

## ORIGINAL ARTICLE

# Autoantibodies against Inosine-5'-Monophosphate Dehydrogenase 2 – Characteristics and Prevalence in Patients with HCV-Infection

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### SUMMARY

**Background:** Indirect immunofluorescence (IIFT) on in house HEp-2 cell preparations revealed a novel antibody giving a granular cytoplasmic pattern not described before, which on two commercial cell preparations revealed a “rings and rods” pattern. This pattern was also observed in four HCV-RNA carriers and prompted the identification of the reactive antigen and the evaluation of the antibody prevalence in HCV-RNA carriers and control groups.

**Methods:** The antigen's molecular weight was determined by radioimmunoprecipitation of <sup>35</sup>S-methionine labeled cell proteins. Expression library screening and sequencing was performed by standard techniques using an oligo(dT)-primed human HeLa cell cDNA expression library. Antibodies against the novel antigen Inositol-5'-monophosphatdehydrogenase 2 (IMPDH2) were analyzed by IIFT, western blot, line blot, and radioimmunoprecipitation assay (RIPA). IIFT was performed on commercial HEp-2 cells and cells cultivated in house for 24 – 60 hours, with or without the IMPDH2 inhibitors mycophenolic acid (MPA) or ribavirin, and subjected to various fixation conditions. Western and line blots were performed with IMPDH2 synthesized in E. coli, RIPA with <sup>35</sup>S-methionine-IMPDH2 from in vitro transcription/translation products. Sera screened were positive for HCV-RNA (108), HBV-DNA (100), anti-mitochondrial (31), anti-actin (42), and anti-nuclear antibodies (51) and negative for HCV-RNA (100) and blood donors (100).

**Results:** IMPDH2 is capable of considerable intracellular rearrangements (upon action of inhibitors like MPA and ribavirin), which explains the contrasting immunofluorescence patterns in cells from different sources. By RIPA, proven to be the sole assay suitable for screening of anti-IMPDH2 in human sera, autoantibodies were found in 35.2 % of HCV-RNA carriers and in low concentrations in 31 % of anti-actin positive patients suspicious of autoimmune hepatitis. Antibodies reacted preferentially with conformational epitopes. Compared to the low concentration of anti-IMPDH2 found in other disease groups, high antibody concentrations were observed in HCV-RNA carriers.

**Conclusions:** The common occurrence of anti-IMPDH2 in HCV-RNA carriers may be related to ribavirin therapy, causing intracellular aggregation of IMPDH2 thereby altering its immunogenicity. In this study the “rods and rings” immunofluorescence pattern observed could be ascribed to anti-IMPDH2. Anti-IMPDH2 may cause difficulties in interpretation of immunofluorescence patterns in routine autoantibody testing.

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### ABBREVIATIONS

IMPDH2: Inosine-5'-Monophosphate Dehydrogenase 2 (NCBI Acc. No. NM\_000884, GI: 66933015, Swiss-Prot. No.: P12268). According to the NCBI nomenclature the adenine nucleotide of the first methionine en-

coding ATG is defined as position +93). ABR: antibody ratio.

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## INTRODUCTION

In the course of routine screening for autoantibodies against nuclear antigens by means of indirect immunofluorescence test (IIFT) with HEp-2-cells, we detected in the serum of a female patient an unusual cytoplasmic staining not described before. The antigenic target of this reaction was identified, by screening an expression library, as type 2 inosine-5'-monophosphate dehydrogenase (1) the key enzyme in the synthesis of purine nucleotides. Inosine-5'-monophosphate dehydrogenase (IMPDH), catalyzes the transition of inosine monophosphate to xanthine monophosphate, which constitutes the rate limiting step in the *de novo* synthesis of guanine nucleotides, precursors of RNA and DNA. In mammalian species there exist two isoforms, IMPDH1 and IMPDH2 (2), each of which is composed of 514 amino acids (55.8 kDa) with a homology of 84 % on amino acid level. Whilst IMPDH1 is constitutively expressed in normal cells, the expression and activity of IMPDH2, however, is augmented in proliferating normal and malignant tissues and cells, in malignant transformation, and differentiation (3–9). The possible occurrence of autoantibodies against IMPDH2 was reported in the serum of one out of 21 patients with autoimmune hepatitis (10), studied by immunoproteomic analysis of HepG2 cell proteins. Contemporaneously to our findings it has been shown, that IMPDH2 probably acts as a tumor associated antigen (11). Our patient, however, showed no evidence of a disease related to cell proliferation or neoplasm nor could her clinical symptoms be associated to the presence of autoimmune hepatitis or another autoimmune disease. Subsequently, we detected IMPDH2 autoantibodies by IIFT in four patients who suffered from chronic hepatitis C virus (HCV) infections. HCV infection may be involved in the pathogenesis of various autoimmune and rheumatic disorders and chronically infected HCV patients have been reported to produce a plethora of autoantibodies (12–17). We determined the presence of anti-IMPDH2 in patients with HCV infections, hepatitis B virus (HBV) infections, autoimmune liver diseases, and rheumatic diseases.

Anti-IMPDH2 was detected in patients harboring HCV infections and autoimmune liver diseases. We could show that the so-called “rods and rings” fluorescence pattern (18), which sometimes can be seen within the cytoplasm of some commercial HEp-2 cell preparations used for screening of autoantibodies, is caused by anti-IMPDH2. This finding may be important particularly with regard to considerations concerning the pathogenesis of IMPDH2 autoantibodies since it has been shown that the IMPDH2 inhibitor mycophenolic acid causes reversible intracellular aggregations of IMPDH2 molecules (19), which impress as “rods and rings” shaped structures. As could be shown here, ribavirin, an inhibitor of IMPDH2 used in treatment of HCV infections, also causes an aggregation of IMPDH2 in “rods and rings” structures. Possibly the aggregated enzyme attains a higher degree of immunogenicity, promoting the

development of autoantibodies in patients treated with IMPDH2 inhibitors. The preferred origination of autoantibodies against constituents of subcellular aggregates and particles is debated and evidenced since more than two decades (20–24).

## MATERIALS AND METHODS

### Patient and control sera

The index patient was a 71-year-old female (HCV-RNA negative), suffering for 15 years from extended osteoporosis who underwent pain management therapy because of major osteoporotic ache. To exclude systemic rheumatic disease, a search for antinuclear antibodies was performed with a negative result. However, the current investigation by indirect immunofluorescence tests (IIFT) revealed a distinct cytoplasmic staining in HEp-2-cells not attributable to a known autoantibody. The serum precipitated a 55 kDa protein from <sup>35</sup>S-methionine labeled HEp-2 cell proteins and an expression library was screened, which revealed IMPDH2 as the reactive antigen.

To establish the prevalence of anti-IMPDH2, we screened (by IIFT, immunoblot, and radioimmunoprecipitation assay (RIPA)) the sera of patients positive for HCV-RNA (n = 108) and of disease controls (hospitalized patients) negative for HCV-RNA (n = 100), sera of patients positive for HBV-DNA (n = 113), with mitochondrial antibodies (AMA Type M2, suspected primary biliary cirrhosis; n = 31) or actin antibodies (suspected autoimmune hepatitis; n = 42), with overt or suspected rheumatic diseases showing antibodies against nuclear antigens (n = 51) with different nuclear staining patterns (homogeneous, speckled, nucleolar, rim), and the sera of healthy blood donors (n = 100).

Screening of autoantibodies (actin, tubulin, mitochondria, microsomes, ribosomes, lysosomes, endosomes, tRNA synthetases, Golgi proteins), HCV-RNA, and HBV-DNA was performed by approved, CE labeled in house tests according to Quality Systems of the Council Directive 98/79/EC. All samples were coded and analysed for the presence of anti-IMPDH2 without knowledge of virus and autoantibody status.

### cDNA library screening and synthesis of recombinant His(6)-tag IMPDH2 in E. coli

Expression library screening and sequencing was performed by standard techniques using an oligo(dT)-primed human HeLa cell cDNA expression library in Lambda ZAP (Stratagene, Amsterdam, The Netherlands) in conjunction with the index patient's serum. Following two rounds of screening, three clones were selected as positive and subjected to sequence analysis using ABI Prism 3130XL and the Cycle Sequencing Kit Big dye Terminator from Applied Biosystems (Darmstadt, Germany). Sequence homologies were identified using the NCBI BLAST program.

Full length IMPDH2 containing a His(6)-tag was syn-

thesized in *E. coli* BL21 Star cells (NovageneLlc) according to standard techniques and purified from inclusion bodies by  $\text{Ni}_2^+$ -agarose (Quiagen, Hilden, Germany). Purity was checked after sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5 % gels stained with Coomassie Brilliant Blue.

### Rabbit antibodies against IMPDH2

Rabbit anti-IMPDH2 were either obtained commercially (Sigma, Munich, Germany) containing 0.07 mg/mL anti-IMPDH2 (anti-IMPDH2<sub>si</sub>) or produced in house by immunization of a female New Zealand White rabbit (Charles River Laboratories, Kisslegg, Germany) with 0.3 mg His(6)-tag-IMPDH2 in PBS followed by 3 injections (0.3 mg protein) within 2.5 months (anti-IMPDH2<sub>ih</sub>). Antibody synthesis was monitored on line blots spotted with His(6)-tag-IMPDH2.

### Antibody detection

#### Indirect immunofluorescence test (IIFT)

For antibody screening by IIFT, HEp-2 cells were cultivated on glass microscope slides for 20 hours and fixed in methanol -20 °C, 5 minutes, acetone 20 °C, 1 minute. They were incubated with patient serum diluted 1:80 in PBS as described (25). For detection of bound antibodies FITC labeled rabbit anti-human-IgG, Fc specific (Invitrogen, Karlsruhe, Germany) was used in optimal dilutions determined by checker board titration. The serum of the index patient was also screened on seven different commercial HEp-2 cell preparations (Inova Diagnostics, San Diego, CA, USA; Orgentec, Mainz, Germany; Euroimmun, Lübeck, Germany; Menarini, Berlin, Germany; Generic Assays, Berlin, Germany; AESKU, Wendelsheim, Germany; AID, Straßberg, Germany).

To explore a possible influence of culture time or kind of cell fixation on the intracellular localization of IMPDH2, HEp-2 cells were harvested after growing periods of 16 – 60 hours and fixed with acetone (-20 °C, 5 minutes), methanol (-20 °C, 5 minutes), ethanol-glacial acetic acid (95:5 [vol:vol], 4 °C, 5 minutes), methanol-acetone (1:1 [vol:vol], -20 °C, 5 minutes), methanol (-20 °C, 5 minutes, permeabilization by acetone at 20 °C, 1 minute) methanol-ethanol (1:1 [vol:vol], -20 °C, 5 minutes), formalin (4 %, 4 °C, 10 minutes), paraformaldehyde (4 %, 20 °C, 10 minutes, permeabilization by Triton X-100, 0.5 %, 20 °C, 10 minutes), paraformaldehyde (4 %, 20 °C, 10 minutes, and methanol (-20 °C, 5 minutes), ethanol (95 %, 20 °C, 5 minutes).

The effect of either mycophenolic acid or ribavirin on the intracellular localization of IMPDH2 was monitored 24 hours after addition of 12.49 µmol mycophenolic acid (Sigma) or 16.38 µmol ribavirin (Sigma) to the cell culture media.

### Radioimmunoprecipitation of <sup>35</sup>S-methionine labeled cell proteins

HEp-2 cells ( $5 \times 10^6$ ) in methionine-free medium (RPMI1640, Sigma) were incubated with 25 µL L-<sup>35</sup>S-methionine (PerkinElmer, Rodgau, Germany) for 16 hours, washed (3 x in 10 mL ice cold PBS), and incubated with 3 mL of ice-cold RIP1 buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 % Igepal, 2 mM PMSF) for 20 minutes. Cells were harvested, sonified on ice (Soni-fier Cell Disruptor B15; Branson, Danbury, CT, USA) for 3 x 30 seconds at output level 7, pulsed, 55 % duty cycle and centrifuged (4000 x g, 10 minutes). Aliquots of the supernatant containing labelled cell proteins were stored frozen at -80 °C until used.

The patient's serum and well characterized control sera (20 µL each) were incubated with 30 µL protein-A coated magnetic beads (Invitrogen, Darmstadt, Germany) for 4 hours on a rotating mixer at 4 °C. After washing (4 x 700 µL RIP1 buffer) 100 µL <sup>35</sup>S-methionine labelled HEp-2 cell proteins (10<sup>7</sup> cpm) were added and incubated on a rotating mixer (16 hours, 4 °C). After washing as stated above the beads were heated (95 °C, 5 minutes) in 30 µL sampling buffer (10 mmol Tris-HCl, pH 6.8, 2 % SDS, 2.5 % β-mercaptoethanol, 10 % glycerol, 0.1 %, bromophenol blue). After brief centrifugation (10000 x g) supernatants were subjected to 9 % SDS-PAGE followed by autoradiography using Hyperfilm MP films (GE Healthcare, Munich, Germany) for 24 – 48 hours.

### Immunoblot

(1) Western blots: Separation of IMPDH2 on 12.5 % SDS-PAGE was done as described (26) in a Mini Protean II electrophoresis chamber (BioRad, Munich, Germany). Per lane 0.2 – 0.5 µg purified His(6)-tag-IMPDH2 or commercially available IMPDH2 (0.13 mg/mL in 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT; Sigma) were applied. After electrophoresis, proteins were transferred to nitrocellulose Protran BA85 membranes (VWR International, Darmstadt, Germany). Membranes were blocked in blocking buffer for 0.5 hours and after washing 3 times according to Towbin et al. (27) incubated with human or rabbit sera diluted 1:100 in blocking buffer for 1 hour, washed 3 times in wash buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Triton X-100) and consecutively incubated with alkaline phosphatase conjugated goat anti-human immunoglobulins (Dianova, Hamburg, Germany) or goat anti-rabbit immunoglobulins (Dianova). After washing 3 times in wash buffer, bound antibodies were visualized using BCIP with NBT as substrate.

(2) Line blots were prepared by using a Nanoplotter 2.0 (GeSiM, Großberkmannsdorf, Germany) with a four channel print head and applying different amounts of IMPDH2 on nitrocellulose BA85 membranes. After spotting, membranes were blocked as described above and cut in strips of 3 mm width. Routinely, protein solutions with concentrations between 12.5 µg/mL and 100 µg/mL in 0.1 mol borate buffer, pH 9.0 were used for

spotting, resulting in four lanes containing 4, 8, 16 or 32 ng IMPDH2 per strip. Membranes were blocked and processed as described above. Human and rabbit sera to be tested were diluted 1:100 in blocking buffer.

#### Radioimmunoprecipitation assay (RIPA)

For production of radiolabelled IMPDH2-tracer, the DNA sequence encoding amino acids 1 - 514 of human IMPDH2 was cloned into a pCITE4a vector (Novagen). In vitro transcription/translation (ivTT) was performed using a T7 promoter controlled coupled transcription/translation system based on rabbit reticulocyte lysate (Promega, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 25  $\mu$ L rabbit reticulocyte lysate, 2  $\mu$ L reaction buffer, 1  $\mu$ L amino acid mixture (without methionine), 1  $\mu$ L T7 polymerase, 3.5  $\mu$ L L-<sup>35</sup>S-methionine (PerkinElmer), 1  $\mu$ L RNasin (40 U; Lonza, Verviers, Belgium) and 1  $\mu$ g template DNA were pipetted into a nuclease free 1.5 mL reaction tube. Nuclease free water was added to a final volume of 50  $\mu$ L. After 90 minutes incubation at 30 °C, unincorporated <sup>35</sup>S-methionine was removed on a Sephadex G-50 MicroSpin column (GE Healthcare). Aliquots were stored at -80 °C until used. The percentage of <sup>35</sup>S-methionine incorporated into the protein was calculated after liquid scintillation counting. Mean incorporation rates were 9.5 % (SD 2.5 %). The quality of the <sup>35</sup>S-methionine-IMPDH2 was checked on SDS-PAGE (12.5 %, 200 V), sample preparation and autoradiography was performed as described above.

For antibody screening, <sup>35</sup>S-methionine labelled IMPDH2 (tracer) was diluted in dilution buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.15 % Tween-20, 100000 KIE Aprotinin [Bayer, Leverkusen, Germany], 10 mM benzamidine, 0.1 % BSA) to an activity of 40000 cpm in 50  $\mu$ L. Duplicates of patient and control sera (5  $\mu$ L) were pipetted into the wells of a 96-well microtiter plate. 50  $\mu$ L tracer were added to each well. After incubation for 3 hours at 4 °C on a plate shaker, 20  $\mu$ L recombinant protein A bound to fractogel (Merck, Darmstadt, Germany) were added (4 °C, 60 minutes). The reaction mixes were transferred to a 0.65  $\mu$ m Durapore 96-well filter plate (Millipore, Schwalbach, Germany). Unbound antigen was removed by vacuum filtration and washing the filter plates 30 times with 100  $\mu$ L RIP2 buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05 % Tween-20). After drying the plates overnight, 20  $\mu$ L liquid scintillation mix MicroScintO (PerkinElmer) were added to each well and the plates were counted using a Topcount NXT 96-well liquid scintillation counter (PerkinElmer).

All assays were performed in duplicate. Five negative controls (healthy blood donors) were used to calculate the antibody concentrations (antibody ratio, ABR) according to the following formula: Antibody ratio (ABR) = (cpm (sample)/[mean cpm (negative controls) + 3.041 SD (negative controls)]) x 10 (28). Sera positive for anti-IMPDH2 were retested with a homologous RIP assay, used for determination of antibodies against ste-

roid-21-hydroxylase to exclude a possible non-specific binding of the tracer.

#### Statistical methods

Receiver operating characteristic (ROC) curve analysis was conducted to determine the cut-off range between sera regarded as positive or negative for anti-IMPDH2. Each of the patient groups was compared with the control group of healthy blood donors. The specificity was regarded to be 98 %. The area under the ROC curve (AUC) with 95 % confidence interval (CI) was calculated from the AUC, SE, CI according to Hanley (29).

## RESULTS

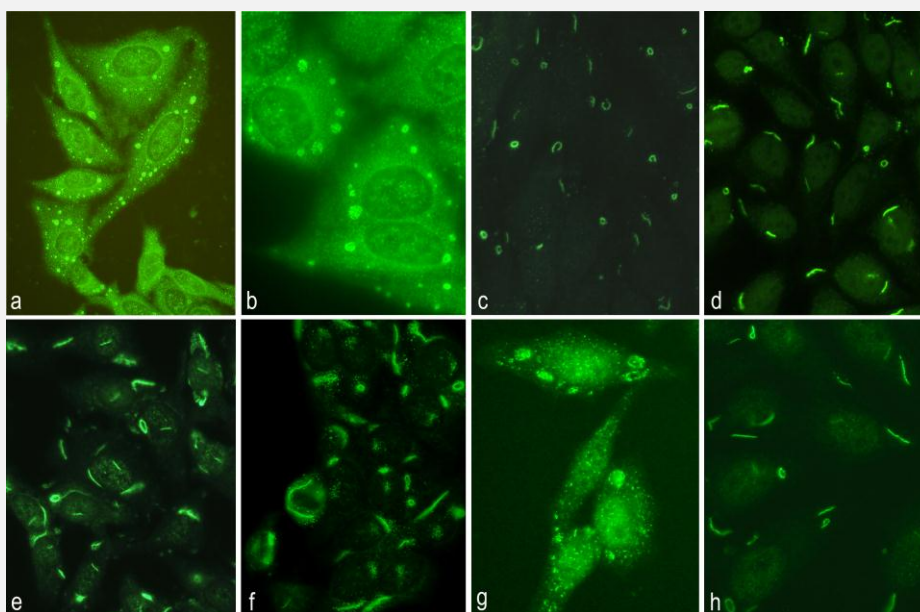
#### Different immunofluorescence patterns evoked by patient serum

The serum of the index patient screened on in house cultivated HEp-2 cells exhibited an intense staining of distinct granular particles diffusely dispersed within the cytoplasm (Figure 1, a, b). In many cells the granules appeared as condensed globular-shaped aggregates reaching numbers from one per cell up to more than a dozen (antibody titer 1:1280). Only a very weak granular cytoplasmic fluorescence could be detected in five different commercial HEp-2 cell preparations (Orgentec, Menarini, Generic Assays, AESKU, AID), indicating that these cell preparations actually are not suitable for the identification of anti-IMPDH2 in human sera.

A rather different immunofluorescence pattern, however, was obtained when the patient's serum was reacted with two other commercial HEp-2 cell preparations (Inova Diagnostics, Euroimmun). Instead of a granular staining within the cytoplasm, one to three thin, smooth fluorescent filaments and/or one to three small plain rings with central apertures (Figure 1c, 1d) were seen. This uncommon fluorescence pattern was only recently described as "rods and rings" pattern in the context of proficiency tests for antinuclear antibodies (18). Possible antibody specificities associated with this pattern, however, were not identified.

Subsequently, sera of four additional patients with this pattern of reactivity (granular within the cytoplasm on in house and "rods and rings" on the commercial HEp-2 cell preparations (Euroimmun, Inova Diagnostics)) were found. Examination of the clinical data revealed that all four patients had past or ongoing HCV infections, which prompted the exploration of the prevalence of these antibodies in HCV-RNA positive patients.

To examine whether the two different fluorescence patterns evoked by the same serum depended on variations in culture conditions or cell fixation, HEp-2 cells cultivated between 16 and 60 hours and thereafter treated with various fixatives as described above, were reacted with the patient's serum. However, neither culture time nor kind of fixative could be linked to a possible transition of the two contrasting staining patterns. There was



**Figure 1.** IIFT of index patient's serum: a, b: In house cultivated HEp-2 cells. Anti-IMPDH2 reacts with distinct cytoplasmic granules and condensed granular aggregates. c, d: Two commercial HEp-2 cell preparations (c: Euroimmun, d: Inova Diagnostics) exhibiting the "rods and rings" fluorescence pattern. e, f: In house cultivated HEp-2 cells addition of (e) mycophenolic acid or (f) ribavirin into the culture medium. Both mycophenolic acid and ribavirin shift the granular fluorescence pattern (seen in a and b) to "rods and rings". g, h: IIFT of rabbit anti-IMPDH2<sub>si</sub> on (g) in house cultivated and (h) commercial (Inova Diagnostics) HEp-2 cells, exhibiting identical fluorescence patterns as seen with the serum of the index patient. Zeiss Axioplan 2, magnifications 400 x.

only a slight increase of granular aggregates within the cytoplasm of cells cultivated for prolonged periods (>20 hours).

#### IMPDH2 constitutes the target antigen

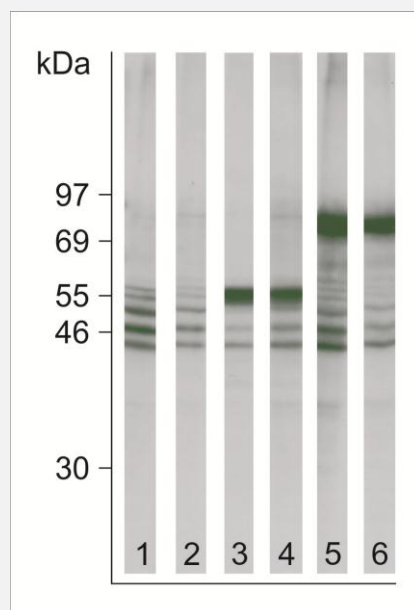
The immunofluorescence pattern observed with the index patient's serum could not be attributed to antibodies against one of the major known cytoplasmic autoantigens (actin, tubulin, mitochondria, microsomes, ribosomes, lysosomes, endosomes, tRNA synthetases, Golgi proteins) either by IIFT or by appropriate antibody specific assays. However, the serum precipitated a substantial amount of a 55 kDa <sup>35</sup>S-methionine-labeled protein from HEp-2 cell extract (Figure 3). Screening a cDNA expression library revealed three clones encoding IMPDH2 and spanning the complete coding region of 1545 nucleotides including 74 nucleotides of the 5'-untranslated region and 52 nucleotides of the 3'-untranslated sequence (positions 19 to 1689).

Direct confirmation of IMPDH2 as target antigen was made by the positive reaction of the index patient's serum with recombinant IMPDH2 in western and line blots (Figures 3a, 3b) and with <sup>35</sup>S-methionine-IMPDH2 in RIPA (Figure 4). As indirect confirmation, a

commercial rabbit anti-IMPDH2 (anti-IMPDH2<sub>si</sub>) reacted essentially in the same way in IIFT as the index patient's serum (Figure 1g, 1h). The in house rabbit anti-IMPDH2 (anti-IMPDH2<sub>ih</sub>) irrespective of its reactivity with His(6)-tag-IMPDH2 (Figure 3a, lane 7), commercial IMPDH2 and <sup>35</sup>S-methionine-IMPDH2 (Figure 4) was, however, unable to recognize IMPDH2 in any preparation of HEp-2 cells. This observation suggests that this antibody was unable to recognize epitopes being presented by intracellular IMPDH2 (Table 1).

#### Mycophenolic acid and ribavirin rearrange intracellular IMPDH2

The reason for the different arrangements of IMPDH2 observed within the cytoplasm of in house cultivated HEp-2 cells and the two commercial preparations is unknown. It may be caused by additives within the culture media, not disclosed by the manufacturers. As could be shown recently (19), addition of mycophenolic acid to culture cells induced a rearrangement of cytoplasmic IMPDH2 from granular particles into the "rods and rings". We could show this transition of IMPDH2 after addition of therapeutic concentrations (12.49 μmol) of



**Figure 2.** Radioimmunoprecipitation of  $^{35}\text{S}$ -methionine labeled HEP-2 cell proteins by the serum of the index patient (lanes 3, 4) precipitating a substantial amount of a 55 kDa protein. Control sera from two healthy blood donors (lanes 1, 2) and from two patients harboring antibodies against threonyl tRNA synthetase (PL7) with a calculated molecular weight of 82.1 kDa (lanes 5, 6). SDS page followed by autoradiography for 24 hours.

mycophenolic acid to the cell cultures (Figure 1e). Because of the suspected prevalence of anti-IMPDPH2 antibodies in patients with HCV infections and the therapeutic use of another IMPDPH2 inhibitor, ribavirin, in these patients, we were also interested, whether ribavirin can cause an identical rearrangement of cytoplasmic IMPDPH2. As can be seen from Figure 1f, ribavirin in concentrations of therapeutic serum levels (16.38  $\mu\text{mol}$ ) also induces a prompt rearrangement of IMPDPH2 in “rods and rings” in cultivated HEP-2 cells studied 24 hours after addition.

#### **Evaluation of possible assays for detection of anti-IMPDPH2 in human sera**

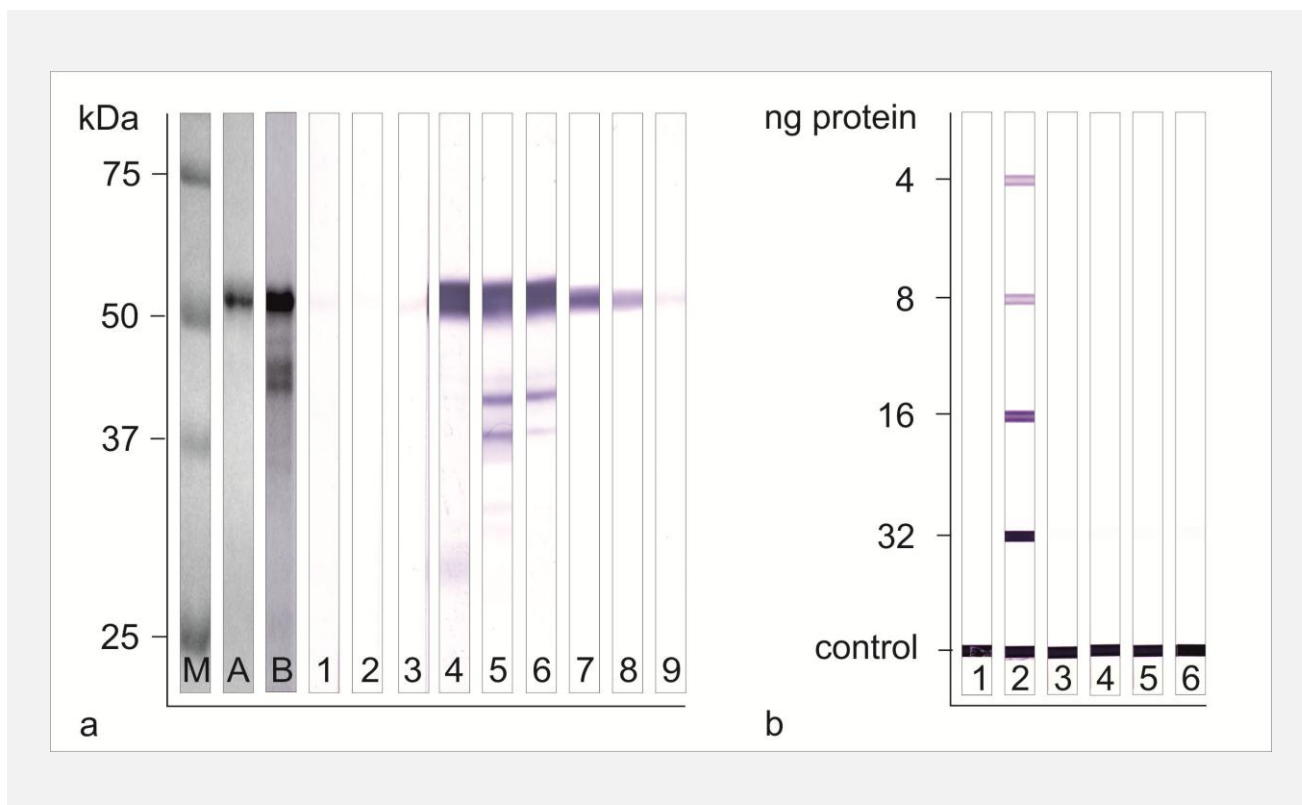
Searching for an appropriate assay for screening of anti-IMPDPH2 in human sera, the following approaches were followed:

First, His(6)-tag-IMPDPH2, synthesized in *E. coli* and affinity purified on  $\text{Ni}_2^+$ -agarose was used for western and line blots. Second, full length  $^{35}\text{S}$ -methionine labeled IMPDPH2 was synthesized by in vitro transcription/translation and applied in RIPA.

As shown by SDS-PAGE (Figure 3a, lane A) the purity of His(6)-tag-IMPDPH2 was >95 %, comparable to that of a commercially available recombinant enzyme. The electrophoretic mobility corresponded well with the calculated molecular weight of 55 kDa.

Blotted on nitrocellulose (Figure 3a, lanes 1 - 9), His(6)-tag-IMPDPH2 was reactive with the index patient's serum (lane 5), rabbit anti-IMPDPH2<sub>si</sub> (lane 7), rabbit anti-IMPDPH2<sub>ih</sub> (lane 8) and rabbit anti-His(6)-tag (lane 9) but not with sera of three healthy blood donors (lanes 1 - 3) and a serum of a HCV-RNA positive but IIFT negative patient (lane 4). As compared to the index patient (lane 5), the serum of one of the four HCV-RNA positive patients (lane 6) exhibiting the same two-tiered immunofluorescence pattern, suggestive of anti-IMPDPH2, showed a rather weak reactivity irrespective of IIFT antibody titer comparable to that of the index serum (1:1280). Identical results were obtained with a commercial IMPDPH2 preparation, except for a missing reaction with anti-His(6)-tag. The disparity of reaction strength of the index serum and that of the HCV-RNA positive patient seen in Figure 3a (lanes 5, 6) is also related to the recombinant commercial antigen lacking the His(6)-tag.

Applying this kind of western blot for screening of anti-IMPDPH2 in 108 HCV-RNA positive patients only one borderline and two weakly positive reacting sera could be detected. As revealed by IIFT and RIPA (vide infra) one of these two sera (Figure 3a, line 6) showed the typical anti-IMPDPH2 immunofluorescence pattern (titer 1:1280) and a positive RIPA (ABR 87.7). The second serum and the borderline one, however, were nonreac-



**Figure 3. a:** SDS-Page of His(6)-tag-IMPDH2 (lane A) and <sup>35</sup>S-methionine labeled IMPDH2 synthesized by in vitro translation/transcription (lane B) for purity assessment (lane M = molecular weight markers). Lanes 1 – 9 depict His(6)-tag-IMPDH2 blotted on nitrocellulose and reacted with control sera of three healthy blood donors (lanes 1-3), serum of a HCV-RNA positive patient with negative immunofluorescence staining (lane 4), serum of index patient (lane 5), serum of a HCV-RNA positive patient showing an immunofluorescence pattern suspicious of anti-IMPDH2 (lane 6), rabbit anti-IMPDH2<sub>ih</sub> (lane 7), rabbit anti-IMPDH2<sub>si</sub> (lane 8), and rabbit anti His(6)-tag (lane 9). The purity of the His(6)-tag-IMPDH2 (lane A) is >95 %. Whereas the reaction of the index patient (lane 5) is rather strong, the reaction with the other IIFT positive patient (lane 6) is rather weak.

<sub>ih</sub> = in house, <sub>si</sub> = Sigma.

**b:** Line blots containing four lines of 4, 8, 16, and 32 ng spotted His(6)-tag-IMPDH2 respectively and reacted with serum of a blood donor (lane 1), serum of the index patient (lane 2), and sera of HCV-RNA positive patients showing an immunofluorescence pattern suspicious for anti-IMPDH2 (lanes 3 – 6). The control line contains spotted anti-human IgG. Only the index patient reacts with the spotted His(6)-tag-IMPDH2. No reaction was seen with the 4 other sera, also containing anti-IMPDH2 as demonstrated by RIPA (Figure 4).

tive in IIFT as well as in RIPA.

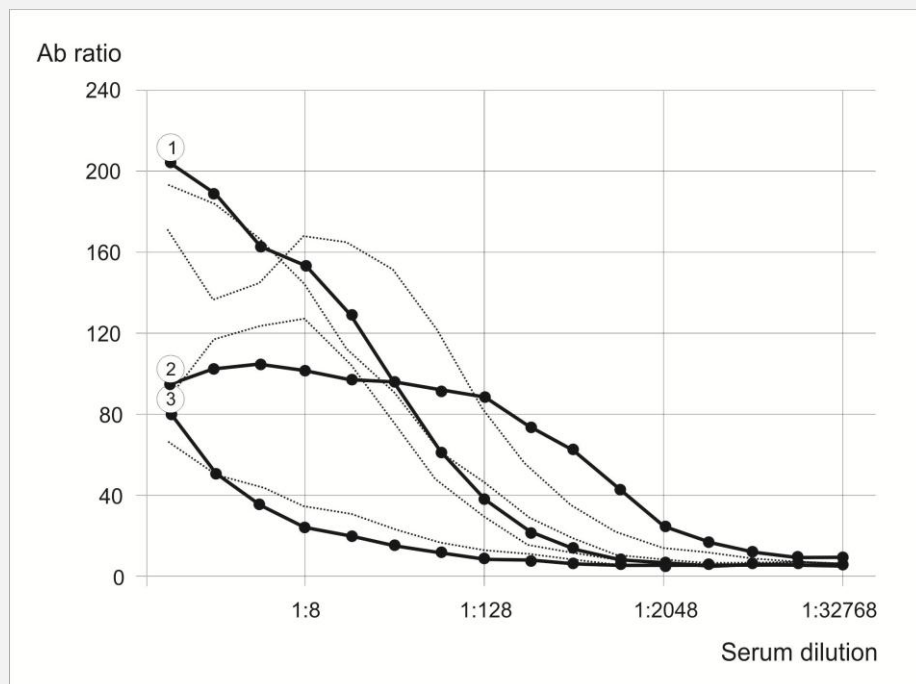
The disparity of the reaction strength of His(6)-tag-IMPDH2 with the serum of the index patient and that of IIFT and HCV-RNA positive patients increased considerably when using line blots. Whereas an unchanged strong reaction was revealed by the index patient (Figure 3b, lane 2), neither the four HCV-RNA positive sera detected by immunofluorescence (lanes 3 – 6) nor any one of the other 104 HCV-RNA containing sera reacted with spotted IMPDH2 in line blots.

The disappointing results obtained with western and line blots prompted the design of a RIPA using <sup>35</sup>S-methionine labeled IMPDH2 produced by in vitro transcription/translation. Using this kind of antigen not only the index patient's serum but also the four HCV-RNA positive sera, depicted in Figure 3b (lanes 3 - 6) as well

as the two rabbit sera (anti-IMPDH2<sub>si</sub>, anti-IMPDH2<sub>ih</sub>) precipitated <sup>35</sup>S-methionine-IMPDH2 in a dose dependent manner (Figure 4). This data strongly suggests that the RIPA is more suitable for anti-IMPDH2 screening in human sera than western or line blots. A summary of the reactivity of the various antigens with human sera and rabbit antibodies under different test conditions is given in Table 1.

#### High prevalence of anti-IMPDH2 in HCV-RNA carriers

To evaluate the prevalence of anti-IMPDH2 in HCV-RNA carriers, 108 HCV-RNA positive sera were screened by RIPA and compared with sera of patients negative for HCV-RNA, patients suffering from HBV infections, autoimmune liver disease, and autoimmune



**Figure 4.** RIPA of serial dilutions of the serum of the index patient (2), of rabbit anti-IMP2<sub>Si</sub> (1), rabbit anti-IMP2<sub>ih</sub> (3) and of four HCV-RNA positive sera (thin dotted lines) showing an immunofluorescence pattern suspicious of containing anti-IMP2H2. Rabbit sera were diluted with preimmune serum, human sera with that of a blood donor. All sera show a dose-dependent precipitation of <sup>35</sup>S-methionine-IMP2H2.

rheumatic diseases and healthy controls. The cut-off between anti-IMP2H2 positive and negative patients was determined by receiver operator curves of 108 HCV-RNA carriers and 100 healthy blood donors (Figure 5). The area under the curve (AUC) was calculated 0.762 representing fairly good test conditions. The 95 % confidence interval was 0.698 to 0.819. Defining an assay specificity of 98 % the cut off was calculated at an antibody ratio (ABR) of 10.5. Under these settings anti-IMP2H2 could be detected in HCV-RNA positive sera with a sensitivity of 35.2 %. However, there was no correlation between HCV-virus load and the presence or absence of anti-IMP2H2 in HCV-RNA carriers. The mean virus load in anti-IMP2H2 positive HCV-RNA-carriers was  $1.4 \times 10^6$  IU/mL, mean virus load in anti-IMP2H2 negative HCV-RNA carriers was  $1.6 \times 10^6$  IU/mL. There was also no correlation between the concentration of anti-IMP2H2 (given as antibody ratio) and virus load ( $R^2$  of the linear regression = 0.0026).

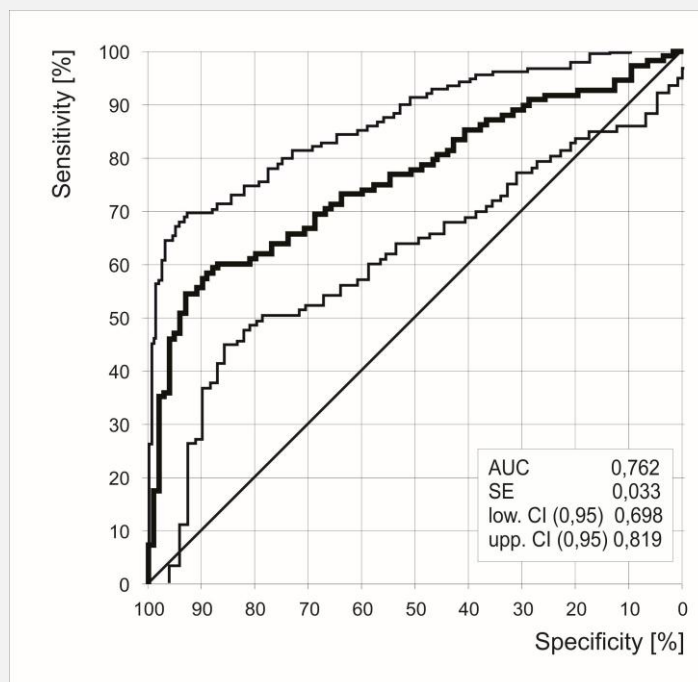
As depicted in Figure 6, anti-IMP2H2 also could be detected in blood donors (2.0 %), HCV-RNA-negative patients (5.0 %), HBV-DNA positive patients (6.2 %), in patients positive for antinuclear antibodies (13.7 %), and in a high number of patients with actin antibodies

(31.0 %). The prevalence of anti IMP2H2 in patients with mitochondrial antibodies of anti-M2 type (3.2 %) was relatively low. Regarding antibody concentrations, high ABRs >20 were found only in HCV-RNA positive patients except for one HCV-RNA negative (ABR 44.9), one blood donor (ABR 39.6) and the index patient (ABR 61.9), who also was negative for HCV-RNA. When anti-IMP2H2 positive sera (ABR >20) were retested by IIFT only sera harboring antibody concentrations of ABR >35 showed a positive immunofluorescence test with a granular pattern on in house and a "rings and rods" pattern on the two commercial HEp-2 cell preparations.

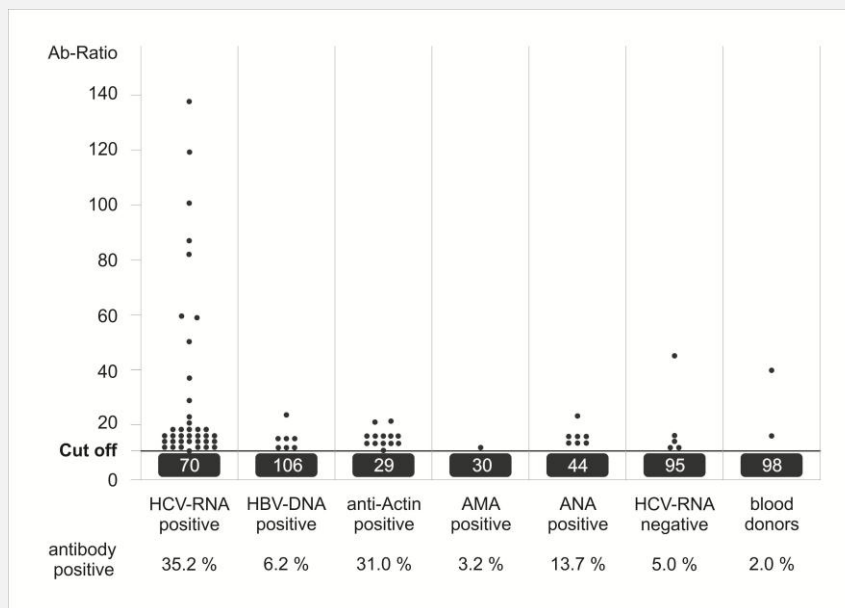
An ABR >20 was seen only in 11.1 % of the HCV-RNA positive patients. There were no major differences regarding age and sex of the antibody positive and antibody negative HCV-RNA positive patients. Mean age of anti-IMP2H2 negative patients was 49.6 years (67 % male, 33 % female), mean age of anti-IMP2H2-positive patients was 50.6 years (75 % male, 25 % female). All patients were subjected to a therapy of interferon and ribavirin either actually or months or years before obtaining the sera used for this screening.

**Table 1. Overview of reactivity of anti-IMPDH2 from index patient, HCV-RNA carriers, and rabbits seen with different IMPDH2 antigens studied by IIFT, western blot, line blot, and RIPA. Only 52.7 % of RIPA positive sera exhibiting an ABR > 20 revealed a positive IIFT and only two of 38 RIPA positive anti-IMPDH2 sera of HCV-RNA carriers showed a rather poor reaction on western blot (+) and none of these 38 sera reacted on line blots. There was one serum weakly positive in the western blot, which, however, did not react in RIPA (ABR = 6,8). [RIPA] = radioimmunoprecipitation assay; [iv TT] = IMPDH2 generated by in vitro transcription – translation.**

Method	Antigens	Serum Index patient	Sera HCV-RNA +	Rabbit anti-IMPDH <sub>Si</sub>	Rabbit anti-IMPDH <sub>ih</sub>
IIFT pattern	HEp-2 cells [in house]	granular	granular	granular	∅
IIFT pattern	HEp-2 cells [commercial]	rings/rods	rings/rods	rings/rods	∅
Western blot	IMPDH2 [His(6)-tag]	+++	∅ -+	+++	+++
Western blot	IMPDH2 [commercial]	+++	∅ -+	+++	+++
Line blot	IMPDH2 [His(6)-tag]	+++	∅	+++	+++
RIPA	IMPDH2 [iv TT]	+++	+++	+++	+++



**Figure 5. Receiver operator curve of anti-IMPDH2 measured by RIPA and expressed in antibody ratios (ABR) of HCV-RNA negative control sera (healthy blood donors) as compared to HCV-RNA positive patients. Assuming an assay specificity of 98 % the sensitivity for detection of anti-IMPDH2 in 108 HCV-RNA carriers was 35 %. Diagonal line indicates no discrimination, AUC = area under the curve, SE = standard error of AUC, CI = lower and upper confidence interval.**



**Figure 6. Prevalence of anti-IMP2H2 in different groups of patients. Black boxes indicate the absolute numbers of negative patients (cut off: ABR <10.5) black dots indicate the anti-IMP2H2 positive patients. High anti-IMP2H2 concentrations prevail within the group of HCV-RNA carriers. Mean virus load of HCV-RNA carriers with anti-IMP2H2 values of <20 ABR was  $1.58 \times 10^6$  IU/mL, of those with anti-IMP2H2 values >20 ABR  $0.96 \times 10^6$  IU/mL.**

## DISCUSSION

There are many examples of accidental detection of autoantibodies simply by means of routine immunofluorescence microscopy and the number detected in this way may be far from being at an end. The molecular characterization of the corresponding antigens nowadays has been improved by screening of cDNA expression libraries. A second approach to identify new autoantibodies utilizes immunoproteomics of cellular proteins with disease specific patient sera followed by identification of reactive proteins by matrix-assisted laser desorption ionization time-of-flight mass spectrometric analysis (MALDITOF). Following the first line, we have identified an autoantibody against IMP2H2 in a female patient by virtue of its distinct immunofluorescence pattern in HEp-2 cells (1). Using the second approach, antibodies against IMP2H2 were found in one of 21 patients with autoimmune hepatitis (18) and in 32 % of 25 patients with colorectal cancer (11).

In this study, anti-IMP2H2 is present in about 35 % of HCV-RNA carriers and, in low concentrations, in 31 % of anti-actin positive patients with suspicion of autoimmune hepatitis. The occurrence of anti-IMP2H2 is, however, not confined to these disease groups. It can also be detected in some patients positive for HBV-DNA, for nuclear or mitochondrial antibodies, in HCV-

RNA negative patients and in healthy blood donors (Figure 6). High concentrations of anti-IMP2H2, however, were found primarily in HCV-RNA carriers. Furthermore, antibodies against IMP2H2 are responsible for the “rings and rods” immunofluorescence pattern (18), found in certain commercial cell preparations used for routine antibody screening. The intracellular distribution of IMP2H2 can be considerably influenced by some of its pharmaceutical inhibitors. The noncompetitive inhibitor mycophenolic acid (MPA), used in organ transplantation, graft versus host disease, vasculitis or also in autoimmune hepatitis (30 - 32), induces intracellular aggregation of IMP2H2 into perinuclear linear arrays and annular configurations (“rods and rings”), which are not associated with intracellular organelles, structures or other interacting proteins (19). Treatment of IMP2H2 with MPA *ex vivo* results in similar aggregations, which proceed most likely through conformational changes (33). MPA binds directly at the NAD site of the enzyme.

As shown in this study, the competitive inhibitor ribavirin, an antiviral drug used in the treatment of hepatitis C in combination with interferon-alpha (34, 35), also induces intracellular aggregations of IMP2H2 in “rods and rings”. Ribavirin, after having entered the cell is converted into ribavirin 5'-monophosphate (RMP), which binds into the IMP binding cleft replacing IMP

(36). Obviously, this state of molecule rearrangement may be induced also by other yet unknown factors as exemplified by its existence in certain commercial diagnostic HEp-2 cell preparations, unlikely having been subjected to prior MPA or ribavirin exposure. In this latter case the HEp-2 cell cultures may have been manipulated in an unknown manner, inducing the transition of IMPDH2 into the "rods and rings" state. As far as routine immunofluorescence tests are concerned, these observations and the fact that five out of seven commercial HEp-2 cell preparations were not able to detect anti-IMPDH2 exemplify to what extent different commercial cell substrates impinge on results and interpretations of the tests (Figure 1).

As far as anti-IMPDH2 itself is concerned, certain differences may exist in its epitope specificity. Antibodies found in HCV-RNA carriers differ at least from those of the index patient in that they react preferentially with conformational epitopes.

This is indicated by the fact that the index patient recognized recombinant IMPDH2 expressed in *E. coli* and subjected to denaturing conditions of western blot, whereas the antibodies found in HCV-RNA carriers, despite high antibody titers, showed only a very weak (western blots) or no reaction (line blots) with this source of antigen. However, the antibodies recognize IMPDH2 synthesized by *in vitro* transcription and translation in rabbit reticulocyte lysate and therefore probably displaying a higher degree of conformational structure. For this reason RIPA was used in this study for the evaluation of the prevalence of anti-IMPDH2. The existence of a broad diversity in epitope and conformational recognition also may be seen in the observation that the *in house* rabbit anti-IMPDH2 recognized the recombinant antigens in western blot and RIPA but neither the natural nor the "rods and rings" forms of IMPDH2 in HEp-2 cells. Differences in epitope specificity also may be responsible for the low prevalence of anti-IMPDH2 (4.76 %) in patients with autoimmune hepatitis detailed by Quing *et al.* (10) using immunoproteomics, principally based on the western blot technology, which has been shown in this study to be less suited than the RIPA assay for detection of these antibodies. Using the immunoprecipitation assay, we detected anti-IMPDH2 in low concentrations in 31 % of the patients with suspected autoimmune hepatitis. The high prevalence of anti-IMPDH2 in patients with colorectal carcinoma demonstrated by immunoproteomics by He *et al.* (11), on the other hand, may speak in favor of the existence in those patients of anti-IMPDH2 preferentially reacting with denatured antigens. To clarify these presumptions, however, detailed studies of epitope specificities in various groups of anti-IMPDH2 positive patients are necessary.

The reason for the high prevalence of anti-IMPDH2 in HCV-RNA carriers remains obscure. Generally, the occurrence of a new autoantibody in patients with HCV-infections is not very surprising since an augmentation of immunological disorders (e. g. mixed cryoglobulin-

emia, thyroid disorders, arthritis, vasculitis, Sjögren syndrome, lung fibrosis) (37 - 39), and of autoantibodies such as smooth muscle antibodies, rheumatoid factor, liver-kidney-microsomal antibodies, anticardiolipin, antinuclear antibodies, cryoglobulins, thyroid antibodies or ribosomal P protein antibodies (12-17,40,41) as well as a correlation between the occurrence of antibodies and disease activity (42,43) have been proposed. Furthermore, treatment with interferon-alpha commonly used in HCV-RNA carriers may render them more susceptible for developing autoantibodies and less frequently autoimmune diseases (e. g. Hashimoto thyroiditis, lupus erythematosus or type 1 diabetes) (47-49).

The reported figures for the prevalence of autoantibodies in HCV infections vary considerably (e. g. antinuclear antibodies 10 - 21 %, smooth muscle antibodies 10 - 55 %) whereas in patients lacking active liver disease the prevalence of autoantibodies seems not to be significantly increased as compared to healthy controls (43-46). It has been suggested, that for antibody development host features especially continuous liver cell damage and hepatocyte necrosis are likely to be more important than viral factors, e. g. HCV-genotype (46). In line with this, we also did not find a correlation between the development of anti-IMPDH2 and virus load. Ribavirin, widely used in therapy for HCV infection, introduces profound alterations of the intracellular distribution of IMPDH2 and therefore possibly enhances its immunogenicity. According to our information all anti-IMPDH2 positive patients were subjected to ribavirin therapy, but detailed data concerning the time elapsed between therapy and antibody testing or the duration of therapy etc. could not be made available. A vague link between ribavirin and anti-IMPDH2 may be seen in the trend of a lower HCV-RNA load in patients exhibiting higher anti-IMPDH2 levels (Figure 6). To answer these questions, prospective studies considering therapy, activity of liver disease, and concomitant autoimmune phenomena are needed.

A high prevalence (31 %) of anti-IMPDH2 in low concentrations (Figure 6) also was found in patients with clinical suspicion of autoimmune hepatitis (AIH) and harboring actin-antibodies (titer 1: >80), demonstrated by IIFT (liver of phalloidin treated mice and vinblastine treated HEp-2). However, detailed information concerning the state of liver disease (activity, remissions) and therapy were not available, neither from these patients nor from those with the suspicion of primary biliary cirrhosis (PBC) harboring mitochondrial antibodies of type M2-specificity. In general, the casual occurrence of anti-IMPDH2 or other autoantibodies (10) in patients with AIH or PBC, intrinsically prone to exhibit autoimmune phenomena, may not be surprising. Based on our more "hypothetical" diagnoses, it seems that in AIH with a rather high prevalence of low titer anti-IMPDH2 specific pathological processes may govern the formation of anti-IMPDH2 not prevailing in PBC where the prevalence of anti-IMPDH does not exceed that of con-

trols (HCV-RNA negatives, blood donors). Whether the development of anti-IMPDH2 in AIH may be caused by particular therapeutic management, e. g. mycophenolate, remains obscure. Sole liver damage, on the other side, may not be sufficient for the generation of anti-IMPDH2, since its prevalence in HBV-DNA carriers did not exceed the controls. As demonstrated by the occurrence of the “rings and rods” fluorescence pattern in culture cells, the intracellular aggregation of IMPDH2 may also be caused by further unknown pharmacological or pathological processes. Thus one might speculate with regard to the therapeutic interventions of inhibition of IMPDH2 by ribavirin in patients with chronic HCV infections and in some autoimmune liver diseases by mycophenolic acid (30, 31), that the development of autoantibodies against IMPDH2 may be related to therapeutic procedures or to other hitherto unknown pathological processes causing intracellular aggregation of IMPDH2.

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#### Declaration of Interest:

The authors declare that to the best of their knowledge there are no current or potential conflicts of interests.

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