves the proteolytic cleavage of a polyprotein into functional constituent HCV viral proteins. The main targets for these inhibitors have been the HCV NS3/4A serine protease and the NS5B viral polymerase (54). The NS3/4A protease performs vital functions, cleaving the HCV polyprotein at four sites, inactivating host proteins involved in the IFN-mediated host antiviral response, and acting as a cofactor for the viral polymerase and for an RNA helicase, among others (262). The viral polymerase, which performs an indispensable function and bears only a slight homology to host polymerases, is a clear target. The development of inhibitors that are specific for these enzymes was made possible by the determination of their three-dimensional structures,

which enabled the rational design of inhibitory molecules (e.g. 50, 265, 266). For example, the serine protease inhibitors, telaprevir (also known as TVR and VX-950) and boceprevir (SCH503034), were discovered via structure-based drug-design techniques (267) and resulted in the elimination of HCV RNA in subgenomic replicon studies (268, 269), eventually leading to current Phase III clinical trials (264). Telaprevir and boceprevir are at the most advanced stage of development (262), and are expected to be approved for use in 2011 (270). Notably, *in vitro* resistance studies involving the use of subgenomic replicons have been of crucial importance in determining the likelihood of the development of HCV strains which are resistant to these drugs, and in comparing different antiviral therapies to assess the potential efficacies of their combination (271). There are no published reports that these therapies have been tested in chimpanzees. Ciluprevir (BILN 2061) was effective against HCV replicons *in vitro*, and in clinical trials (240, 272), though the clinical trials were halted when cardiac toxicity was reported in chimpanzees (50), and also in monkeys (262). TMC435350 has shown efficacy in patients, and is currently in Phase II trials, as are ITMN-191 and MK-7009 (42, 198, 237).

- *Other agents:* Other agents in clinical trials include an inhibitor of cyclophilin B (an important co-factor for the HCV polymerase; 273), Debio 025 (274), which showed significant antiviral activity in subgenomic replicon systems, as well as in full-length infectious HCV systems (275, 276), and is currently in Phase I and II clinical trials (274, 275). The thiazolidine-signalling modulator, Nitazoxanide, was serendipitously found to harbour anti-HCV properties during its use for its intended purpose as a treatment for intestinal parasites (277), and it may therefore prove to be useful in the treatment of HCV infections. Further agents undergoing trials include modified IFNs with greater efficacy and reduced adverse effects, such as Albuferon, Locteron and Omega-IFN (278, 279), a number of Toll-like receptor (TLR) agonists that have exhibited moderate antiviral activity (280, 281), and ribavirin analogues, such as Viramidine (taribavirin), which causes fewer anaemic adverse reactions, but is less effective (282). Sakamoto and Watanabe (237) list almost 20 other agents, including IFN formulations, immune modulators and host-targeted agents and anti-steatosis drugs, which are in current clinical trials (237). Many of these agents are reviewed in detail in other contemporary reports, which are replete with *in vitro*, *in silico*, molecular and *ex vivo* references, and almost devoid (if not entirely devoid)

of references to chimpanzee studies (see, for example, 42, 283, 284).

In common with establishing the scale of animal use in preclinical testing generally, it is difficult to assess the degree of chimpanzee use in the preclinical development of HCV antiviral agents. While Internet searches suggest that only a small proportion of agents have been tested in chimpanzees, a smaller proportion still are reported in the peer-reviewed scientific literature. Of greater than 60 HCV antiviral agents, identified via a cursory scan of review articles and via Internet searches, just eight were readily identifiable as being associated with chimpanzee testing. For example, chimpanzees were involved in preclinical studies of Viramidine/taribavirin (a modified form of ribavirin), and, along with rodent experiments, they provided pharmacological and toxicological data (285). Antiviral activity was found in chimpanzees for the nucleoside analogue, valopicitabine (NM-283), as well as in an HCV replicon system (286), and Phase III clinical trials are ongoing. The polymerase inhibitor, A-848837 (Abbott), was tested for its pharmacokinetic (PK) properties in chimpanzees, following efficacy studies *in vitro* (261, 287). The polymerase inhibitors, ANA 598 (288), IDX102 and IDX184 (289), and Merck's S282T (290, 291) and MK-0608, all demonstrated antiviral efficacy in chimpanzees (292), as did Merck's protease inhibitor, R155K (290, 291). In late 2009, it was reported that Santaris Pharma's SPC3649 exhibited efficacy in chimpanzees by interacting with miRNA-122, thus affecting HCV replication (293, 294).

Mention of chimpanzee use is also notably absent in reviews detailing drugs whose development has been discontinued, suggesting a lack of importance and utility of the chimpanzee model in the development of drugs for the treatment of hepatitis C. For example, reviews of discontinued anti-infective drugs published in the last three years (2008–2010, inclusive), list a total of 14 anti-HCV drugs (including two vaccines), many of which were in clinical trials, that were discontinued from 2006–2008 inclusive (295–297), for reasons of toxicity, poor absorption, and insufficient efficacy. Chimpanzee data and/or testing are not cited for any of these drugs, nor was any other published evidence of chimpanzee use able to be located.

Overall, it is clear that chimpanzees are used only infrequently in the development of HCV antiviral drugs, and it follows that the latter cannot be dependent on the former. In fact, the literature reveals that current regulatory requirements for preclinical PK and toxicological data from two animal species have been fulfilled in the majority of cases — as is the case for HCV vaccine development — without recourse to chimpanzee use. For example, prior to arguably superfluous experi-

ments in chimpanzees, the nucleoside analogue, MK-0608, exhibited efficacy *in vitro*, and its pharmacokinetics were determined in rats, dogs and rhesus macaques (94). The nucleoside inhibitor, B102, and the Heptazyme HCV-translation inhibitor were similarly tested in rats and monkeys (255, 298–300). A number of modified murine models have also been developed and have been widely utilised (91), such as the uPA-SCID mouse model, in which human hepatocytes are transplanted into immunodeficient mice, to provide a chimaeric liver that can be infected with HCV (301–303). These mice have been used, for example, to test the cyclophilin inhibitor, Debio 025 (275).

Where chimpanzee experiments have been performed, in addition to those in other species, it can be argued that they were redundant. This can be illustrated for the recently reported miRNA drug, SPC3649. The role of the target molecule of the drug, miRNA-122, in HCV replication was described four years previously, via tissue culture experiments with human liver cells (304). Subsequent *in vitro* experiments demonstrated the therapeutic potential of other agents, very similar to SPC3649, in decreasing the level of HCV during infection (305). Clinical trials of the drug had begun 18 months prior to the chimpanzee reports, and clearly before the chimpanzee experiments had commenced. Single-dose Phase I trials were completed in 2009, for which the results are imminent (306), and another multi-dose Phase I human trial is in progress and is due to finish in 2010 (307). Furthermore, the testing of SPC3649 had already complied with regulatory toxicology requirements, having been tested in African green monkeys (308), as well as in mice (309). Therefore, from a Three Rs and regulatory perspective, there is more than ample scope for the *in vivo* testing of HCV antivirals by using species other than chimpanzees. However, from a scientific perspective, there is evidence that all non-human species, not just chimpanzees, are superfluous to requirements for establishing the efficacy and pharmacokinetics of new antiviral drugs for the treatment of hepatitis C in humans (see the companion paper [17]).

Scientific, Ethical and Practical Problems With the Use of Chimpanzees in HCV Research

In addition to the arguments presented above, there are further fundamental scientific arguments for the lack of human relevance and redundancy of the chimpanzee model in HCV research. Important practical and ethical matters also support a move away from chimpanzee use, toward the widespread adoption of superior alternatives. For instance, there are major and fundamental

disparities between HCV infection and disease in humans and chimpanzees. Recently, striking differences in gene expression have begun to emerge; for example, treatment of HCV infection with IFN in humans leads to a decrease in expression of the SOCS3 (Suppression Of Cytokine Signalling) gene, which is involved in the regulation of IFN-signalling pathways. In chimpanzees, by contrast, IFN treatment increases SOCS3 expression, preventing the activation of ISGs and ‘blunting’ the IFN response (180). It therefore appears that, despite some similarities between HCV infections in humans and chimpanzees, profound differences exist, which indicate significantly different pathological processes and immune responses (94). In some respects, it could be argued that chimpanzees suffer a different disease altogether (59). This is supported, for example, by the fact that the JFH-1 strain of HCV is “not particularly infectious” in chimpanzees (310), yet it was isolated from a patient with fulminant hepatitis C (311). Further evidence that HCV infections in the two species display critical differences comes from the failure of the chimpanzee model to positively impact vaccine development and the understanding of hepatocellular damage (312), and it suggests that the majority of the progress has emanated from *in vitro* and clinical studies (313).

Practically, there is the basic issue of chimpanzee availability and cost. Chimpanzee use is expensive, so chimpanzee-based projects reduce the funds available for other avenues of research. The availability of chimpanzees (particularly naïve individuals) is also severely limited, which restricts the number of investigations that can be conducted (32, 77, 243). Breeding for new availability would further contribute to the current so-called ‘surplus’ of chimpanzees, and to the enormous costs of lifetime care of federally supported chimpanzees in US laboratories. These costs alone, for the chimpanzees that are already supported by the US National Institutes of Health, have been estimated at \$312 million. This figure compares to an estimated \$139 million for superior care in sanctuary (T. Capaldo & M. Owens [2010], submitted for publication). Further, most studies involve just two to four animals, meaning that the statistical significance of the data is highly questionable, and apparent differences can often be due to inherent biological variation (86) — a factor that cannot be addressed due to the lack of availability and the expense of acquiring more individuals. Rather, there is ample evidence that HCV drug leads progress solely on the basis of *in vitro* efficacy models (314).

Obvious and widely documented ethical aspects are often cited (e.g. 32, 91), which are considered in more detail in the Discussion section, below. To illustrate the capacity of chimpanzees, both in and from research, to suffer greatly, studies have revealed the existence of post-traumatic stress dis-

order in ex-research chimpanzees now in sanctuaries (2). These studies have detailed physical and psychological traumas suffered by chimpanzees that were raised in various human/chimpanzee contexts and then used in research. They have also reported on the compromised ability of the chimpanzees to recover from such trauma, once in a sanctuary (3). From a pragmatic perspective, it must be appreciated that, worldwide, chimpanzees may only be used in research in the USA. The last facility in Europe closed in 2004, following a decision by the Dutch government that chimpanzee research was of “limited importance” and that the chimpanzee was an “unsuitable model” (9, 10). In the USA, a similar implication emanated from the National Center for Research Resources (NCRR), a centre within the National Institutes of Health, which made a ten-year breeding moratorium for NCRR-owned chimpanzees permanent in 2007 (315). The apparent lack of value of chimpanzee research, as viewed by these institutions, is reflected in public opinion — for every US citizen who opposes a ban on chimpanzee research, almost two individuals support the ban (11).

Other Animal Models

While this report focuses on evaluating the need for chimpanzees in HCV research, and the *in vitro* and clinical alternatives to chimpanzee use, it is important to highlight the existence of other animal models for HCV studies. While the position of this review (based on the data it presents) is that the needs of HCV research and drug development can be met without recourse to any *in vivo* model, it must be accepted that some scientists believe that an animal model for HCV research is useful, and that current regulatory requirements look toward animal data when approving new drug applications.

A number of *in vivo* alternatives to chimpanzees are already in use. There are rodent models bearing human hepatocytes (reviewed in 316), which include immunocompetent fetal rats (317), immunodeficient trimera mice (318, 319), uPA mice (302, 303, 320), and several other transgenic mice (e.g. 316, 321). It is claimed that these models allow the evaluation of the efficacy of new HCV antiviral drugs and monoclonal antibodies, and the investigation of the expression of HCV genes upon liver injury (91). New World monkeys infected with GB virus-B (GBV-B), which is related to HCV, are also being used (91), including tamarins (genus *Saguinus*), the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*). These monkeys develop subacute self-resolving hepatitis following GBV-B infection (322, 323) — chronicity, as for HCV-infected chimpanzees, is relatively uncommon. Chronicity must be induced

in GBV-B infected monkeys via immunosuppression, or by the use of an engineered virus (324, 325). Various species of tree shrew (genus *Tupaia*) have been infected with HCV under severe immunosuppression (326), though persistent infection was not possible.

Discussion and Conclusions

The *GAPA* is a current bill in the US House of Representatives and in the Senate which seeks to end invasive biomedical research and testing on the estimated 500 federally-owned chimpanzees remaining in US laboratories, and to retire them to sanctuaries. The foundations of the bill are that chimpanzee research is ethically unacceptable and scientifically unjustifiable, assertions that have been augmented by comprehensive, insightful and robust peer-reviewed publications in recent years. While these papers have addressed concerns and claims regarding the impact of invasive research on captive chimpanzees, and of the efficacy and human relevance of chimpanzee research generally, as well as specifically in AIDS and cancer research, the question of hepatitis C had not been substantially addressed. Further, there are concerns that almost four decades of HCV research have had little tangible impact: the incidence of hepatitis C continues to increase markedly, there is no vaccine yet available, and there is only one non-specific and partly-ineffective treatment. This review of chimpanzee-based research is timely, as it provides important information concerning the scientific ramifications of the potential passage of the *GAPA* for research into hepatitis C, and collates prominent examples of all aspects of HCV research to inform debate and deliberation over the best future path for HCV investigation. These issues are of serious ethical concern, not just for chimpanzees in US laboratories, but also for human beings who are relying on science to provide treatments and cures for this grave disease.

In considering the current and future need for chimpanzees in HCV research, the salient issue had to be a deliberation of what alternative scientific techniques and research methods, currently and in the near future, could deliver equal or better results. Could chimpanzee experimentation add any data of gravity that could not be obtained by using other approaches? If chimpanzee use could inform specific areas not investigable by other means, how likely is this data to be crucial or redundant, and is its speculative importance mitigated by the ethical and financial costs of obtaining it? If chimpanzee use is not necessary, given the alternative investigative methods available (see companion article [17]), then chimpanzee experimentation is redundant and cannot be justi-

fied. However, it is informative to consider the past use of chimpanzees in HCV research to help estimate the relevance of chimpanzee data, and therefore to assess the likelihood that any future chimpanzee experiments will be truly germane to knowledge concerning human HCV infection. Therefore, this was a significant part of this review.

A major reason offered for the use of chimpanzees was the absence of any other option. Simply put, chimpanzees were used because neither another animal model, nor a full *in vitro* culture system, was available until recently. The desire for an alternative animal model may have been due to any combination of the following: the ethical and financial costs of relying on chimpanzees; scientific reasons for the lack of relevance of the chimpanzee model to human HCV infection; and practical considerations. Many researchers have readily highlighted serious caveats in chimpanzee use, and many have stressed the urgent need for *in vitro* viral culture systems, to accelerate discovery and to lead to true translational research and clinical benefit, as had occurred for other human viral diseases, such as polio and measles.

Nevertheless, claims of the critical nature of chimpanzee use in historic HCV research are widespread, and many claims regarding the future importance of the chimpanzee in HCV research continue to be made. These assertions, however, have been subject to little or no critical and detailed analysis. The mere involvement of chimpanzees in research is not sufficient justification for their use. In any valid argument for their necessity, it must be asked whether, and demonstrated how, the use of chimpanzees did indeed provide unique and reliable data.

Therefore, this review revisited and scrutinised various claims of chimpanzee necessity in HCV research, and found, in most cases, that such claims were exaggerated and overstated — and, in the case of future chimpanzee need, incorrect and unjustifiable.

While HCV research has constituted the main area of chimpanzee experimentation for some time, chimpanzees have actually been used in only a relatively small number of research projects. This is true from the early days of HCV research — when the disease and the virus were being discovered and characterised through analyses of immune responses, and the factors influencing viral clearance and progression to chronicity — right through to the determination of mechanisms of antiviral therapy and new therapeutic targets, and then through to the ongoing design, screening development and testing of antivirals and vaccines. This review illustrates that any argument for their essential involvement is highly contentious, if not erroneous. Historically, many instances of chim-

panzee use produced data that had been similarly and contemporarily provided by non-chimpanzee (indeed non-animal) means, rendering those chimpanzee studies entirely redundant, as well as cases where the chimpanzee data confounded or conflicted with human studies.

Of all the claims of chimpanzee experimentation necessity, the assertion that chimpanzees were vital for the identification of the HCV itself (at a time when many of the molecular methods available now were not able to circumvent the problems involved) has the most substance. However, in retrospect, it seems that chimpanzees were perhaps not required to characterise the high-titre human serum samples used in the process. The only other substantial argument was for the use of chimpanzees in determining the infectivity of HCV clones. This appears to have been for reasons of practicability (chimpanzees were already being used for infectivity studies with other viruses and were readily available), and the appropriate alternative methods available at the time had been too readily dismissed. Further, the development of *in vivo* infectious clones did not constitute a key step in the development of the sought-after *in vitro* replicon, VLP, HCVpp and HCVcc systems in any case, as described in the companion article (17). Even if it *were* the case that chimpanzees had been crucial in these instances, these discoveries took place relatively long ago and have no bearing on the suitability and necessity of chimpanzees for HCV research now and in the future. It must therefore be concluded that the impact of chimpanzee experimentation on HCV research is relatively negligible compared to non-chimpanzee methods, certainly during its recent history, which adds weight to the conviction that HCV research would not suffer if chimpanzee experiments were ended.

When considered alongside the major caveats of the chimpanzee model, including their expense, their lack of availability and practicability, and the notable significant biological differences between HCV infection and pathology in chimpanzees and in humans, the use of the chimpanzee model in HCV research holds little or no current value. Serious ethical issues must also be considered, especially in light of the *US Animal Welfare Act* requirements for the psychological wellbeing of primates (327), of US public opinion, and of overwhelming support for not employing chimpanzees in harmful research when alternatives are available (328). Ethical concerns include life-long behavioural disorders, physical and psychological problems, such as post-traumatic stress disorder resulting from the consequences of long-term captivity, anaesthetic-dart knockdowns, and subjection to multiple invasive procedures. The proposed merits of chimpanzee use are called into further question when one considers other

avenues and options currently at our disposal, and the comprehensive data they can, and do, deliver. Many of the myriad *in vitro* methods of inquiry, including a significant number at the cutting edge of technology, have been detailed in the companion report (17).

In summary, there is a very strong argument against any scientific requirement for the use of chimpanzees in hepatitis C research. Given recent opinions that the chimpanzee is a poor model in other areas, such as HIV/AIDS and cancer research, and more generally as a model organism, this is not an unexpected conclusion. Chimpanzee HCV research cannot be considered a necessity, and indeed, prohibiting it would accelerate progress against hepatitis C by releasing funds currently appropriated to expensive chimpanzee projects, enabling those funds to support more-productive methods. The 'hepatitis C necessity argument' should therefore not dissuade changes in public policy with regard to the confinement and use of chimpanzees in US laboratories, as it has little or no foundation, but it should support changes in funding priorities. Continuing unsubstantiated claims of the necessity of chimpanzees in HCV research, such as those that this paper attempts to address, are adversely affecting humans, chimpanzees and scientific progress. The in-depth investigation of these claims, by using the actual data from, and statistics of, chimpanzee hepatitis C studies, suggests an ethical and scientific movement away from captive and invasive chimpanzee experimentation. Therefore, it lends scientific support to efforts such as the GAPA, and an end to the USA remaining the only country in the world that currently uses chimpanzees in research to any significant degree. This investigation adds further explanation as to why many scientifically advanced countries have banned or severely limited chimpanzee use. For the hundreds of millions of human beings infected by HCV, or at risk of being infected, as well as for the approximate 1,000 chimpanzees in US laboratories, the results of this study, and of the associated investigation (Paper 2 [17]), clearly support the replacement of chimpanzees in HCV research with superior alternatives.

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References

1. NEAVS (2008). *End Chimpanzee Research: Overview — International Bans*. Boston, MA, USA: New England Anti-Vivisection Society (Project R&R). Available at: <http://www.releasechimps.org/mission/end-chimpanzee-research/country-bans> (Accessed 20.07.10).
2. Bradshaw, G.A., Capaldo, T., Lindner, L. & Grow, G. (2008). Building an inner sanctuary: Complex PTSD in chimpanzees. *Journal of Trauma & Dissociation* **9**, 9–34.
3. Bradshaw, G.A., Capaldo, T., Lindner, L. & Grow, G. (2009). Developmental context effects on bi-cultural post-trauma self repair in chimpanzees. *Developmental Psychology* **45**, 1376–1388.
4. Sauer, U.G. (2000). Reasons for not using primates in research. [In German] *ALTEX* **17**, 217–220.
5. Thew, M. (2002). Are results of primate research worth the suffering it causes? *Nature, London* **418**, 273.
6. Bailey, J. (2008). An assessment of the role of chimpanzees in AIDS vaccine research. *ATLA* **36**, 381–428.
7. Bailey, J., Balcombe, J. & Capaldo, T. (2007). *Chimpanzee Research: An Examination of Its Contribution to Biomedical Knowledge and Efficacy in Combating Human Diseases*, 47pp. Boston, MA, USA: New England Anti-Vivisection Society (Project R&R). Available at: <http://www.releasechimps.org/pdfs/chimp-efficacy-paper-main.pdf> (Accessed 17.08.10).
8. Knight, A. (2007). The poor contribution of chimpanzee experiments to biomedical progress. *Journal of Applied Animal Welfare Science* **10**, 281–308.
9. de Kok, W. (2002). *Dutch Lab Chimps to be Retired*. Summerville, SC, USA: International Primate Protection League. Available at: <http://www.ippl.org/2002-dutch-chimps.php> (Accessed 20.07.10).
10. Vermij, P. (2003). Europe's last research chimps to retire. *Nature Medicine* **9**, 981.
11. NEAVS (2005). *End Chimpanzee Research: Overview — Public Opinion*. Boston, MA, USA: New England Anti-Vivisection Society (Project R&R). Available at: <http://www.releasechimps.org/mission/end-chimpanzee-research/public-opinion> (Accessed 18.08.10).
12. US Government Printing Office (2007). *Public Law 110–170 — Dec 26. 121 Stat 2465*. 110th Congress (S. 1916), 2pp. Washington, DC, USA: Government Printing Office. Available at: http://frwebgate.access.gpo.gov/cgi-bin/getdoc.cgi?dbname=110_cong_public_laws&docid=f:publ170.110.pdf (Accessed 18.08.10).
13. OpenCongress (2009). *H.R.1326 Great Ape Protection Act*. New York, NY, USA: OpenCongress. Available at: <http://www.opencongress.org/bill/111-h1326/show> (Accessed 18.08.10).
14. Cohen, J. (2007). Biomedical research. The endan-

- gered lab chimp. *Science, New York* **315**, 450–452.
15. VandeBerg, J.L. & Zola, S.M. (2005). A unique biomedical resource at risk. *Nature, London* **437**, 30–32.
 16. Bailey, J. (2009). An examination of the use of chimpanzees in human cancer research. *ATLA* **37**, 399–416.
 17. Bailey, J. (2010). An assessment of the use of chimpanzees in hepatitis C research past, present and future: 2. Alternative replacement methods. *ATLA* [In press].
 18. Seeff, L.B. (2009). The history of the “natural history” of hepatitis C (1968–2009). *Liver International* **29**, Suppl. 1, 89–99.
 19. Feinstone, S.M., Kapikian, A.Z., Purcell, R.H., Alter, H.J. & Holland, P.V. (1975). Transfusion-associated hepatitis not due to viral hepatitis type A or B. *New England Journal of Medicine* **292**, 767–770.
 20. Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science, New York* **244**, 359–362.
 21. Alter, H.J., Purcell, R.H., Shih, J.W., Melpolder, J.C., Houghton, M., Choo, Q.L. & Kuo, G. (1989). Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *New England Journal of Medicine* **321**, 1494–1500.
 22. Kuo, G., Choo, Q.L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., Tegtmeier, G.E., Bonino, F., Colombo, M., Lee, W-S., Kuo, C., Berger, K., Shuster, J.R., Overby, L.R., Bradley, D.W. & Houghton, M. (1989). An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science, New York* **244**, 362–364.
 23. Gottwein, J.M. & Bukh, J. (2008). Cutting the Gordian knot-development and biological relevance of hepatitis C virus cell culture systems. *Advances in Virus Research* **71**, 51–133.
 24. Marcellin, P. (2009). Hepatitis B and hepatitis C in 2009. *Liver International* **29**, Suppl. 1, 1–8.
 25. Soriano, V., Madejon, A., Vispo, E., Labarga, P., Garcia-Samaniego, J., Martin-Carbonero, L., Sheldon, J., Bottecchia, M., Tuma, P. & Barreiro, P. (2008). Emerging drugs for hepatitis C. *Expert Opinion on Emerging Drugs* **13**, 1–19.
 26. Shepard, C.W., Finelli, L. & Alter, M.J. (2005). Global epidemiology of hepatitis C virus infection. *Lancet Infectious Diseases* **5**, 558–567.
 27. Brown, R.S. (2005). Hepatitis C and liver transplantation. *Nature, London* **436**, 973–978.
 28. Agnello, V. & De Rosa, F.G. (2004). Extrahepatic disease manifestations of HCV infection: Some current issues. *Journal of Hepatology* **40**, 341–352.
 29. Lerat, H., Berby, F., Trabaud, M.A., Vidalin, O., Major, M., Trepo, C. & Inchauspé, G. (1996). Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *Journal of Clinical Investigation* **97**, 845–851.
 30. Barria, M.I., Vera-Otarola, J., Leon, U., Vollrath, V., Marsac, D., Riquelme, A., Lopez-Lastra, M. & Soza, A. (2008). Influence of extrahepatic viral infection on the natural history of hepatitis C. *Annals of Hepatology* **7**, 136–143.
 31. Dienstag, J.L. & McHutchison, J.G. (2006). American Gastroenterological Association technical review on the management of hepatitis C. *Gastroenterology* **130**, 231–264; quiz 214–217.
 32. Brass, V., Moradpour, D. & Blum, H.E. (2007). Hepatitis C virus infection: *In vivo* and *in vitro* models. *Journal of Viral Hepatitis* **14**, Suppl. 1, 64–67.
 33. Walters, K-A. & Katze, M.G. (2009). Using high-throughput genomics to study hepatitis C: What determines the outcome of infection? *Antiviral Research* **81**, 198–208.
 34. Poynard, T., Bedossa, P. & Opolon, P. (1997). Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* **349**, 825–832.
 35. Lauer, G.M. & Walker, B.D. (2001). Hepatitis C virus infection. *New England Journal of Medicine* **345**, 41–52.
 36. Albert, M.L., Decalf, J. & Pol, S. (2008). Plasmacytoid dendritic cells move down on the list of suspects: In search of the immune pathogenesis of chronic hepatitis C. *Journal of Hepatology* **49**, 1069–1078.
 37. Kim, J.W. & Wang, X.W. (2003). Gene expression profiling of preneoplastic liver disease and liver cancer: A new era for improved early detection and treatment of these deadly diseases? *Carcinogenesis* **24**, 363–369.
 38. Simmonds, P. (2004). Genetic diversity and evolution of hepatitis C virus — 15 years on. *Journal of General Virology* **85**, 3173–3188.
 39. Anon. (2002). National Institutes of Health consensus development conference statement: Management of hepatitis C. *Hepatology* **36**, Suppl. 1, S3–S20.
 40. Dhumeaux, D., Marcellin, P. & Lerebours, E. (2003). Treatment of hepatitis C. The 2002 French consensus. *Gut* **52**, 1784–1787.
 41. Fried, M.W. & Hadziyannis, S.J. (2004). Treatment of chronic hepatitis C infection with peginterferons plus ribavirin. *Seminars in Liver Disease* **24**, Suppl. 2, 47–54.
 42. Manns, M.P., Foster, G.R., Rockstroh, J.K., Zeuzem, S., Zoulim, F. & Houghton, M. (2007). The way forward in HCV treatment — finding the right path. *Nature Reviews Drug Discovery* **6**, 991–1000.
 43. Hadziyannis, S.J., Sette, H.J., Morgan, T.R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer, H.J., Bernstein, D., Rizzetto, M., Zeuzem, S., Pockros, P.J., Lin, A. & Ackrill, A.M. (2004). Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. *Annals of Internal Medicine* **140**, 346–355.
 44. Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales, F.L.J., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. & Yu, J. (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *New England Journal of Medicine* **347**, 975–982.
 45. Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M. & Albrecht, J.K. (2001). Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. *Lancet* **358**, 958–965.

46. [McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S. & Albrecht, J.K. \(1998\). Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *New England Journal of Medicine* **339**, 1485–1492.](#)
47. [Muir, A.J., Bornstein, J.D. & Killenberg, P.G. \(2004\). Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *New England Journal of Medicine* **350**, 2265–2271.](#)
48. [Marcellin, P., Boyer, N., Gervais, A., Martinot, M., Pouteau, M., Castelnau, C., Kilani, A., Areias, J., Auperin, A., Benhamou, J.P., Degott, C. & Erlinger, S. \(1997\). Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. *Annals of Internal Medicine* **127**, 875–881.](#)
49. [Maylin, S., Martinot-Peignoux, M., Moucari, R., Boyer, N., Ripault, M.P., Cazals-Hatem, D., Giuily, N., Castelnau, C., Cardoso, A.C., Asselah, T., Feray, C., Nicolas-Chanoine, M.H., Bedossa, P. & Marcellin, P. \(2008\). Eradication of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Gastroenterology* **135**, 821–829.](#)
50. [Asselah, T., Benhamou, Y. & Marcellin, P. \(2009\). Protease and polymerase inhibitors for the treatment of hepatitis C. *Liver International* **29**, Suppl. 1, 57–67.](#)
51. [Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspé, G., Kuiken, C., Maertens, G., Mizokami, M., Murphy, D.G., Okamoto, H., Pawlotsky, J.M., Penin, F., Sablon, E., Shin-I, T., Stuyver, L.J., Thiel, H.J., Viazov, S., Weiner, A.J. & Widell, A. \(2005\). Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* **42**, 962–973.](#)
52. [Houghton, M. & Abrignani, S. \(2005\). Prospects for a vaccine against the hepatitis C virus. *Nature, London* **436**, 961–966.](#)
53. [Falck-Ytter, Y., Kale, H., Mullen, K.D., Sarbah, S.A., Sorescu, L. & McCullough, A.J. \(2002\). Surprisingly small effect of antiviral treatment in patients with hepatitis C. *Annals of Internal Medicine* **136**, 288–292.](#)
54. [De Francesco, R. & Migliaccio, G. \(2005\). Challenges and successes in developing new therapies for hepatitis C. *Nature, London* **436**, 953–960.](#)
55. [Feld, J.J. & Hoofnagle, J.H. \(2005\). Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature, London* **436**, 967–972.](#)
56. [Manns, M.P., Wedemeyer, H. & Cornberg, M. \(2006\). Treating viral hepatitis C: Efficacy, side effects, and complications. *Gut* **55**, 1350–1359.](#)
57. [Bowen, D.G. & Walker, C.M. \(2005\). Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature, London* **436**, 946–952.](#)
58. [Lang, K. & Weiner, D.B. \(2008\). Immunotherapy for HCV infection: Next steps. *Expert Review of Vaccines* **7**, 915–923.](#)
59. [Bettauer, R.H. \(2010\). Chimpanzees in hepatitis C virus research: 1998–2007. *Journal of Medical Primatology* **39**, 9–23.](#)
60. [Prince, A.M., Brotman, B., Lee, D.H., Pfahler, W., Tricoche, N., Andrus, L. & Shata, M.T. \(2005\). Protection against chronic hepatitis C virus infection after rechallenge with homologous, but not heterologous, genotypes in a chimpanzee model. *Journal of Infectious Diseases* **192**, 1701–1709.](#)
61. [Sakai, A., Takikawa, S., Thimme, R., Meunier, J.C., Spangenberg, H.C., Govindarajan, S., Farci, P., Emerson, S.U., Chisari, F.V., Purcell, R.H. & Bukh, J. \(2007\). *In vivo* study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone \(pHC-TN\) are infectious in chimpanzees but not in Huh7.5 cells. *Journal of Virology* **81**, 7208–7219.](#)
62. [Yanagi, M., St Claire, M., Shapiro, M., Emerson, S.U., Purcell, R.H. & Bukh, J. \(1998\). Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious *in vivo*. *Virology* **244**, 161–172.](#)
63. [NEAVS \(2008\). *Fearful Knockdowns*. Boston, MA, USA: New England Anti-Vivisection Society \(Project R&R\). Available at: <http://www.releasechimps.org/lab-life-traumas-g/fearful-knockdowns> \(Accessed 11.08.10\).](#)
64. [NEAVS \(2009\). *Hepatitis Detour*. Boston, MA, USA: New England Anti-Vivisection Society \(Project R&R\). Available at: <http://www.releasechimps.org/harm-suffering/research-current/hepatitis-detour> \(Accessed 11.08.10\).](#)
65. [Bigger, C.B., Brasky, K.M. & Lanford, R.E. \(2001\). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *Journal of Virology* **75**, 7059–7066.](#)
66. [Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R.E., Govindarajan, S., Shapiro, M., St Claire, M. & Bartenschlager, R. \(2002\). Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proceedings of the National Academy of Sciences of the USA* **99**, 14,416–14,421.](#)
67. [Sakai, A., St Claire, M., Faulk, K., Govindarajan, S., Emerson, S.U., Purcell, R.H. & Bukh, J. \(2003\). The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proceedings of the National Academy of Sciences of the USA* **100**, 11,646–11,651.](#)
68. [Shimizu, Y.K., Igarashi, H., Kiyohara, T., Shapiro, M., Wong, D.C., Purcell, R.H. & Yoshikura, H. \(1998\). Infection of a chimpanzee with hepatitis C virus grown in cell culture. *Journal of General Virology* **79**, 1383–1386.](#)
69. [Su, A.I., Pezacki, J.P., Wodicka, L., Brideau, A.D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R.H., Schultz, P.G. & Chisari, F.V. \(2002\). Genomic analysis of the host response to hepatitis C virus infection. *Proceedings of the National Academy of Sciences of the USA* **99**, 15,669–15,674.](#)
70. [Maillard, P., Krawczynski, K., Nitkiewicz, J., Bronnert, C., Sidorkiewicz, M., Gounon, P., Dubuisson, J., Faure, G., Crainic, R. & Budkowska, A. \(2001\). Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. *Journal of Virology* **75**, 8240–8250.](#)
71. [Caldwell, S.H. \(2001\). Controlling pain in liver biopsy, or “we will probably need to repeat the biopsy in a year or two to assess the response”. *American Journal of Gastroenterology* **96**, 1327–1329.](#)

72. Major, M.E., Mihalik, K., Puig, M., Rehmann, B., Nascimbeni, M., Rice, C.M. & Feinstone, S.M. (2002). Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. *Journal of Virology* **76**, 6586–6595.
73. Yanagi, M., Purcell, R.H., Emerson, S.U. & Bukh, J. (1999). Hepatitis C virus: An infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. *Virology* **262**, 250–263.
74. Nascimbeni, M., Mizukoshi, E., Bosmann, M., Major, M.E., Mihalik, K., Rice, C.M., Feinstone, S.M. & Rehmann, B. (2003). Kinetics of CD4⁺ and CD8⁺ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. *Journal of Virology* **77**, 4781–4793.
75. Kolykhalov, A.A., Mihalik, K., Feinstone, S.M. & Rice, C.M. (2000). Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication *in vivo*. *Journal of Virology* **74**, 2046–2051.
76. Esumi, M., Rikihisa, T., Nishimura, S., Goto, J., Mizuno, K., Zhou, Y.H. & Shikata, T. (1999). Experimental vaccine activities of recombinant E1 and E2 glycoproteins and hypervariable region 1 peptides of hepatitis C virus in chimpanzees. *Archives of Virology* **144**, 973–980.
77. Folgori, A., Capone, S., Ruggeri, L., Meola, A., Sporeno, E., Ercole, B.B., Pezzanera, M., Tafi, R., Arcuri, M., Fattori, E., Lahm, A., Luzzago, A., Vitelli, A., Colloca, S., Cortese, R. & Nicosia, A. (2006). A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. *Nature Medicine* **12**, 190–197.
78. Puig, M., Major, M.E., Mihalik, K. & Feinstone, S.M. (2004). Immunization of chimpanzees with an envelope protein-based vaccine enhances specific humoral and cellular immune responses that delay hepatitis C virus infection. *Vaccine* **22**, 991–1000.
79. Alter, H.J., Purcell, R.H., Holland, P.V. & Popper, H. (1978). Transmissible agent in non-A, non-B hepatitis. *Lancet* **312** (8062), 4 March, 459–463.
80. Tabor, E., Gerety, R.J., Drucker, J.A., Seeff, L.B., Hoofnagle, J.H., Jackson, D.R., April, M., Barker, L.F. & Pineda-Tamondong, G. (1978). Transmission of non-A, non-B hepatitis from man to chimpanzee. *Lancet* **312** (8062), 4 March, 463–466.
81. He, L.F., Alling, D., Popkin, T., Shapiro, M., Alter, H.J. & Purcell, R.H. (1987). Determining the size of non-A, non-B hepatitis virus by filtration. *Journal of Infectious Diseases* **156**, 636–640.
82. Feinstone, S.M., Mihalik, K.B., Kamimura, T., Alter, H.J., London, W.T. & Purcell, R.H. (1983). Inactivation of hepatitis B virus and non-A, non-B hepatitis by chloroform. *Infection & Immunity* **41**, 816–821.
83. Farci, P. (2002). Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome [Science 1989; 244:359–362]. *Journal of Hepatology* **36**, 582–585.
84. Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M. & Rice, C.M. (1997). Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science, New York* **277**, 570–574.
85. Yanagi, M., Purcell, R.H., Emerson, S.U. & Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proceedings of the National Academy of Sciences of the USA* **94**, 8738–8743.
86. Bukh, J. (2004). A critical role for the chimpanzee model in the study of hepatitis C. *Hepatology* **39**, 1469–1475.
87. Lanford, R.E., Bigger, C., Bassett, S. & Klimpel, G. (2001). The chimpanzee model of hepatitis C virus infections. *ILAR Journal* **42**, 117–126.
88. Farci, P., Alter, H.J., Govindarajan, S., Wong, D.C., Engle, R., Lesniewski, R.R., Mushahwar, I.K., Desai, S.M., Miller, R.H., Ogata, N. & Purcell, R.H. (1992). Lack of protective immunity against reinfection with hepatitis C virus. *Science, New York* **258**, 135–140.
89. Prince, A.M., Brotman, B., Huima, T., Pascual, D., Jaffery, M. & Inchauspé, G. (1992). Immunity in hepatitis C infection. *Journal of Infectious Diseases* **165**, 438–443.
90. Bukh, J., Forns, X., Emerson, S.U. & Purcell, R.H. (2001). Studies of hepatitis C virus in chimpanzees and their importance for vaccine development. *Intervirology* **44**, 132–142.
91. Akari, H., Iwasaki, Y., Yoshida, T. & Iijima, S. (2009). Non-human primate surrogate model of hepatitis C virus infection. *Microbiology & Immunology* **53**, 53–57.
92. Inchauspé, G. & Michel, M.L. (2007). Vaccines and immunotherapies against hepatitis B and hepatitis C viruses. *Journal of Viral Hepatitis* **14**, Suppl. 1, 97–103.
93. Nuffield Council on Bioethics (2005). *The Ethics of Research Involving Animals*, 376pp. London, UK: Nuffield Council on Bioethics. Available at: http://www.nuffieldbioethics.org/fileLibrary/pdf/RI_A_Report_FINAL-opt.pdf (Accessed 11.08.10).
94. Carroll, S.S., Ludmerer, S., Handt, L., Koeplinger, K., Zhang, N.R., Graham, D., Davies, M.E., MacCoss, M., Hazuda, D. & Olsen, D.B. (2009). Robust antiviral efficacy upon administration of a nucleoside analog to hepatitis C virus-infected chimpanzees. *Antimicrobial Agents & Chemotherapy* **53**, 926–934.
95. Major, M.E. & Feinstone, S.M. (2000). Characterization of hepatitis C virus infectious clones in chimpanzees: Long-term studies. *Current Topics in Microbiology & Immunology* **242**, 279–298.
96. Bassett, S.E., Brasky, K.M. & Lanford, R.E. (1998). Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profiles. *Journal of Virology* **72**, 2589–2599.
97. Bassett, S.E., Thomas, D.L., Brasky, K.M. & Lanford, R.E. (1999). Viral persistence, antibody to E1 and E2, and hypervariable region 1 sequence stability in hepatitis C virus-inoculated chimpanzees. *Journal of Virology* **73**, 1118–1126.
98. Prince, A.M., Brotman, B., Lee, D.H., Ren, L., Moore, B.S. & Scheffel, J.W. (1999). Significance of the anti-E2 response in self-limited and chronic hepatitis C virus infections in chimpanzees and in humans. *Journal of Infectious Diseases* **180**, 987–991.
99. Ray, S.C., Mao, Q., Lanford, R.E., Bassett, S., Laeyendecker, O., Wang, Y.M. & Thomas, D.L. (2000). Hypervariable region 1 sequence stability during hepatitis C virus replication in chimpanzees. *Journal of Virology* **74**, 3058–3066.

100. Zanetti, A.R., Tanzi, E., Paccagnini, S., Principi, N., Pizzocolo, G., Caccamo, M.L., D'Amico, E., Cambie, G. & Vecchi, L. (1995). Mother-to-infant transmission of hepatitis C virus. Lombardy Study Group on Vertical HCV Transmission. *Lancet* **345**, 289–291.
101. Houghton, M. (2009). Discovery of the hepatitis C virus. *Liver International* **29**, Suppl. 1, 82–88.
102. Alter, H.J. & Houghton, M. (2000). Clinical Medical Research Award. Hepatitis C virus and eliminating post-transfusion hepatitis. *Nature Medicine* **6**, 1082–1086.
103. Blumberg, B.S., Alter, H.J. & Visnich, S. (1965). A “new” antigen in leukemia sera. *JAMA* **191**, 541–546.
104. Alter, H.J., Holland, P.V., Morrow, A.G., Purcell, R.H., Feinstone, S.M. & Moritsugu, Y. (1975). Clinical and serological analysis of transfusion-associated hepatitis. *Lancet* **306** (7940), 8 November, 838–841.
105. Hoofnagle, J.H. & Alter, H.J. (1985). Chronic non-A, non-B hepatitis. *Progress in Clinical & Biological Research* **182**, 63–69.
106. Berman, M., Alter, H.J., Ishak, K.G., Purcell, R.H. & Jones, E.A. (1979). The chronic sequelae of non-A, non-B hepatitis. *Annals of Internal Medicine* **91**, 1–6.
107. Aach, R.D., Szmunes, W., Mosley, J.W., Hollinger, F.B., Kahn, R.A., Stevens, C.E., Edwards, V.M. & Werch, J. (1981). Serum alanine aminotransferase of donors in relation to the risk of non-A, non-B hepatitis in recipients: The transfusion-transmitted viruses study. *New England Journal of Medicine* **304**, 989–994.
108. Alter, M.J., Gerety, R.J., Smallwood, L.A., Sampliner, R.E., Tabor, E., Deinhardt, F., Frosner, G. & Matanoski, G.M. (1982). Sporadic non-A, non-B hepatitis: Frequency and epidemiology in an urban U.S. population. *Journal of Infectious Diseases* **145**, 886–893.
109. Alter, H.J. (1989). Chronic sequences of non-A, non-B hepatitis. In *Current Perspectives in Hepatology* (ed. L.B. Seeff & J.H. Lewis), pp. 83–97. New York, NY, USA: Plenum Medical.
110. Gilliam, J.H., 3rd, Geisinger, K.R. & Richter, J.E. (1984). Primary hepatocellular carcinoma after chronic non-A, non-B post-transfusion hepatitis. *Annals of Internal Medicine* **101**, 794–795.
111. Mullis, K.B. & Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**, 335–350.
112. Colombo, M., Kuo, G., Choo, Q.L., Donato, M.F., Del Ninno, E., Tommasini, M.A., Dioguardi, N. & Houghton, M. (1989). Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. *Lancet* **334** (8670), 28 October, 1006–1008.
113. Morita, T., Hada, H., Koide, N., Shiraha, H., Shinji, T., Nakamura, M., Ujike, K., Wato, M., Shimomura, H. & Tsuji, T. (1996). Detection of hepatitis C virus RNA in circulating immune complexes by RT-PCR. *Hepatology* **43**, 582–585.
114. Fujita, N., Kaito, M., Takeo, M., Iwasa, M., Ikoma, J., Watanabe, S. & Adachi, Y. (2003). Nonimmune complexed HCV RNA titer in serum as a predictor of interferon response in patients with chronic hepatitis C. *American Journal of Gastroenterology* **98**, 645–652.
115. Gale, M.J. & Beard, M.R. (2001). Molecular clones of hepatitis C virus: Applications to animal models. *ILAR Journal* **42**, 139–151.
116. Duverlie, G. & Wychowski, C. (2007). Cell culture systems for the hepatitis C virus. *World Journal of Gastroenterology* **13**, 2442–2445.
117. Moormann, R.J., van Gennip, H.G., Miedema, G.K., Hulst, M.M. & van Rijn, P.A. (1996). Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. *Journal of Virology* **70**, 763–770.
118. Bartenschlager, R. (2006). Hepatitis C virus molecular clones: From cDNA to infectious virus particles in cell culture. *Current Opinion in Microbiology* **9**, 416–422.
119. Higashi, Y., Kakumu, S., Yoshioka, K., Wakita, T., Mizokami, M., Ohba, K., Ito, Y., Ishikawa, T., Takayanagi, M. & Nagai, Y. (1993). Dynamics of genome change in the E2/NS1 region of hepatitis C virus *in vivo*. *Virology* **197**, 659–668.
120. Yagi, S., Mori, K., Tanaka, E., Matsumoto, A., Sunaga, F., Kiyosawa, K. & Yamaguchi, K. (2005). Identification of novel HCV subgenome replicating persistently in chronic active hepatitis C patients. *Journal of Medical Virology* **77**, 399–413.
121. Sheehy, P., Mullan, B., Moreau, I., Kenny-Walsh, E., Shanahan, F., Scallan, M. & Fanning, L.J. (2007). *In vitro* replication models for the hepatitis C virus. *Journal of Viral Hepatitis* **14**, 2–10.
122. Yoo, B.J., Selby, M.J., Choe, J., Suh, B.S., Choi, S.H., Joh, J.S., Nuovo, G.J., Lee, H.S., Houghton, M. & Han, J.H. (1995). Transfection of a differentiated human hepatoma cell line (Huh7) with *in vitro*-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *Journal of Virology* **69**, 32–38.
123. Dash, S., Halim, A.B., Tsuji, H., Hiramatsu, N. & Gerber, M.A. (1997). Transfection of HepG2 cells with infectious hepatitis C virus genome. *American Journal of Pathology* **151**, 363–373.
124. Okamoto, H., Okada, S., Sugiyama, Y., Kurai, K., Iizuka, H., Machida, A., Miyakawa, Y. & Mayumi, M. (1991). Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. *Journal of General Virology* **72**, 2697–2704.
125. Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. & Okayama, H. (1991). Structure and organization of the hepatitis C virus genome isolated from human carriers. *Journal of Virology* **65**, 1105–1113.
126. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. & Shimotohno, K. (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proceedings of the National Academy of Sciences of the USA* **87**, 9524–9528.
127. Bartenschlager, R. & Sparacio, S. (2007). Hepatitis C virus molecular clones and their replication capacity *in vivo* and in cell culture. *Virus Research* **127**, 195–207.
128. Beard, M.R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D.V. & Lemon, S.M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30**, 316–324.
129. Lanford, R.E., Lee, H., Chavez, D., Guerra, B. &

- Brasky, K.M. (2001). Infectious cDNA clone of the hepatitis C virus genotype 1 prototype sequence. *Journal of General Virology* **82**, 1291–1297.
130. Yanagi, M., St Claire, M., Emerson, S.U., Purcell, R.H. & Bukh, J. (1999). *In vivo* analysis of the 3' untranslated region of the hepatitis C virus after *in vitro* mutagenesis of an infectious cDNA clone. *Proceedings of the National Academy of Sciences of the USA* **96**, 2291–2295.
 131. Myung, J., Khalap, N., Kalkeri, G., Garry, R. & Dash, S. (2001). Inducible model to study negative strand RNA synthesis and assembly of hepatitis C virus from a full-length cDNA clone. *Journal of Virological Methods* **94**, 55–67.
 132. Lim, S.P., Soo, H.M., Tan, Y.H., Brenner, S., Horstmann, H., MacKenzie, J.M., Ng, M.L., Lim, S.G. & Hong, W. (2002). Inducible system in human hepatoma cell lines for hepatitis C virus production. *Virology* **303**, 79–99.
 133. Moradpour, D., Wakita, T., Wands, J.R. & Blum, H.E. (1998). Tightly regulated expression of the entire hepatitis C virus structural region in continuous human cell lines. *Biochemical & Biophysical Research Communications* **246**, 920–924.
 134. Moradpour, D., Kary, P., Rice, C.M. & Blum, H.E. (1998). Continuous human cell lines inducibly expressing hepatitis C virus structural and non-structural proteins. *Hepatology* **28**, 192–201.
 135. Kalkeri, G., Khalap, N., Akhter, S., Garry, R.F., Fermin, C.D. & Dash, S. (2001). Hepatitis C viral proteins affect cell viability and membrane permeability. *Experimental & Molecular Pathology* **71**, 194–208.
 136. Huang, Y., Uchiyama, Y., Fujimura, T., Kanamori, H., Doi, T., Takamizawa, A., Hamakubo, T. & Kodama, T. (2001). A human hepatoma cell line expressing hepatitis C virus nonstructural proteins tightly regulated by tetracycline. *Biochemical & Biophysical Research Communications* **281**, 732–740.
 137. Qi, Z.T., Kalkeri, G., Hanible, J., Prabhu, R., Bastian, F., Garry, R.F. & Dash, S. (2003). Stem-loop structures II-IV of the 5' untranslated sequences are required for the expression of the full-length hepatitis C virus genome. *Archives of Virology* **148**, 449–467.
 138. Prabhu, R., Joshi, V., Garry, R.F., Bastian, F., Haque, S., Regenstein, F., Thung, S. & Dash, S. (2004). Interferon alpha-2b inhibits negative-strand RNA and protein expression from full-length HCV1a infectious clone. *Experimental & Molecular Pathology* **76**, 242–252.
 139. Chung, R.T., He, W., Saquib, A., Contreras, A.M., Xavier, R.J., Chawla, A., Wang, T.C. & Schmidt, E.V. (2001). Hepatitis C virus replication is directly inhibited by IFN-alpha in a full-length binary expression system. *Proceedings of the National Academy of Sciences of the USA* **98**, 9847–9852.
 140. Harada, T., Kim, D.W., Sagawa, K., Suzuki, T., Takahashi, K., Saito, I., Matsuura, Y. & Miyamura, T. (1995). Characterization of an established human hepatoma cell line constitutively expressing non-structural proteins of hepatitis C virus by transfection of viral cDNA. *Journal of General Virology* **76**, 1215–1221.
 141. Heim, M.H., Moradpour, D. & Blum, H.E. (1999). Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *Journal of Virology* **73**, 8469–8475.
 142. Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C.M., Blum, H.E. & Moradpour, D. (2001). Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *Journal of Biological Chemistry* **276**, 44,052–44,063.
 143. Wolk, B., Sansonno, D., Krausslich, H.G., Damacco, F., Rice, C.M., Blum, H.E. & Moradpour, D. (2000). Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *Journal of Virology* **74**, 2293–2304.
 144. Cooper, S., Erickson, A.L., Adams, E.J., Kansopon, J., Weiner, A.J., Chien, D.Y., Houghton, M., Parham, P. & Walker, C.M. (1999). Analysis of a successful immune response against hepatitis C virus. *Immunity* **10**, 439–449.
 145. Koziel, M.J., Dudley, D., Afdhal, N., Choo, Q.L., Houghton, M., Ralston, R. & Walker, B.D. (1993). Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes recognize epitopes in the core and envelope proteins of HCV. *Journal of Virology* **67**, 7522–7532.
 146. Bronowicki, J.P., Vetter, D., Uhl, G., Hudziak, H., Uhrlicher, A., Vetter, J.M. & Doffoel, M. (1997). Lymphocyte reactivity to hepatitis C virus (HCV) antigens shows evidence for exposure to HCV in HCV-seronegative spouses of HCV-infected patients. *Journal of Infectious Diseases* **176**, 518–522.
 147. Scognamiglio, P., Accapezzato, D., Casciaro, M.A., Cacciani, A., Artini, M., Bruno, G., Chircu, M.L., Sidney, J., Southwood, S., Abrignani, S., Sette, A. & Barnaba, V. (1999). Presence of effector CD8⁺ T cells in hepatitis C virus-exposed healthy seronegative donors. *Journal of Immunology* **162**, 6681–6689.
 148. Takaki, A., Wiese, M., Maertens, G., Depla, E., Seifert, U., Liebetrau, A., Miller, J.L., Manns, M.P. & Rehermann, B. (2000). Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nature Medicine* **6**, 578–582.
 149. Barrett, S., Ryan, E. & Crowe, J. (1999). Association of the HLA-DRB1*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin. *Journal of Hepatology* **30**, 979–983.
 150. Chisari, F.V. (1997). Cytotoxic T cells and viral hepatitis. *Journal of Clinical Investigation* **99**, 1472–1477.
 151. Gruner, N.H., Gerlach, T.J., Jung, M.C., Diepolder, H.M., Schirren, C.A., Schraut, W.W., Hoffmann, R., Zachoval, R., Santantonio, T., Cucchiari, M., Cerny, A. & Pape, G.R. (2000). Association of hepatitis C virus-specific CD8⁺ T cells with viral clearance in acute hepatitis C. *Journal of Infectious Diseases* **181**, 1528–1536.
 152. Cerny, A. & Chisari, F.V. (1999). Pathogenesis of chronic hepatitis C: Immunological features of hepatic injury and viral persistence. *Hepatology* **30**, 595–601.
 153. Diepolder, H.M., Zachoval, R., Hoffmann, R.M., Jung, M.C., Gerlach, T. & Pape, G.R. (1996). The role of hepatitis C virus specific CD4⁺ T lymphocytes in acute and chronic hepatitis C. *Journal of*

- Molecular Medicine* **74**, 583–588.
154. Lechner, F., Wong, D.K., Dunbar, P.R., Chapman, R., Chung, R.T., Dohrenwend, P., Robbins, G., Phillips, R., Klenerman, P. & Walker, B.D. (2000). Analysis of successful immune responses in persons infected with hepatitis C virus. *Journal of Experimental Medicine* **191**, 1499–1512.
 155. Timme, R., Oldach, D., Chang, K.M., Steiger, C., Ray, S.C. & Chisari, F.V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. *Journal of Experimental Medicine* **194**, 1395–1406.
 156. Wedemeyer, H., He, X.S., Nascimbeni, M., Davis, A.R., Greenberg, H.B., Hoofnagle, J.H., Liang, T.J., Alter, H. & Rehermann, B. (2002). Impaired effector function of hepatitis C virus-specific CD8⁺ T cells in chronic hepatitis C virus infection. *Journal of Immunology* **169**, 3447–3458.
 157. Bukh, J., Timme, R., Meunier, J.-C., Faulk, K., Spangenberg, H.C., Chang, K.-M., Satterfield, W., Chisari, F.V. & Purcell, R.H. (2008). Previously infected chimpanzees are not consistently protected against reinfection or persistent infection after reexposure to the identical hepatitis C virus strain. *Journal of Virology* **82**, 8183–8195.
 158. Lanford, R.E. & Bigger, C. (2002). Advances in model systems for hepatitis C virus research. *Virology* **293**, 1–9.
 159. Bassett, S.E., Guerra, B., Brasky, K., Miskovsky, E., Houghton, M., Klimpel, G.R. & Lanford, R.E. (2001). Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology* **33**, 1479–1487.
 160. Lanford, R.E., Guerra, B., Chavez, D., Bigger, C., Brasky, K.M., Wang, X.H., Ray, S.C. & Thomas, D.L. (2004). Cross-genotype immunity to hepatitis C virus. *Journal of Virology* **78**, 1575–1581.
 161. Kaplan, D.E., Sugimoto, K., Newton, K., Valiga, M.E., Ikeda, F., Aytaman, A., Nunes, F.A., Lucey, M.R., Vance, B.A., Vonderheide, R.H., Reddy, K.R., McKeating, J.A. & Chang, K.M. (2007). Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology* **132**, 654–666.
 162. von Hahn, T., Yoon, J.C., Alter, H., Rice, C.M., Rehermann, B., Balfe, P. & McKeating, J.A. (2007). Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection *in vivo*. *Gastroenterology* **132**, 667–678.
 163. Neumann, A.U., Lam, N.P., Dahari, H., Gretsch, D.R., Wiley, T.E., Layden, T.J. & Perelson, A.S. (1998). Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-alpha therapy. *Science, New York* **282**, 103–107.
 164. Lam, N.P., Neumann, A.U., Gretsch, D.R., Wiley, T.E., Perelson, A.S. & Layden, T.J. (1997). Dose-dependent acute clearance of hepatitis C genotype 1 virus with interferon alfa. *Hepatology* **26**, 226–231.
 165. Bigger, C.B., Guerra, B., Brasky, K.M., Hubbard, G., Beard, M.R., Luxon, B.A., Lemon, S.M. & Lanford, R.E. (2004). Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. *Journal of Virology* **78**, 13,779–13,792.
 166. Lanford, R.E., Guerra, B., Lee, H., Chavez, D., Brasky, K.M. & Bigger, C.B. (2006). Genomic response to interferon-alpha in chimpanzees: Implications of rapid downregulation for hepatitis C kinetics. *Hepatology* **43**, 961–972.
 167. Jaeckel, E., Cornberg, M., Wedemeyer, H., Santantonio, T., Mayer, J., Zankel, M., Pastore, G., Dietrich, M., Trautwein, C. & Manns, M.P. (2001). Treatment of acute hepatitis C with interferon alfa-2b. *New England Journal of Medicine* **345**, 1452–1457.
 168. Castet, V., Fournier, C., Soulier, A., Brillet, R., Coste, J., Larrey, D., Dhumeaux, D., Maurel, P. & Pawlotsky, J.M. (2002). Alpha interferon inhibits hepatitis C virus replication in primary human hepatocytes infected *in vitro*. *Journal of Virology* **76**, 8189–8199.
 169. Shimizu, Y.K. & Yoshikura, H. (1994). Multicycle infection of hepatitis C virus in cell culture and inhibition by alpha and beta interferons. *Journal of Virology* **68**, 8406–8408.
 170. Frese, M., Pietschmann, T., Moradpour, D., Haller, O. & Bartenschlager, R. (2001). Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. *Journal of General Virology* **82**, 723–733.
 171. Guo, J.T., Bichko, V.V. & Seeger, C. (2001). Effect of alpha interferon on the hepatitis C virus replicon. *Journal of Virology* **75**, 8516–8523.
 172. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science, New York* **285**, 110–113.
 173. Lanford, R.E., Guerra, B., Lee, H., Averett, D.R., Pfeiffer, B., Chavez, D., Notvall, L. & Bigger, C. (2003). Antiviral effect and virus–host interactions in response to alpha interferon, gamma interferon, poly(i)-poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. *Journal of Virology* **77**, 1092–1104.
 174. Blight, K.J., Kolykhalov, A.A. & Rice, C.M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science, New York* **290**, 1972–1974.
 175. Helbig, K.J., Lau, D.T., Semendric, L., Harley, H.A. & Beard, M.R. (2005). Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. *Hepatology* **42**, 702–710.
 176. Smith, M.W., Yue, Z.N., Korth, M.J., Do, H.A., Boix, L., Fausto, N., Bruix, J., Carithers, R.L.J. & Katze, M.G. (2003). Hepatitis C virus and liver disease: Global transcriptional profiling and identification of potential markers. *Hepatology* **38**, 1458–1467.
 177. Ji, X., Cheung, R., Cooper, S., Li, Q., Greenberg, H.B. & He, X.S. (2003). Interferon alfa regulated gene expression in patients initiating interferon treatment for chronic hepatitis C. *Hepatology* **37**, 610–621.
 178. Chen, L., Borozan, I., Feld, J., Sun, J., Tannis, L.L., Coltescu, C., Heathcote, J., Edwards, A.M. & McGilvray, I.D. (2005). Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology* **128**, 1437–1444.
 179. MacQuillan, G.C., Mamotte, C., Reed, W.D., Jeffrey, G.P. & Allan, J.E. (2003). Upregulation of endogenous intrahepatic interferon stimulated genes during chronic hepatitis C virus infection. *Journal of Medical Virology* **70**, 219–227.
 180. Huang, Y., Feld, J.J., Sapp, R.K., Nanda, S., Lin, J.H., Blatt, L.M., Fried, M.W., Murthy, K. & Liang,

- T.J. (2007). Defective hepatic response to interferon and activation of suppressor of cytokine signaling 3 in chronic hepatitis C. *Gastroenterology* **132**, 733–744.
181. Der, S.D., Zhou, A., Williams, B.R. & Silverman, R.H. (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proceedings of the National Academy of Sciences of the USA* **95**, 15,623–15,628.
 182. Tan, H., Derrick, J., Hong, J., Sanda, C., Grosse, W.M., Edenberg, H.J., Taylor, M., Seiwert, S. & Blatt, L.M. (2005). Global transcriptional profiling demonstrates the combination of type I and type II interferon enhances antiviral and immune responses at clinically relevant doses. *Journal of Interferon & Cytokine Research* **25**, 632–649.
 183. Meyer, M.F., Lehmann, M., Cornberg, M., Wieg, J., Manns, M.P., Klade, C. & Wedemeyer, H. (2007). Clearance of low levels of HCV viremia in the absence of a strong adaptive immune response. *Virology Journal* **4**, 58.
 184. Hassan, M., Selimovic, D., Ghozlan, H. & Abdelkader, O. (2009). Hepatitis C virus core protein triggers hepatic angiogenesis by a mechanism including multiple pathways. *Hepatology* **49**, 1469–1482.
 185. Pfeffer, S. & Baumert, T.F. (2009). Unravelling the importance of microRNAs during hepatitis C virus infection in the human liver. *Journal of Hepatology* **51**, 606–609.
 186. Smith, M.W., Walters, K.A., Korth, M.J., Fitzgibbon, M., Proll, S., Thompson, J.C., Yeh, M.M., Shuhart, M.C., Furlong, J.C., Cox, P.P., Thomas, D.L., Phillips, J.D., Kushner, J.P., Fausto, N., Carithers, R.L.J. & Katze, M.G. (2006). Gene expression patterns that correlate with hepatitis C and early progression to fibrosis in liver transplant recipients. *Gastroenterology* **130**, 179–187.
 187. Lau, D.T., Luxon, B.A., Xiao, S.Y., Beard, M.R. & Lemon, S.M. (2005). Intrahepatic gene expression profiles and alpha-smooth muscle actin patterns in hepatitis C virus induced fibrosis. *Hepatology* **42**, 273–281.
 188. Ryder, S.D., Irving, W.L., Jones, D.A., Neal, K.R. & Underwood, J.C. (2004). Progression of hepatic fibrosis in patients with hepatitis C: A prospective repeat liver biopsy study. *Gut* **53**, 451–455.
 189. Hui, C.K., Belaye, T., Montegrando, K. & Wright, T.L. (2003). A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. *Journal of Hepatology* **38**, 511–517.
 190. Boccato, S., Pistis, R., Noventa, F., Guido, M., Benvegna, L. & Alberti, A. (2006). Fibrosis progression in initially mild chronic hepatitis C. *Journal of Viral Hepatitis* **13**, 297–302.
 191. Ghany, M.G., Kleiner, D.E., Alter, H., Doo, E., Khokar, F., Promrat, K., Herion, D., Park, Y., Liang, T.J. & Hoofnagle, J.H. (2003). Progression of fibrosis in chronic hepatitis C. *Gastroenterology* **124**, 97–104.
 192. Levine, R.A., Sanderson, S.O., Ploutz-Snyder, R., Murray, F., Kay, E., Hegarty, J., Nolan, N., Kelleher, D., McDonald, G., O'Keane, J.C. & Crowe, J. (2006). Assessment of fibrosis progression in untreated Irish women with chronic hepatitis C contracted from immunoglobulin anti-D. *Clinical Gastroenterology & Hepatology* **4**, 1271–1277.
 193. Persico, M., Persico, E., Suozzo, R., Conte, S., De Seta, M., Coppola, L., Palmentieri, B., Sasso, F.C. & Torella, R. (2000). Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels. *Gastroenterology* **118**, 760–764.
 194. Martinot-Peignoux, M., Boyer, N., Cazals-Hatem, D., Pham, B.N., Gervais, A., Le Breton, V., Levy, S., Degott, C., Valla, D.C. & Marcellin, P. (2001). Prospective study on anti-hepatitis C virus-positive patients with persistently normal serum alanine transaminase with or without detectable serum hepatitis C virus RNA. *Hepatology* **34**, 1000–1005.
 195. Stevenson, F.K. & Rosenberg, W. (2001). DNA vaccination: A potential weapon against infection and cancer. *Vox Sanguinis* **80**, 12–18.
 196. Elmowalid, G.A., Qiao, M., Jeong, S-H., Borg, B.B., Baumert, T.F., Sapp, R.K., Hu, Z., Murthy, K. & Liang, T.J. (2007). Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. *Proceedings of the National Academy of Sciences of the USA* **104**, 8427–8432.
 197. Stoll-Keller, F., Barth, H., Fafi-Kremer, S., Zeisel, M.B. & Baumert, T.F. (2009). Development of hepatitis C virus vaccines: Challenges and progress. *Expert Review of Vaccines* **8**, 333–345.
 198. de Bruijne, J., Weegink, C.J., Jansen, P.L.M. & Reesink, H.W. (2009). New developments in the antiviral treatment of hepatitis C. *Vox Sanguinis* **97**, 1–12.
 199. Choo, Q.L., Kuo, G., Ralston, R., Weiner, A., Chien, D., Van Nest, G., Han, J., Berger, K., Thudium, K., Kuo, C., Kansopon, J., McFarland, J., Tabrizi, A., Ching, K., Moss, B., Cummins, L.B., Houghton, M. & Muchmore, E. (1994). Vaccination of chimpanzees against infection by the hepatitis C virus. *Proceedings of the National Academy of Sciences of the USA* **91**, 1294–1298.
 200. Rollier, C., Depla, E., Drexhage, J.A., Verschoor, E.J., Verstrepen, B.E., Fatmi, A., Brinster, C., Fournillier, A., Whelan, J.A., Whelan, M., Jacobs, D., Maertens, G., Inchauspé, G. & Heeney, J.L. (2004). Control of heterologous hepatitis C virus infection in chimpanzees is associated with the quality of vaccine-induced peripheral T-helper immune response. *Journal of Virology* **78**, 187–196.
 201. Jeong, S.H., Qiao, M., Nascimbeni, M., Hu, Z., Rehermann, B., Murthy, K. & Liang, T.J. (2004). Immunization with hepatitis C virus-like particles induces humoral and cellular immune responses in nonhuman primates. *Journal of Virology* **78**, 6995–7003.
 202. Abraham, J.D., Himoudi, N., Kien, F., Berland, J.L., Codran, A., Bartosch, B., Baumert, T., Paranhos-Baccala, G., Schuster, C., Inchauspé, G. & Kieny, M.P. (2004). Comparative immunogenicity analysis of modified vaccinia Ankara vectors expressing native or modified forms of hepatitis C virus E1 and E2 glycoproteins. *Vaccine* **22**, 3917–3928.
 203. Brinster, C., Chen, M., Boucreux, D., Paranhos-Baccala, G., Liljestrom, P., Lemmonier, F. & Inchauspé, G. (2002). Hepatitis C virus non-structural protein 3-specific cellular immune responses following single or combined immunization with DNA or recombinant Semliki Forest virus particles. *Journal of General Virology* **83**, 369–381.
 204. O'Hagan, D.T., Singh, M., Dong, C., Ugozzoli, M., Berger, K., Glazer, E., Selby, M., Winingar, M., Ng,

- P., Crawford, K., Paliard, X., Coates, S. & Houghton, M. (2004). Cationic microparticles are a potent delivery system for a HCV DNA vaccine. *Vaccine* **23**, 672–680.
205. Wuest, T., Both, G.W., Prince, A.M., Hofmann, C. & Loser, P. (2004). Recombinant ovine adenovirus induces a strong and sustained T cell response against the hepatitis C virus NS3 antigen in mice. *Vaccine* **22**, 2717–2721.
206. Perri, S., Greer, C.E., Thudium, K., Doe, B., Legg, H., Liu, H., Romero, R.E., Tang, Z., Bin, Q., Dubensky, T.W.J., Vajdy, M., Otten, G.R. & Polo, J.M. (2003). An alphavirus replicon particle chimera derived from Venezuelan equine encephalitis and Sindbis viruses is a potent gene-based vaccine delivery vector. *Journal of Virology* **77**, 10,394–10,403.
207. Pancholi, P., Perkus, M., Tricoche, N., Liu, Q. & Prince, A.M. (2003). DNA immunization with hepatitis C virus (HCV) polycistronic genes or immunization by HCV DNA priming–recombinant canarypox virus boosting induces immune responses and protection from recombinant HCV-vaccinia virus infection in HLA-A2.1-transgenic mice. *Journal of Virology* **77**, 382–390.
208. Youn, J.W., Hu, Y.W., Tricoche, N., Pfahler, W., Shata, M.T., Dreux, M., Cosset, F.L., Folgori, A., Lee, D.H., Brotman, B. & Prince, A.M. (2008). Evidence for protection against chronic hepatitis C virus infection in chimpanzees by immunization with replicating recombinant vaccinia virus. *Journal of Virology* **82**, 10,896–10,905.
209. Ma, X., Fornis, X., Gutierrez, R., Mushahwar, I.K., Wu, T., Payette, P.J., Bukh, J., Purcell, R.H. & Davis, H.L. (2002). DNA-based vaccination against hepatitis C virus (HCV): Effect of expressing different forms of HCV E2 protein and use of CpG-optimized vectors in mice. *Vaccine* **20**, 3263–3271.
210. Song, M.K., Lee, S.W., Suh, Y.S., Lee, K.J. & Sung, Y.C. (2000). Enhancement of immunoglobulin G2a and cytotoxic T-lymphocyte responses by a booster immunization with recombinant hepatitis C virus E2 protein in E2 DNA-primed mice. *Journal of Virology* **74**, 2920–2925.
211. Matsui, M., Moriya, O. & Akatsuka, T. (2003). Enhanced induction of hepatitis C virus-specific cytotoxic T lymphocytes and protective efficacy in mice by DNA vaccination followed by adenovirus boosting in combination with the interleukin-12 expression plasmid. *Vaccine* **21**, 1629–1639.
212. Makimura, M., Miyake, S., Akino, N., Takamori, K., Matsuura, Y., Miyamura, T. & Saito, I. (1996). Induction of antibodies against structural proteins of hepatitis C virus in mice using recombinant adenovirus. *Vaccine* **14**, 28–36.
213. Engler, O.B., Schwendener, R.A., Dai, W.J., Wolk, B., Pichler, W., Moradpour, D., Brunner, T. & Cerny, A. (2004). A liposomal peptide vaccine inducing CD8⁺ T cells in HLA-A2.1 transgenic mice, which recognise human cells encoding hepatitis C virus (HCV) proteins. *Vaccine* **23**, 58–68.
214. Gehring, S., Gregory, S.H., Kuzushita, N. & Wands, J.R. (2005). Type I interferon augments DNA-based vaccination against hepatitis C virus core protein. *Journal of Medical Virology* **75**, 249–257.
215. Gordon, E.J., Bhat, R., Liu, Q., Wang, Y.F., Tackney, C. & Prince, A.M. (2000). Immune responses to hepatitis C virus structural and nonstructural proteins induced by plasmid DNA immunizations. *Journal of Infectious Diseases* **181**, 42–50.
216. Inchauspé, G., Major, M.E., Nakano, I., Vitvitski, L. & Trepo, C. (1997). DNA vaccination for the induction of immune responses against hepatitis C virus proteins. *Vaccine* **15**, 853–856.
217. Inchauspé, G., Major, M.E., Nakano, I., Vivitski, L., Maisonnas, M. & Trepo, C. (1998). Immune responses against hepatitis C virus structural proteins following genetic immunisation. *Developments in Biological Standardization* **92**, 163–168.
218. Major, M.E., Vitvitski, L., Mink, M.A., Schleef, M., Whalen, R.G., Trepo, C. & Inchauspé, G. (1995). DNA-based immunization with chimeric vectors for the induction of immune responses against the hepatitis C virus nucleocapsid. *Journal of Virology* **69**, 5798–5805.
219. Youn, J.W., Park, S.H., Cho, J.H. & Sung, Y.C. (2003). Optimal induction of T-cell responses against hepatitis C virus E2 by antigen engineering in DNA immunization. *Journal of Virology* **77**, 11,596–11,602.
220. Zhu, L.X., Liu, J., Ye, Y., Xie, Y.H., Kong, Y.Y., Li, G.D. & Wang, Y. (2004). A candidate DNA vaccine elicits HCV specific humoral and cellular immune responses. *World Journal of Gastroenterology* **10**, 2488–2492.
221. Yu, H., Babiuk, L.A. & van Drunen Littel-van den Hurk, S. (2004). Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3. *Journal of General Virology* **85**, 1533–1543.
222. Pancholi, P., Liu, Q., Tricoche, N., Zhang, P., Perkus, M.E. & Prince, A.M. (2000). DNA prime–canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. *Journal of Infectious Diseases* **182**, 18–27.
223. Park, S.H., Yang, S.H., Lee, C.G., Youn, J.W., Chang, J. & Sung, Y.C. (2003). Efficient induction of T helper 1 CD4⁺ T-cell responses to hepatitis C virus core and E2 by a DNA prime–adenovirus boost. *Vaccine* **21**, 4555–4564.
224. Netter, H.J., Macnaughton, T.B., Woo, W.P., Tindle, R. & Gowans, E.J. (2001). Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes. *Journal of Virology* **75**, 2130–2141.
225. Lee, S.W., Cho, J.H., Lee, K.J. & Sung, Y.C. (1998). Hepatitis C virus envelope DNA-based immunization elicits humoral and cellular immune responses. *Molecules & Cells* **8**, 444–451.
226. Lee, S.W., Cho, J.H. & Sung, Y.C. (1998). Optimal induction of hepatitis C virus envelope-specific immunity by bicistronic plasmid DNA inoculation with the granulocyte-macrophage colony-stimulating factor gene. *Journal of Virology* **72**, 8430–8436.
227. Rollier, C.S., Paranhos-Baccala, G., Verschoor, E.J., Verstrepen, B.E., Drexhage, J.A., Fagrouch, Z., Berland, J.L., Komurian-Pradel, F., Duverger, B., Himoudi, N., Staib, C., Meyr, M., Whelan, M., Whelan, J.A., Adams, V.C., Larrea, E., Riezu, J.L., Lasarte, J.J., Bartosch, B., Cosset, F.L., Spaan, W.J., Diepolder, H.M., Pape, G.R., Sutter, G., Inchauspé, G. & Heeney, J.L. (2007). Vaccine-induced early control of hepatitis C virus infection in chimpanzees fails to impact on hepatic PD-1 and chronicity. *Hepatology* **45**, 602–613.

228. Youn, J.W., Park, S.H., Lavillette, D., Cosset, F.L., Yang, S.H., Lee, C.G., Jin, H.T., Kim, C.M., Shata, M.T., Lee, D.H., Pfahler, W., Prince, A.M. & Sung, Y.C. (2005). Sustained E2 antibody response correlates with reduced peak viremia after hepatitis C virus infection in the chimpanzee. *Hepatology* **42**, 1429–1436.
229. Polakos, N.K., Drane, D., Cox, J., Ng, P., Selby, M.J., Chien, D., O'Hagan, D.T., Houghton, M. & Paliard, X. (2001). Characterization of hepatitis C virus core-specific immune responses primed in rhesus macaques by a nonclassical ISCOM vaccine. *Journal of Immunology* **166**, 3589–3598.
230. Franzusoff, A., Duke, R.C., King, T.H., Lu, Y. & Rodell, T.C. (2005). Yeasts encoding tumour antigens in cancer immunotherapy. *Expert Opinion on Biological Therapy* **5**, 565–575.
231. Leroux-Roels, G., Depla, E., Hulstaert, F., Tobback, L., Dincq, S., Desmet, J., Desombere, I. & Maertens, G. (2004). A candidate vaccine based on the hepatitis C E1 protein: Tolerability and immunogenicity in healthy volunteers. *Vaccine* **22**, 3080–3086.
232. Nevens, F., Roskams, T., Van Vlierberghe, H., Horsmans, Y., Sprengers, D., Elewaut, A., Desmet, V., Leroux-Roels, G., Quinaux, E., Depla, E., Dincq, S., Vander Stichele, C., Maertens, G. & Hulstaert, F. (2003). A pilot study of therapeutic vaccination with envelope protein E1 in 35 patients with chronic hepatitis C. *Hepatology* **38**, 1289–1296.
233. Firbas, C., Jilma, B., Tauber, E., Buerger, V., Jelovcan, S., Lingnau, K., Buschle, M., Frisch, J. & Klade, C.S. (2006). Immunogenicity and safety of a novel therapeutic hepatitis C virus (HCV) peptide vaccine: A randomized, placebo controlled trial for dose optimization in 128 healthy subjects. *Vaccine* **24**, 4343–4353.
234. Klade, C.S., Wedemeyer, H., Berg, T., Hinrichsen, H., Cholewinska, G., Zeuzem, S., Blum, H., Buschle, M., Jelovcan, S., Buerger, V., Tauber, E., Frisch, J. & Manns, M.P. (2008). Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology* **134**, 1385–1395.
235. Schlaphoff, V., Klade, C.S., Jilma, B., Jelovcan, S.B., Cornberg, M., Tauber, E., Manns, M.P. & Wedemeyer, H. (2007). Functional and phenotypic characterization of peptide-vaccine-induced HCV-specific CD8⁺ T cells in healthy individuals and chronic hepatitis C patients. *Vaccine* **25**, 6793–6806.
236. Yutani, S., Yamada, A., Yoshida, K., Takao, Y., Tamura, M., Komatsu, N., Ide, T., Tanaka, M., Sata, M. & Itoh, K. (2007). Phase I clinical study of a personalized peptide vaccination for patients infected with hepatitis C virus (HCV) 1b who failed to respond to interferon-based therapy. *Vaccine* **25**, 7429–7435.
237. Sakamoto, N. & Watanabe, M. (2009). New therapeutic approaches to hepatitis C virus. *Journal of Gastroenterology* **44**, 643–649.
238. Foy, E., Li, K., Sumpter, R.J., Loo, Y.M., Johnson, C.L., Wang, C., Fish, P.M., Yoneyama, M., Fujita, T., Lemon, S.M. & Gale, M.J. (2005). Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proceedings of the National Academy of Sciences of the USA* **102**, 2986–2991.
239. Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C., Ikeda, M., Ray, S.C., Gale, M.J. & Lemon, S.M. (2005). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proceedings of the National Academy of Sciences of the USA* **102**, 2992–2997.
240. Lamarre, D., Anderson, P.C., Bailey, M., Beaulieu, P., Bolger, G., Bonneau, P., Bos, M., Cameron, D.R., Cartier, M., Cordingley, M.G., Faucher, A.M., Goudreau, N., Kawai, S.H., Kukolj, G., Lagace, L., LaPlante, S.R., Narjes, H., Poupard, M.A., Rancourt, J., Sentjens, R.E., St George, R., Simoneau, B., Steinmann, G., Thibeault, D., Tsantrizos, Y.S., Weldon, S.M., Yong, C.L. & Llinas-Brunet, M. (2003). An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature, London* **426**, 186–189.
241. Crotta, S., Stilla, A., Wack, A., D'Andrea, A., Nuti, S., D'Oro, U., Mosca, M., Filliponi, F., Brunetto, R.M., Bonino, F., Abrignani, S. & Valiante, N.M. (2002). Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *Journal of Experimental Medicine* **195**, 35–41.
242. Tseng, C.T. & Klimpel, G.R. (2002). Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *Journal of Experimental Medicine* **195**, 43–49.
243. Forns, X., Bukh, J. & Purcell, R.H. (2002). The challenge of developing a vaccine against hepatitis C virus. *Journal of Hepatology* **37**, 684–695.
244. Pestka, J.M., Zeisel, M.B., Blaser, E., Schurmann, P., Bartosch, B., Cosset, F.L., Patel, A.H., Meisel, H., Baumert, J., Viazov, S., Rispeter, K., Blum, H.E., Roggendorf, M. & Baumert, T.F. (2007). Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proceedings of the National Academy of Sciences of the USA* **104**, 6025–6030.
245. Lavillette, D., Morice, Y., Germanidis, G., Donot, P., Soulier, A., Pagkalos, E., Sakellariou, G., Intrator, L., Bartosch, B., Pawlotsky, J.M. & Cosset, F.L. (2005). Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *Journal of Virology* **79**, 6023–6034.
246. Haberstroh, A., Schnober, E.K., Zeisel, M.B., Carolla, P., Barth, H., Blum, H.E., Cosset, F.L., Koutsoudakis, G., Bartenschlager, R., Union, A., Depla, E., Owsianka, A., Patel, A.H., Schuster, C., Stoll-Keller, F., Doffoel, M., Dreux, M. & Baumert, T.F. (2008). Neutralizing host responses in hepatitis C virus infection target viral entry at postbinding steps and membrane fusion. *Gastroenterology* **135**, 1719–1728.e1.
247. De Francesco, R., Neddermann, P., Tomei, L., Steinkuhler, C., Gallinari, P. & Folgori, A. (2000). Biochemical and immunologic properties of the non-structural proteins of the hepatitis C virus: Implications for development of antiviral agents and vaccines. *Seminars in Liver Disease* **20**, 69–83.
248. Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A. & Rice, C.M. (2005). Complete replication of hepatitis C virus in cell culture. *Science, New York* **309**, 623–626.
249. HCV Advocate (2009). *Hepatitis C Treatments in Current Clinical Development* (ed. A. Franciscus),

- 21pp. San Francisco, CA, USA: HCV Advocate. Available at: <http://www.hcvadvocate.org/hepatitis/hepC/HCVDrugs.html> (Accessed 17.08.10).
250. Davis, G.L., Nelson, D.R., Terrault, N., Pruetz, T.L., Schiano, T.D., Fletcher, C.V., Sapan, C.V., Riser, L.N., Li, Y., Whitley, R.J. & Gnann, J.W.J. (2005). A randomized, open-label study to evaluate the safety and pharmacokinetics of human hepatitis C immune globulin (Civacir) in liver transplant recipients. *Liver Transplantation* **11**, 941–949.
251. Schiano, T.D., Charlton, M., Younossi, Z., Galun, E., Pruetz, T., Tur-Kaspa, R., Eren, R., Dagan, S., Graham, N., Williams, P.V. & Andrews, J. (2006). Monoclonal antibody HCV-AbXTL68 in patients undergoing liver transplantation for HCV: Results of a phase 2 randomized study. *Liver Transplantation* **12**, 1381–1389.
252. Yu, M.Y., Bartosch, B., Zhang, P., Guo, Z.P., Renzi, P.M., Shen, L.M., Granier, C., Feinstone, S.M., Cosset, F.L. & Purcell, R.H. (2004). Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. *Proceedings of the National Academy of Sciences of the USA* **101**, 7705–7710.
253. Krawczynski, K., Alter, M.J., Tankersley, D.L., Beach, M., Robertson, B.H., Lambert, S., Kuo, G., Spelbring, J.E., Meeks, E., Sinha, S. & Carson, D.A. (1996). Effect of immune globulin on the prevention of experimental hepatitis C virus infection. *Journal of Infectious Diseases* **173**, 822–828.
254. Soler, M., McHutchison, J.G., Kwoh, T.J., Dorr, F.A. & Pawlotsky, J.M. (2004). Virological effects of ISIS 14803, an antisense oligonucleotide inhibitor of hepatitis C virus (HCV) internal ribosome entry site (IRES), on HCV IRES in chronic hepatitis C patients and examination of the potential role of primary and secondary HCV resistance in the outcome of treatment. *Antiviral Therapy* **9**, 953–968.
255. Usman, N. & Blatt, L.M. (2000). Nuclease-resistant synthetic ribozymes: Developing a new class of therapeutics. *Journal of Clinical Investigation* **106**, 1197–1202.
256. ClinicalTrials.gov (2009). *Antiviral Activity and Safety of 3 Different Doses of Mifepristone in Hepatitis C Infected Patients*. Bethesda, MD, USA: US National Library of Medicine. Available at: <http://clinicaltrials.gov/ct2/show/NCT00255177?term=VGX&rank=4> (Accessed 17.08.10).
257. ClinicalTrials.gov (2008). *A Study to Evaluate the Safety, Antiviral Effect, and Pharmacokinetics of Celgosivir in Combination With Peginterferon Alfa-2b and Ribavirin in Treatment-Naïve Patients With Chronic Hepatitis C*. Bethesda, MD, USA: US National Library of Medicine. Available at: <http://clinicaltrials.gov/ct2/show/NCT00332176?term=celgosivir&rank=1> (Accessed 17.08.10).
258. Del Vecchio, A.M. & Sarisky, R.T. (2006). Small molecule and biologic inhibitors of hepatitis C virus: A symbiotic approach. *Mini Reviews in Medicinal Chemistry* **6**, 1263–1268.
259. Mo, H., Lu, L., Pilot-Matias, T., Pithawalla, R., Mondal, R., Masse, S., Dekhtyar, T., Ng, T., Koev, G., Stoll, V., Stewart, K.D., Pratt, J., Donner, P., Rockway, T., Maring, C. & Molla, A. (2005). Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor *in vitro*. *Antimicrobial Agents & Chemotherapy* **49**, 4305–4314.
260. Lu, L., Dekhtyar, T., Masse, S., Pithawalla, R., Krishnan, P., He, W., Ng, T., Koev, G., Stewart, K., Larson, D., Bosse, T., Wagner, R., Pilot-Matias, T., Mo, H. & Molla, A. (2007). Identification and characterization of mutations conferring resistance to an HCV RNA-dependent RNA polymerase inhibitor *in vitro*. *Antiviral Research* **76**, 93–97.
261. Chen, C.M., He, Y., Lu, L., Lim, H.B., Tripathi, R.L., Middleton, T., Hernandez, L.E., Beno, D.W., Long, M.A., Kati, W.M., Bosse, T.D., Larson, D.P., Wagner, R., Lanford, R.E., Kohlbrenner, W.E., Kempf, D.J., Pilot-Matias, T.J. & Molla, A. (2007). Activity of a potent hepatitis C virus polymerase inhibitor in the chimpanzee model. *Antimicrobial Agents & Chemotherapy* **51**, 4290–4296.
262. Kronenberger, B. & Zeuzem, S. (2008). Future treatment options for HCV: Double, triple, what is the optimal combination? *Best Practice & Research Clinical Gastroenterology* **22**, 1123–1136.
263. Coelmont, L., Paeshuysse, J., Windisch, M.P., De Clercq, E., Bartenschlager, R. & Neyts, J. (2006). Ribavirin antagonizes the *in vitro* anti-hepatitis C virus activity of 2'-C-methylcytidine, the active component of valopicitabine. *Antimicrobial Agents & Chemotherapy* **50**, 3444–3446.
264. Soriano, V., Peters, M.G. & Zeuzem, S. (2009). New therapies for hepatitis C virus infection. *Clinical Infectious Diseases* **48**, 313–320.
265. Yao, N., Hesson, T., Cable, M., Hong, Z., Kwong, A.D., Le, H.V. & Weber, P.C. (1997). Structure of the hepatitis C virus RNA helicase domain. *Nature Structural Biology* **4**, 463–467.
266. Kim, J.L., Morgenstern, K.A., Lin, C., Fox, T., Dwyer, M.D., Landro, J.A., Chambers, S.P., Markland, W., Lepre, C.A., O'Malley, E.T., Harbeson, S.L., Rice, C.M., Murcko, M.A., Caron, P.R. & Thomson, J.A. (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**, 343–355.
267. Lin, C., Kwong, A.D. & Perni, R.B. (2006). Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3,4A serine protease. *Infectious Disorders Drug Targets* **6**, 3–16.
268. Lin, K., Perni, R.B., Kwong, A.D. & Lin, C. (2006). VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCV replicon cells. *Antimicrobial Agents & Chemotherapy* **50**, 1813–1822.
269. Malcolm, B.A., Liu, R., Lahser, F., Agrawal, S., Belanger, B., Butkiewicz, N., Chase, R., Gheyas, F., Hart, A., Hesk, D., Ingravalle, P., Jiang, C., Kong, R., Lu, J., Pichardo, J., Prongay, A., Skelton, A., Tong, X., Venkatraman, S., Xia, E., Girijavallabhan, V. & Njoroge, F.G. (2006). SCH 503034, a mechanism-based inhibitor of hepatitis C virus NS3 protease, suppresses polyprotein maturation and enhances the antiviral activity of alpha interferon in replicon cells. *Antimicrobial Agents & Chemotherapy* **50**, 1013–1020.
270. Neukam, K., Macías, J., Mira, J.A. & Pineda, J.A. (2009). A review of current anti-HCV treatment regimens and possible future strategies. *Expert Opinion on Pharmacotherapy* **10**, 417–433.
271. Lin, C., Lin, K., Luong, Y.P., Rao, B.G., Wei, Y.Y., Brennan, D.L., Fulghum, J.R., Hsiao, H.M., Ma, S., Maxwell, J.P., Cottrell, K.M., Perni, R.B., Gates, C.A. & Kwong, A.D. (2004). *In vitro* resist-

- ance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: Structural analysis indicates different resistance mechanisms. *Journal of Biological Chemistry* **279**, 17,508–17,514.
272. Hinrichsen, H., Benhamou, Y., Wedemeyer, H., Reiser, M., Sentjens, R.E., Calleja, J.L., Forns, X., Erhardt, A., Cronlein, J., Chaves, R.L., Yong, C.L., Nehmiz, G. & Steinmann, G.G. (2004). Short-term antiviral efficacy of BILN 2061, a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. *Gastroenterology* **127**, 1347–1355.
273. Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y. & Shimotohno, K. (2005). Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Molecular Cell* **19**, 111–122.
274. Flisiak, R., Horban, A., Gallay, P., Bobardt, M., Selvarajah, S., Wiercinska-Drapalo, A., Siwak, E., Cielniak, I., Higersberger, J., Kierkus, J., Aeschlimann, C., Grosgrurin, P., Nicolas-Métral, V., Dumont, J.M., Porchet, H., Crabbe, R. & Scalfaro, P. (2008). The cyclophilin inhibitor Debio-025 shows potent anti-hepatitis C effect in patients coinfecting with hepatitis C and human immunodeficiency virus. *Hepatology* **47**, 817–826.
275. Crabbé, R., Vuagniaux, G., Dumont, J.-M., Nicolas-Métral, V., Marfurt, J. & Novaroli, L. (2009). An evaluation of the cyclophilin inhibitor Debio 025 and its potential as a treatment for chronic hepatitis C. *Expert Opinion on Investigational Drugs* **18**, 211–220.
276. Paeshuyse, J., Kaul, A., De Clercq, E., Rosenwirth, B., Dumont, J.M., Scalfaro, P., Bartenschlager, R. & Neyts, J. (2006). The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication *in vitro*. *Hepatology* **43**, 761–770.
277. Rossignol, J.F., Elfert, A., El-Gohary, Y. & Keeffe, E.B. (2009). Improved virologic response in chronic hepatitis C genotype 4 treated with nitazoxanide, peginterferon, and ribavirin. *Gastroenterology* **136**, 856–862.
278. De Leede, L.G., Humphries, J.E., Bechet, A.C., Van Hoogdale, E.J., Verrijck, R. & Spencer, D.G. (2008). Novel controlled-release Lemna-derived IFN-alpha2b (Locteron): Pharmacokinetics, pharmacodynamics, and tolerability in a phase I clinical trial. *Journal of Interferon & Cytokine Research* **28**, 113–122.
279. Rustgi, V.K. (2009). Albinterferon alfa-2b, a novel fusion protein of human albumin and human interferon alfa-2b, for chronic hepatitis C. *Current Medical Research & Opinion* **25**, 991–1002.
280. Horsmans, Y., Berg, T., Desager, J.P., Mueller, T., Schott, E., Fletcher, S.P., Steffy, K.R., Bauman, L.A., Kerr, B.M. & Averett, D.R. (2005). Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. *Hepatology* **42**, 724–731.
281. McHutchison, J.G., Bacon, B.R., Gordon, S.C., Lawitz, E., Shiffman, M., Afdhal, N.H., Jacobson, I.M., Muir, A., Al-Adhami, M., Morris, M.L., Lekstrom-Himes, J.A., Efler, S.M. & Davis, H.L. (2007). Phase 1B, randomized, double-blind, dose-escalation trial of CPG 10101 in patients with chronic hepatitis C virus. *Hepatology* **46**, 1341–1349.
282. Gish, R.G., Arora, S., Rajender Reddy, K., Nelson, D.R., O'Brien, C., Xu, Y. & Murphy, B. (2007). Virological response and safety outcomes in therapy-naïve patients treated for chronic hepatitis C with taribavirin or ribavirin in combination with pegylated interferon alfa-2a: A randomized, phase 2 study. *Journal of Hepatology* **47**, 51–59.
283. Hirata, Y., Sudoh, M. & Kohara, M. (2008). Suppression of hepatitis C virus with the reagent targeting host factors. [In Japanese] *Uirusu* **58**, 207–213.
284. Krawczyk, M., Wasowska-Lukawska, M., Oszczapowicz, I. & Boguszewska-Chachulska, A.M. (2009). Amidinoanthracyclines — a new group of potential anti-hepatitis C virus compounds. *Biological Chemistry* **390**, 351–360.
285. Pawlotsky, J.M. & McHutchison, J.G. (2004). Hepatitis C. Development of new drugs and clinical trials: Promises and pitfalls. Summary of an AASLD hepatitis single topic conference. *Hepatology* **39**, 554–567.
286. Standring, D.N., Lanford, R., Wright, T., Chung, R.T., Bichko, V., Cretton-Scott, E., Pan-Zhou, X., Bergelson, S., Qu, L., Tausek, M., Bridges, E., Moussa, A., Storer, R., Pierra, C., Benzaria, S., Gosselin, G., La Colla, P. & Sommadossi, J.P. (2003). NM 283 has potent antiviral activity against genotype 1 chronic hepatitis C virus (HCV-1) infection in the chimpanzee. *Journal of Hepatology* **38**, 3–64.
287. HCV Advocate (2007). *Newsletter May 2007*, 10pp. San Francisco, CA, USA: HCV Advocate. Available at: <http://www.hcvadvocate.org/news/newsLetter/2007/advocate0507.html> (Accessed 17.08.10).
288. Pockros, P.J. (2009). *New Frontiers in Therapy Clinical Symposium: New Direct Acting Antivirals (DAAs) in Development for HCV Infection*. Bethesda, MD, USA: Digestive Disease Week. Available at: http://www.ddw.org/user-assets/documents/PDF/01_program/2009/Handouts/Sp508%20Paul%20Pockros.pdf (Accessed 17.08.10).
289. Standring, D.N., Lanford, R., Li, B., Panzo, R.J., Seifer, M., Larsson, M., Good, S.S. & Zhou, X.J. (2009). *Antiviral Activity of the Liver-Targeted Nucleotide HCV Polymerase Inhibitor IDX184 Correlates with Trough Serum Levels of the Nucleoside Metabolite in HCV-infected Chimpanzees*. New York, NY, USA: National AIDS Treatment Advocacy Project. Available at: http://www.natap.org/2009/EASL/EASL_63.htm (Accessed 17.08.10).
290. Olsen, D.B., Carroll, S.S., Handt, L., Ludmerer, S., Graham, D., Fandozzi, C., DeLuca, L., Liverton, N., Vacca, J. & Hazuda, D. (2009). *HCV Antiviral Activity and Resistance Analysis in Chronically Infected Chimpanzees Treated with Merck NS3/4A Protease and NS5B Polymerase Inhibitors*. New York, NY, USA: National AIDS Treatment Advocacy Project. Available at: http://www.natap.org/2007/EASL/EASL_59.htm (Accessed 17.08.10).
291. Olsen, D.B., Carroll, S.S., Handt, L., Ludmerer, S., Graham, D., Fandozzi, C., DeLuca, L., Liverton, N., Vacca, J. & Hazuda, D. (2009). *Merck HCV Protease & Polymerase Inhibitor Viral Load Reduction & Resistance in Chimps*. New York, NY, USA: National AIDS Treatment Advocacy Project. Available at: http://www.natap.org/2007/EASL/EASL_14.htm (Accessed 17.08.10).
292. Anon. (2009). *The Nucleoside Inhibitor MK-0608 Mediates Suppression of HCV Replication for >30 Days in Chronically Infected Chimpanzees*. New

- York, NY, USA: National AIDS Treatment Advocacy Project. Available at: http://www.natap.org/2006/ICAAC/ICAAC_13.htm (Accessed 17.08.10).
293. Southwest Foundation for Biomedical Research (2009). *New drug technology produces marked improvement in hepatitis C therapy in animals; may be useful for a wide range of diseases*. San Antonio, TX, USA: Southwest Foundation for Biomedical Research. Available at: <http://www.sfbr.org/News/detail.aspx?id=167> (Accessed 17.08.10).
294. Lanford, R.E., Hildebrandt-Eriksen, E.S., Petri, A., Persson, R., Lindow, M., Munk, M.E., Kauppinen, S. & Orum, H. (2009). Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science, New York* **327**, 198–201.
295. Ryder, N.S. (2007). Discontinued drugs in 2006: Anti-infectives. *Expert Opinion on Investigational Drugs* **16**, 1867–1878.
296. Ryder, N.S. (2009). Discontinued drugs in 2007: Anti-infectives. *Expert Opinion on Investigational Drugs* **18**, 1–11.
297. Ryder, N.S. (2010). Discontinued drugs in 2008: Anti-infectives. *Expert Opinion on Investigational Drugs* **19**, 1–21.
298. Brown-Augsburger, P., Yue, X.M., Lockridge, J.A., McSwiggen, J.A., Kamboj, D. & Hillgren, K.M. (2004). Development and validation of a sensitive, specific, and rapid hybridization-ELISA assay for determination of concentrations of a ribozyme in biological matrices. *Journal of Pharmaceutical & Biomedical Analysis* **34**, 129–139.
299. Gonzalez-Aseguinolaza, G., Crettaz, J., Ochoa, L., Otano, I., Aldabe, R. & Paneda, A. (2006). Gene therapy for viral hepatitis. *Expert Opinion on Biological Therapy* **6**, 1263–1278.
300. SureChem (undated). *Compounds and pharmaceutical compositions for the treatment of viral infections: Publication Number: 20080286230. Example 43: Demonstration of Potent Antiviral Activity of Second Generation Nucleoside Inhibitors, B102, in HCV-Infected Chimpanzees*. London, UK: SureChem. Available at: http://www.surechem.org/index.php?Action=document&docId=1953837&db=USPTO&tab=desc&lang=&db_query=0%3A%3A0%3A%3A0%3A&markupType=all (Accessed 17.08.10).
301. Meuleman, P. & Leroux-Roels, G. (2008). The human liver-uPA-SCID mouse: A model for the evaluation of antiviral compounds against HBV and HCV. *Antiviral Research* **80**, 231–238.
302. Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L. & Kneteman, N.M. (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nature Medicine* **7**, 927–933.
303. Meuleman, P., Libbrecht, L., De Vos, R., de Hemptinne, B., Gevaert, K., Vandekerckhove, J., Roskams, T. & Leroux-Roels, G. (2005). Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* **41**, 847–856.
304. Jopling, C.L., Yi, M., Lancaster, A.M., Lemon, S.M. & Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science, New York* **309**, 1577–1581.
305. Jopling, C.L., Norman, K.L. & Sarnow, P. (2006). Positive and negative modulation of viral and cellular mRNAs by liver-specific microRNA miR-122. *Cold Spring Harbor Symposia on Quantitative Biology* **71**, 369–376.
306. ClinicalTrials.gov (2009). *Safety Study of SPC3649 in Healthy Men*. Bethesda, MD, USA: US National Library of Medicine. Available at: <http://clinicaltrials.gov/ct2/show/NCT00688012?term=SPC3649&rank=2> (Accessed 17.08.10).
307. ClinicalTrials.gov (2010). *SPC3649 Multiple Dose Study in Healthy Volunteers*. Bethesda, MD, USA: US National Library of Medicine. Available at: <http://clinicaltrials.gov/ct2/show/NCT00979927?term=SPC3649&rank=1> (Accessed 17.08.10).
308. Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H.F., Berger, U., Gullans, S., Kearney, P., Sarnow, P., Straarup, E.M. & Kauppinen, S. (2008). LNA-mediated microRNA silencing in non-human primates. *Nature, London* **452**, 896–899.
309. Elmen, J., Lindow, M., Silaharoglu, A., Bak, M., Christensen, M., Lind-Thomsen, A., Hedtjarn, M., Hansen, J.B., Hansen, H.F., Straarup, E.M., McCullagh, K., Kearney, P. & Kauppinen, S. (2008). Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Research* **36**, 1153–1162.
310. Kato, T., Matsumura, T., Heller, T., Saito, S., Sapp, R.K., Murthy, K., Wakita, T. & Liang, T.J. (2007). Production of infectious hepatitis C virus of various genotypes in cell cultures. *Journal of Virology* **81**, 4405–4411.
311. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Kräusslich, H.G., Mizokami, M., Bartenschlager, R. & Liang, T.J. (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Medicine* **11**, 791–796.
312. Rosen, H.R. & Martin, P. (2000). Hepatitis B and C in the liver transplant recipient. *Seminars in Liver Disease* **20**, 465–480.
313. Bailey, J. (2005). Non-human primates in medical research and drug development: A critical review. *Biogenic Amines* **19**, 235–256.
314. Combes, R.D., Berridge, T., Connelly, J., Eve, M.D., Garner, R.C., Toon, S. & Wilcox, P. (2003). Early microdose drug studies in human volunteers can minimise animal testing: Proceedings of a workshop organised by Volunteers in Research and Testing. *European Journal of Pharmaceutical Sciences* **19**, 1–11.
315. NCCR (undated). *Chimpanzee Management Program*. Bethesda, MD, USA: NCCR. Available at: http://www.nccr.nih.gov/comparative_medicine/chimpanzee_management_program (Accessed 17.08.10).
316. Kremsdorf, D. & Brezillon, N. (2007). New animal models for hepatitis C viral infection and pathogenesis studies. *World Journal of Gastroenterology* **13**, 2427–2435.
317. Wu, G.Y., Konishi, M., Walton, C.M., Olive, D., Hayashi, K. & Wu, C.H. (2005). A novel immunocompetent rat model of HCV infection and hepatitis. *Gastroenterology* **128**, 1416–1423.
318. Ilan, E., Arazi, J., Nussbaum, O., Zauberman, A., Eren, R., Lubin, I., Neville, L., Ben-Moshe, O.,

- Kischitzky, A., Litchi, A., Margalit, I., Gopher, J., Mounir, S., Cai, W., Daudi, N., Eid, A., Jurim, O., Czerniak, A., Galun, E. & Dagan, S. (2002). The hepatitis C virus (HCV)-Trimera mouse: A model for evaluation of agents against HCV. *Journal of Infectious Diseases* **185**, 153–161.
319. Ilan, E., Eren, R., Lubin, I., Nussbaum, O., Zauberman, A. & Dagan, S. (2002). The Trimera mouse: A system for generating human monoclonal antibodies and modeling human diseases. *Current Opinion in Molecular Therapeutics* **4**, 102–109.
320. Kneteman, N.M., Weiner, A.J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z.J., Zhu, Q., Hashash, A., Kline, J., Hsi, B., Schiller, D., Douglas, D., Tyrrell, D.L. & Mercer, D.F. (2006). Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* **43**, 1346–1353.
321. Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T. & Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nature Medicine* **4**, 1065–1067.
322. Bukh, J., Engle, R.E., Govindarajan, S. & Purcell, R.H. (2008). Immunity against the GBV-B hepatitis virus in tamarins can prevent productive infection following rechallenge and is long-lived. *Journal of Medical Virology* **80**, 87–94.
323. Woollard, D.J., Haqshenas, G., Dong, X., Pratt, B.F., Kent, S.J. & Gowans, E.J. (2008). Virus-specific T-cell immunity correlates with control of GB virus B infection in marmosets. *Journal of Virology* **82**, 3054–3060.
324. Nam, J.H., Faulk, K., Engle, R.E., Govindarajan, S., St Claire, M. & Bukh, J. (2004). *In vivo* analysis of the 3' untranslated region of GB virus B after *in vitro* mutagenesis of an infectious cDNA clone: Persistent infection in a transfected tamarin. *Journal of Virology* **78**, 9389–9399.
325. Lanford, R.E., Chavez, D., Notvall, L. & Brasky, K.M. (2003). Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia. *Virology* **311**, 72–80.
326. Xie, Z.C., Riezu-Boj, J.I., Lasarte, J.J., Guillen, J., Su, J.H., Civeira, M.P. & Prieto, J. (1998). Transmission of hepatitis C virus infection to tree shrews. *Virology* **244**, 513–520.
327. US Government Printing Office (2007). Transportation, sale, and handling of certain animals. In *Animal Welfare Act as Amended*, pp. 2131–2159. Washington, DC, USA: Government Printing Office. Available at: <http://frwebgate.access.gpo.gov/cgi-bin/usc.cgi?ACTION=BROWSE&TITLE=7USCC54> (Accessed 17.08.10).
328. Humane Research Council (2005). *U.S. Public Opinion of Chimpanzee Research, Support for a Ban, and Related Issues. Prepared for the New England Anti-Vivisection Society*. Boston, MA, USA: New England Anti-Vivisection Society (Project R&R). Available at: <http://www.releasechimps.org/2006/06/20/poll-reveals-americans-agree-chimpanzees-in-laboratories-for-more-than-10-years-should-be-retired> (Accessed 04.02.10).
329. Forns, X., Payette, P.J., Ma, X., Satterfield, W., Eder, G., Mushahwar, I.K., Govindarajan, S., Davis, H.L., Emerson, S.U., Purcell, R.H. & Bukh, J. (2000). Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV. *Hepatology* **32**, 618–625.
330. Capone, S., Zampaglione, I., Vitelli, A., Pezzanera, M., Kierstead, L., Burns, J., Ruggeri, L., Arcuri, M., Cappelletti, M., Meola, A., Ercole, B.B., Tafi, R., Santini, C., Luzzago, A., Fu, T.-M., Colloca, S., Ciliberto, G., Cortese, R., Nicosia, A., Fattori, E. & Folgori, A. (2006). Modulation of the immune response induced by gene electrotransfer of a hepatitis C virus DNA vaccine in nonhuman primates. *Journal of Immunology* **177**, 7462–7471.