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# Hepatitis C Infection and Viremia in Dutch Hemophilia Patients

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Serum samples from 316 patients visiting the Dutch National Hemophilia Center were collected from 1979 to 1993 and stored at −30°C. Patients were placed into three different groups: 1) patients ever treated with large pool non-hepatitis C virus (HCV)-safe concentrate (n = 179); 2) patients treated with cryoprecipitate (n = 125); and 3) patients treated exclusively with HCV-save concentrate (n = 12). In order to examine the prevalence of HCV infection in the different treatment groups serum samples were tested retrospectively for anti-HCV antibody using second generation enzyme-linked immunosorbent assay (ELISA) and recombinant immunoblot assay (RIBA-2). Significant differences in the prevalence of HCV infection were found between these 3 groups (group 1: 99%, group 2: 66%, group 3: 0%). The safety of currently administered clotting products is demonstrated in 57 patients who remained without HCV markers between 1989 and 1993. To examine the natural course of HCV infection fresh-frozen plasma samples were obtained recently from a subgroup of 277 hemophilia patients for HCV-RNA detection by a well-validated cDNA-PCR assay. In contrast to other reports, no evidence was found for seronegative HCV carriers. None of 52 patients without anti-HCV had detectable HCV-RNA. Of 225 patients with anti-HCV, 182 (81%) were HCV-RNA positive. None of 39 anti-HCV positive patients with a negative HCV-RNA reaction had serum alanine aminotransferase (ALT) levels above 50 U/l, whereas 44% of HCV-RNA positive patients had persistently elevated ALT levels above 50 U/l. These results indicate that 20% of hemophilia patients who have been infected with HCV in the past eliminated the virus or have viral replication below the detection limit of polymerase chain reaction (PCR) without biochemical evidence of liver damage. 

**KEY WORDS:** hepatitis C, HCV-RNA, hemophilia, prevalence

## INTRODUCTION

In most developed countries the majority of patients with hemophilia A or B have been infected with the hepatitis C virus (HCV). Nearly all patients treated with insufficiently virus-inactivated clotting products have been shown to be anti-HCV positive (Esteban et al., 1989; Brettler et al., 1990; Rumi et al., 1990; Allain et al., 1991; Lim et al., 1991; van der Poel et al., 1991; Tedder et al., 1991; Watson et al., 1992). Since the early 1980s physicians involved in hemophilia care and manufacturers of clotting products have been aware of the risk of transmission of non-A, non-B hepatitis virus (Fletcher et al., 1983; Kernoff et al., 1985). Consequently, methods were developed to inactivate this virus. In the early 1980s Behringwerke developed a pasteurization method in liquid state (10 hr at 60°C). It is now known that this method is effective for inactivating both HCV and the human immunodeficiency virus (Kernoff et al., 1987; Schimpf et al., 1987). Dry heat treatment up to 68°C appeared not to be effective in eliminating HCV transmission (Colombo et al., 1985). In contrast, viral inactivation by the so-called solvent detergent (SD) method and dry heat treatment up to 80°C have been shown to be effective (Horowitz et al., 1988; Study Group of the U.K. Haemophilia Centre Directors, 1988; Pasi et al., 1990; Pistello et al., 1991).

In The Netherlands, most patients were treated with local blood products manufactured by the Central Laboratory of The Netherlands Red Cross Blood Transfu-

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sion Service (CLB) or with cryoprecipitate produced by local blood banks. A small group (approximately 10%) of patients were treated with clotting products imported from the United States (Armour, Baxter), Austria (Immuno), or Germany (Behringwerke). Since June 1990, factor VIII concentrate produced by the CLB has been pasteurized and local blood banks produce 80°C heat-treated concentrate since 1991. Prothrombin complex concentrate has been viral-inactivated by the SD method since 1990. Since 1991 patients are no longer expected to be (re)infected with HCV because they are treated with HCV-safe clotting products only.

In a longitudinal study from 1979 to 1993, we examined retrospectively the prevalence and incidence of HCV infection in Dutch hemophilia patients in relation with the type of clotting product used. The natural course of infection in a subgroup of patients who had been exposed to HCV was also studied. The proportion of viremic and non-viremic patients was examined by testing for HCV-RNA in a recently obtained plasma sample with a well-validated cDNA-polymerase chain reaction (PCR) assay (Cuypers et al., 1992; Zaaijer et al., 1993). Because the detection limit of PCR is about 1,000 HCV-RNA molecules/ml plasma, it cannot be established whether anti-HCV positive but PCR negative patients have eliminated virus or have low viral replication below the detection limit of our PCR test (Cuypers et al., 1992). To address this issue the serum alanine aminotransferase (ALT) values and anti-HCV levels in anti-HCV positive patients were compared with and without HCV-RNA reactivity in PCR.

## MATERIALS AND METHODS

### Patients

Since 1979 serum samples were taken routinely once or twice a year from 316 patients visiting the clinic. Sequential serum samples were stored at −30°C before use for retrospective analysis of anti-HCV antibodies. Between 1991 and 1993, all patients received HCV-safe clotting preparations. During this period a fresh-frozen plasma sample for detection of HCV-RNA by cDNA-PCR was taken from a subgroup of 277 patients. Patients were subdivided into three treatment groups: group 1, patients ever treated with large pool non-virus-inactivated product (n = 179); group 2, patients treated exclusively with small pool cryoprecipitate (n = 125; after 1988 some of these patients and after 1990 all of them were treated with HCV-safe concentrate); and group 3, patients treated exclusively with HCV-safe products (n = 12).

### Assays

***Enzyme-linked immunosorbent assay (ELISA).*** All stored sequential serum samples were tested by a second generation anti-HCV ELISA (EIA-2, Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions. This assay detects anti-HCV antibodies to the non-structural proteins C33c and C100 and the structural core protein C22 (Bresters et al., 1992).

***Recombinant immunoblot assay (RIBA-2).*** Concurrently with the PCR test at least one recent sample of each patient was also tested by RIBA-2 (Ortho Diagnostics, Raritan, NJ) according to the manufacturer's instructions. RIBA-2 detects antibody reactivity to C22, C33c, C100, and 5-1-1. Following the interpretation criteria of the manufacturer, antibody reactivity against two or more recombinant antigens is considered a positive result, whereas single antibody reactivity is an indeterminate result (Ebeling et al., 1991; Da Silva Cordoso et al., 1992).

***HCV cDNA-PCR.*** Of 277 patients a recent cell-free and fresh-frozen plasma sample was obtained for HCV-RNA detection. Within 1 hr after venepuncture plasma samples were frozen and stored at −20°C before HCV-RNA detection by PCR, which has been validated extensively (Cuypers et al., 1992; Zaaijer et al., 1993).

***ALT.*** During the entire observation period serum samples were tested for ALT by an automated method two or three times a year. The upper limit of normal is 20 U/l. ALT values presented in this report were studied in the same blood samples used for PCR.

### Definition of HCV Infection

Patients were considered to be anti-HCV positive and infected with HCV if in multiple follow-up samples anti-HCV ELISA reactivity was found, which was confirmed by at least anti-C22 and/or anti-C33 reactivity in RIBA-2. We regarded isolated weak reactivity (<3+) to the C100 and/or 5-1-1 proteins to be non-specific as documented earlier in an extensive validation study of the RIBA-2 test (Bresters et al., 1992). HCV-RNA positivity was regarded to be a marker of HCV replication, whereas HCV-RNA negativity in antibody positive patients was considered to be indicative of either resolved infection or low viremia with HCV-RNA levels below the detection limit of the PCR test. Patients with ALT levels above 50 U/ml were considered to have hepatitis.

### Statistical Analysis

Seroconversion points were defined as the median time between the last anti-HCV negative sample and the first anti-HCV positive sample. Differences in the prevalence of HCV infection or in proportions of patients with HCV-RNA reactivity, elevated ALT values, or ELISA response values were compared by the chi-square test.

## RESULTS

Table I shows the proportions of anti-HCV and HCV-RNA positive hemophilia patients in the three treatment groups. The number of HCV-infected patients according to their antibody status (see Materials and Methods) was significantly higher in the patients receiving insufficiently inactivated clotting factor concentrates than in those treated with cryoprecipitate (98% vs. 66%, $P < 0.0001$). Three of 179 (1.7%) patients who had been infrequently treated with concentrates escaped HCV infection. The prevalence of HCV infection increases with age (see Fig. 1). In HCV-infected

TABLE I. Prevalence of Anti-HCV and HCV-RNA in the Three Treatment Groups of Hemophilia Patients

| Treatment groups | Anti-HCV positives/number of patients tested for anti-HCV | HCV-RNA positives/number of patients tested for HCV-RNA[a] | HCV-RNA positives/number of anti-HCV positive patients |
|---|---|---|---|
| Group 1: clotting factor concentrate | 176/179 (98%)* | 129/155 (83%) | 129/152 (85%)** |
| Group 2: cryoprecipitate | 83/125 (66%)* | 53/112 (47%) | 53/73 (73%)** |
| Group 3: HCV-safe clotting products | 0/12 (0%) | 0/10 (0%) | 0/0 |
| Total | 259/316 (82%) | 182/277 (66%) | 182/225 (81%) |

[a]All HCV-RNA positive patients were also anti-HCV positive.
*$P < 0.0001$.
**$P < 0.044$.

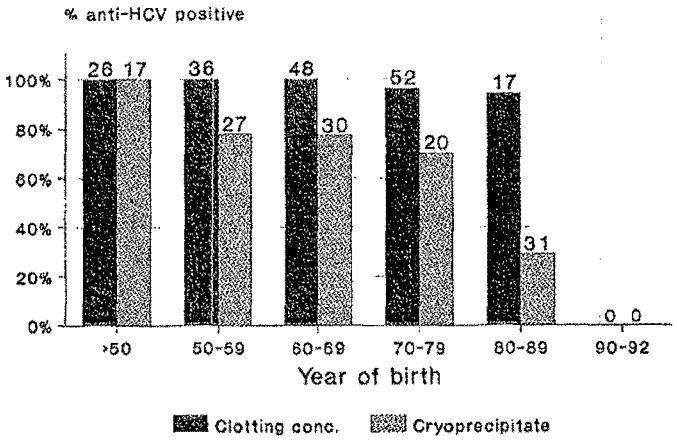


Fig. 1. Prevalence of anti-HCV as a marker for HCV infection in 304 hemophilia patients in relation to the year of birth. The number of patients in each birth year range is indicated at the top of the bars.

patients the proportion of recipients of concentrate with viremia was slightly higher than in recipients of cryoprecipitate 85% vs. 73% ($P < 0.044$). None of the anti-HCV negative patients was HCV-RNA positive. Of 214 RIBA-2 positive patients 178 (83%) were HCV-RNA positive, whereas 4 of 11 (36%) RIBA-2 indeterminate patients with isolated C22 or C33 reactivity were HCV-RNA positive. This difference between RIBA-2 positives and indeterminates is highly significant ($P < 0.0001$). Markers of HCV infection were not found in any of the 12 patients that received only HCV-safe products. The follow-up periods for this group of patients varied from 6 months to 5 years.

During the 14 year observation period the increase in prevalence of HCV infection was examined in 41 recipients of concentrate and 59 recipients of cryoprecipitate who were either anti-HCV negative at the start of follow-up or were born during the study period. In Figure 2 the increasing prevalence of HCV infection over the years is demonstrated in these two treatment groups. Figure 2 shows that in group 1 the prevalence in 1989 leveled off at 93% (38/41). In this group new infections with HCV have not been seen since then. In group 2 the prevalence of HCV infection leveled off at a significantly lower percentage (27%, 16/59; $P < 0.0001$). After 1990, one patient in this group became infected with HCV after treatment with insufficiently inactivated cryoprecipitate.

The ALT values were compared in 267 patients with and without viremia according to PCR. Ten patients were excluded from this analysis because they were either hepatitis B carriers or addicted to alcohol. The percentage of patients with ALT elevations and the distribution of ALT values in subjects with and without HCV viremia are demonstrated in Figure 3. Eight of 39 (21%) of non-viremic patients with anti-HCV antibodies had slight ALT elevations, whereas 5 of 51 (10%) of anti-HCV negative patients had slightly elevated ALT

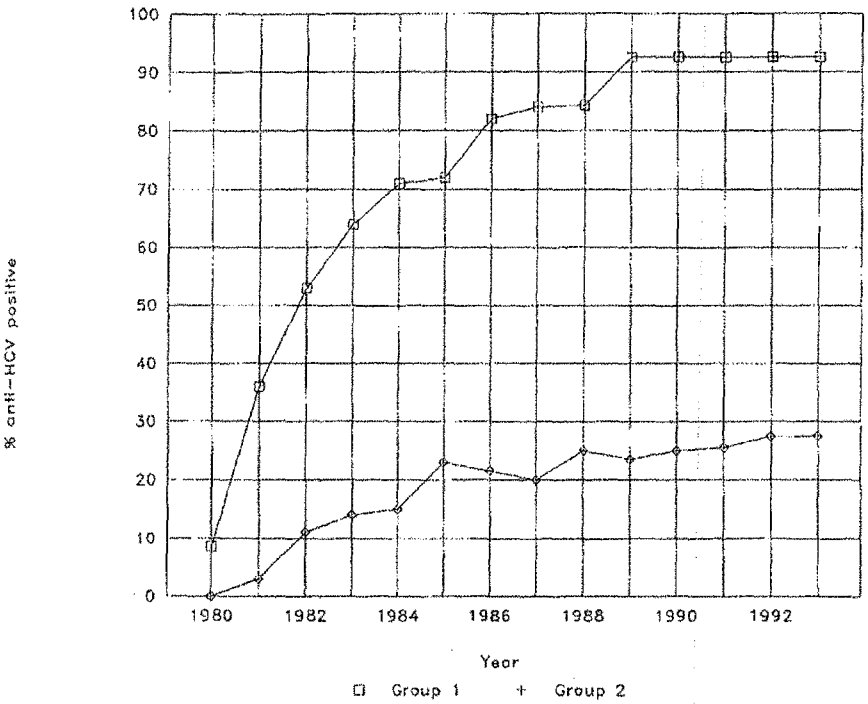


Fig. 2. Increase in prevalence of HCV infection during 14 years of observation in recipients of concentrate (group 1) and cryoprecipitate (group 2) who were anti-HCV negative at the start of follow-up.

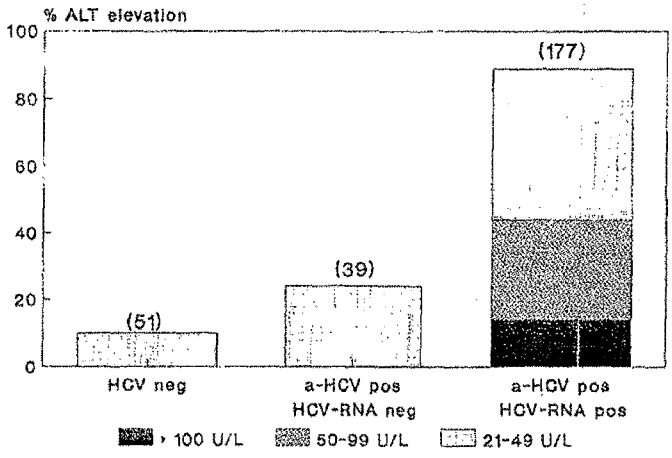


Fig. 3. ALT levels in hemophilia patients with and without HCV viremia according to PCR. The number of patients in each group is indicated at the top of the bars.

values ($P = 0.25$). In HCV-RNA positive patients the proportion of subjects with elevated ALT levels was 158/177 (89%), which was significantly higher ($P < 0.0001$) than the proportion found in HCV-RNA negative patients with anti-HCV antibodies. In viremic patients 78/177 (44%) had ALT levels above the hepati-

tis limit (50 U/l), whereas none of the HCV-RNA negative patients with serological signs of (past) hepatitis C infection had ALT levels above 50 U/l.

We also compared the anti-HCV ELISA response values (OD/cutoff ratio) in anti-HCV positive patients with and without detectable HCV-RNA. The proportion of patients with weak response values (OD/cutoff ratio below 2.0) was significantly higher ($P < 0.0001$) in nonviremic patients (9/43, 21%) than in viremic patients (0/182, 0%).

## DISCUSSION

### HCV Prevalence in Relation to Product

The overall prevalence of anti-HCV antibodies in our population of hemophilia patients is 82%. When we exclude patients who have been treated exclusively with HCV-safe clotting products, the prevalence is 85%. The differences in anti-HCV prevalence found between recipients of clotting concentrate and cryoprecipitate (99% vs. 66%) can be explained by the varying degree of exposure to viral load during the patients' lifetime. At the time of introduction of anti-HCV screening in the Dutch blood banks in 1990, 4 of 10,000 donors were found to be infected with HCV (van der Poel et al., 1991). The virus titers in plasma according to HCV-RNA detection vary between below the detection limit to $10^8$ HCV-RNA molecules/ml (Bresters et al., 1993b). Since concentrates are prepared from large pools of plasma obtained from 1,000 to 10,000 donors many of the previously produced batches must have been HCV-contaminated. Cryoprecipitate is a small pool product prepared from plasma of 2–12 donations. Consequently, the HCV infection rate after exposure to cryoprecipitate is expected to be much lower. However, when patients have been treated frequently with cryoprecipitate over a long period of time, the risk of HCV infection increases (Fig. 2). This is reflected by the 80–100% prevalence of anti-HCV antibodies found in older patients who had received cryoprecipitate exclusively (Fig. 1). Since 1980, the treatment strategy in The Netherlands has gradually changed. Patients were treated with higher dosages of factor VIII and more patients were treated with concentrate instead of cryoprecipitate. This resulted in a sharp increase in the prevalence of HCV infection in recipients of concentrate (see Fig. 2), as we observed in the group of patients who were free of infection at the start of follow-up. Nearly all patients treated with concentrate are anti-HCV antibody positive. This is in accord with the results reported by Allain et al. (1991). Since the large scale introduction of improved viral-inactivation methods such as dry heat treatment at 80°C, the solvent detergent method, and liquid pasteurization, no new HCV infections have been found during 3 years of follow-up. This is in agreement with other studies on the viral safety of these products at least as far as HCV is concerned (Schimpf et al., 1987; Horowitz et al., 1988; Study Group of the U.K. Haemophilia Centre Directors, 1988; Pistello et al., 1991).

### HCV Antibodies and HCV Viremia

In our study a subgroup of 277 patients was tested for HCV-RNA in the period that they were no longer exposed to HCV-unsafe blood products. This enabled us to examine the natural course of HCV infection in these patients. No positive HCV-RNA results were found in anti-HCV negative patients. Some investigators reported seronegative donors or patients that were HCV-RNA positive in their PCR test (Alter et al., 1992; Sugitani et al., 1992). However, false positive results are often observed in PCR methods developed locally (Zaaijer et al., 1993). In this study no evidence was found for the existence of seronegative HCV carriers. In anti-HCV positive patients 85% and 73% of recipients of concentrate and cryoprecipitate, respectively, were viremic by PCR. The small difference in the proportion of viremia between the two treatment groups may be caused by the higher (re)infection risk in concentrate recipients. Hence about 20% of HCV-infected hemophilia patients seem to have a resolved type of HCV infection. A similar percentage of HCV-RNA positives was found among RIBA-2 positive blood donors (Bresters et al., 1993a). Especially when by RIBA-2 only one solitary C22 or C33 band was found to be positive, HCV-RNA was more often negative. In many of these latter patients a decrease in ELISA response values and a loss of RIBA reactivity were observed by testing the previously drawn sequential samples (results not shown). These observations indicate that in HCV-RNA negative patients HCV infection has been resolved. Further evidence for elimination of viral replication is the lower number of patients with elevated ALT levels in the HCV-RNA negative population (see Fig. 3). Forty-four percent of HCV-RNA positive patients had ALT levels above 50 U/l, whereas none of the HCV-RNA negative patients showed ALT levels above 50 U/l.

## CONCLUSIONS

Although low-level viral replication without biochemical evidence of liver damage cannot be excluded, it is considered that about one fifth of HCV-infected patients have cleared the virus. It is recommended that HCV-RNA be examined in HCV-infected patients at intervals of 1 year.

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