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Rapid and preemptive evaluation of individual anti-hepatitis C virus treatment outcome capability by a short-term autologous liver tissue culture system

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SUMMARY

Hepatitis C virus (HCV) standard of care (SOC) therapy is not effective in a large percentage of patients and its efficacy may be evaluated only after several weeks. The aim of this work was to set up an *in vitro* liver culture assay able to preemptively predict SOC outcome by using residual liver samples from HCV patients. The *in vitro* response to SOC was found associated with the *in vivo* treatment outcome with a concordance of 100%. A wider clinical trial on a larger patient group is necessary to fully evaluate the impact of this procedure on the clinical management of untreated HCV patients.

KEY WORDS: HCV standard therapy, Liver biopsies, *In vitro* tissue model, *In vitro* HCV replication.

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Hepatitis C virus (HCV) is a single-stranded RNA virus that infects 170 million people worldwide and is a major cause of cirrhosis, fibrosis and hepatocellular carcinoma (HCC) (Kanwal *et al.,* 2011). About 20% of patients infected with HCV spontaneously clear acute infection whereas 80% progress to chronicity (Hoofnagle *et al.,* 2002). An effective antiviral treatment with pegylated-interferon and ribavirin has been available since early 2000, which in fact cures most infections from HCV genotypes 2 and 3, while it is by far less successful against HCV genotype 1, the prevalent strain infecting patients in Europe, North America and Asia (Manns *et al*., 2001; Hayashi *et al.,* 2006). Under evaluation are new promising drugs (direct antiviral agents) that could be used in association with standard treatment. Although effective, their use has opened the question on drug-resistant HCV variants, and their high costs may make them unsuitable in cost constraint situations (Zeuzem *et al.,* 2011; Lawitz *et al.,* 2013; Abe *et al.,* 2013; Colombo *et al.,* 2013).

An *ex vivo* culture of liver tissue was previously proposed as able to preemptively evaluate antiviral therapy efficacy (Chang *et al.,* 2009). However, that paper analyzed liver tissue response *in vitro* after treatment with IFN-α alone, while *in vivo* therapy was performed using Peg-IFN-α/RBV. Moreover, the complex culture system in the study using feeder cells makes it not readily applicable to a real routine-type scenario.

Aim of our work was to design a simple liver culture protocol able to sustain HCV replica-

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tion, and to verify if it may be used to preemptively evaluate autologous HCV response to antiviral combination treatment.

Twenty-seven chronic HCV patients undergoing liver biopsy for clinical reasons were consecutively enrolled. All patients were negative for HIV and HBV infections. The study was approved by the ethical committee at the "L.

TABLE 1 - *Patient's characteristics.*

Patient	*Gender*	*Age (years)*	*HCV Genotype*	*ALT (IU/ml)*	*Plasma HCV-RNA (copies/ml)*	*Liver fibrosis (Metavir score)*
1	M	44	1b	43	4,012	A1; F1
2	M	48	1b	59	381,101	A1; F1
3	F	59	3a	15	4,518,754	A1; F1
4	M	31	1a	99	48,390	A2; F2
5	F	60	1b	86	9,617,263	A1; F2
6	F	54	1a	43	1,836,572	A1; F2
7	F	43	3	73	168,736	A2; F1
8	F	64	1b	120	14,953,909	A2; F1
9	F	56	1b	22	6,427	A1; F1
10	M	51	1a	78	2,958,874	A2; F2
11	M	61	3a	22	14,551	A1; F1
12	M	59	1a	48	565,109	A1; F2
13	M	42	1a	21	2,280,776	A1; F1
14	F	64	1b	85	56,071	A2; F3
15	M	69	2a/2c	136	678,911	A2; F2
16	M	49	2	33	6,073,235	A1; F1
17	M	57	1b	75	3,188,796	A2; F3
18	M	62	1b	61	865,348	A1; F2
19	M	51	4	92	279,004	A1; F2
20	M	51	4	58	3,981,879	A2; F2
21	M	50	1a	173	13,666,731	A2; F3
22	M	59	1b	72	900,832	A2; F1
23	M	44	1a	41	774,909	A1; F1
24	M	53	1b	33	8,275,090	A2; F2
25	M	61	2	205	3,499,934	A3; F2
26	M	60	1b	78	2,965,300	A2; F2
27	F	68	1b	60	3,895,064	A1; F3

ALT: alanine aminotransferase.

Spallanzani" National Institute for Infectious Diseases, and all individuals provided written informed consent. Clinical features of HCV patients are summarized in Table 1. Plasma HCV-RNA levels and HCV genotype were tested by Abbott assays (Abbott Laboratories).

An easily applicable *in vitro* liver culture assay was set up by culturing residual liver tissue from biopsies in standard medium (RPMI-1640 with 10% FBS, 2 mM L-glutamine, 50 IU/ml Penicillin, 50 µg/ml Streptomycin, EuroClone) in 96 well flat-bottomed plates (Costar, Corning Incorporated) at 37°C and 5%CO_2. In order to evaluate the ability of this system to sustain autologous HCV replication, liver tissue from 5 HCV patients was divided into two equally sized slices and cultured for three (t3) and seven (t7) days, and HCV copies were quantified by RT-qPCR (Table 1, patients 1-5). Briefly, liver tissue was digested with Trizol Reagent (Invitrogen) by Gentle MACS Dissociator (Miltenyi Biotec) and HCV-RNA was quantified by TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems; Foster City, CA) using HCV specific primers and probe as described in (Daniel *et al.,* 2008); β-actin was quantified and was used as a housekeeping reference gene to verify the equal size of liver tissue used in different wells. Results showed that HCV viral load increased from t3 to t7 in each experiment; HCV-RNA increased at t7 on average 3.38 times in respect to t3 (range 1.98-5.02). Data from individual patients were as follows: patient 1 (t7 *vs* t3) 7370.0 cp/µg RNA *vs* 2570.0, index (t7/t3) =2.86; patient 2 (t7 *vs* t3) 990.3 vs. 226.7, index =4.36; patient 3 (t7 *vs* t3) 13175.0 vs. 4875.0, index =2.70; patient 4 (t7 *vs* t3) 3134.0 *vs* 1580.0, index =1.98; patient 5 (t7 *vs* t3) 1960.0 *vs* 390.0, index =5.02. Our data suggest that a low rate of HCV replication is maintained in these culture conditions, and that t7 may represent a good time point to evaluate the antiviral effects of anti-HCV drugs.

To evaluate if *in vitro* culture may affect hepatocyte viability, lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured (Siemens Clinical Chemistry System) in culture supernatants at t7 in four experiments. Irrespective of IFN-α/RBV treatment, similar levels of LDH (Medium 648.7±185.4 *vs* IFN-α/RBV 584.3±124.5), AST (Medium 289±79.2 *vs* IFN-α/RBV 215.8±79.2) and ALT (Medium 61.5±18.2 *vs* IFN-α/RBV 44.5±12) were found. Moreover, liver biopsies were digested for 2 hours at 37°C with an enzymatic mix solution (Collagenase 10 mg/ml, Hyaluronidase 1 mg/ml, DNAse 200 mg/ml) by Gentle MACS Dissociator (Miltenyi Biotec). The hepatic cell suspensions (HCS) were tested for apoptosis/necrosis by Annexin-V-FITC Kit (Bender Med Systems). No apoptotic/necrotic cells were observed at t7 in HCS, independently from IFN-α/RBV treatment (data not shown).

Among 27 patients enrolled (Table 1), 23 patients were tested by the *in vitro* assay (Table 2); 4 samples were excluded due to low RNA quality (patients 9 and 15) or because RNA quantity allowed only the HCV replication test (patients 1 and 5). Briefly, liver tissue was divided in two equally sized slices and cultured as described above, in the absence or presence of IFN-α 1000 IU/ml) and RBV (200 µM). The test result was expressed as a responsiveness index obtained by the ratio between HCV-RNA copies in treated and untreated slices. A preliminary analysis showed that all index values clustered in two different groups, below 0.5 and above 1.0, with no values in between. Therefore, these values were used to evaluate the test outcome:

1) an index ≤0.5 was associated with a reduction of HCV-RNA after treatment, identifying "*in vitro* responder" patients (ivR);
2) an index ≥1 was associated with a lack of Peg-IFN-α/RBV efficacy, identifying "*in vitro* non responder" patients (ivNR).

In vivo treatment efficacy was evaluated as SVR and not-SVR (Non-Responders, Relapsers and Breakthrough) patients (Table 2). Among the 23 patients analyzed, 15 started treatment within six months from biopsy according to the physician's clinical decision. Treatment included Peg-IFN-α and a weight-based dose of RBV for a period of 24 or 48 weeks according to viral genotype. Of the 15 patients treated, 3 reached SVR and 12 were not-SVR. The *in vitro* test was indeed preemptively associated with *in vivo* treatment outcome with a concordance of 100%: 12/12 not-SVR patients were ivNR (100%), and 3/3 SVR patients were ivR (100%) (Fisher exact test p=0.0015).

Among the patients undergoing the *in vitro* test, seven patients had a history of previous failure; interestingly, these patients were confirmed to be non-SVR and failed to respond to the *in vitro* test (Table 2).

RNA replicon systems and replication competent viruses have been developed to test antiviral drugs (Moriishi *et al.,* 2007), but these *in vitro* models do not take into account HCV variability and its ability to replicate in the complex liver architecture, barely simulated by laboratory cell lines.

In this context, some *ex vivo* tissue culture systems have been proposed in the past, allowing the fast evaluation of individual virus/host response to antiviral drugs (Yeh *et al.,* 2003; Chang *et al.,* 2009).

In Chang *et al.* paper (Chang *et al.,* 2009), a poor correlation between *in vitro* test results and *in vivo* SVR occurrence was found. We cannot explain why our test offered a better outcome, albeit in a much smaller cohort.

We may speculate that this is due to the different culture conditions used by Chang *et al.*, including the use of Vero cells as feeders; this experimental setting may allow a more efficient HCV replication, that in turn may conceal or make less evident the effect of antiviral drugs, and cause the very high cell mortality.

The availability of promising new direct antiviral agents for the clinical management of HCV patients may improve their clinical outcome. Nevertheless, several factors have been identified that may limit both access as well as response rates to direct agents (Zeuzem *et al.,* 2011). In this context, the possibility to identify interferon-sensitive patients may represent an important step to support clinicians in treatment decision-making.

In conclusion, we developed an *ex vivo* culture system of liver tissue allowing a fast preemptive evaluation of HCV treatment efficacy. Although we are aware that the small size of the HCV cohort does not allow conclusive statements, we believe that the *in vitro* test has the potential to avoid ineffective treatment, possible serious side-effects, and useless treatment costs.

Overall, our results show a strong correlation between the preemptive *in vitro* test, performed before treatment, and *in vivo* treatment outcome.

However convincing, a coordinate analysis of the impact of this assay, together with other known predictive factors (reviewed in Arnaud *et al.,* 2013), in a wider clinical trial is necessary to fully evaluate the impact of this procedure on the clinical management of untreated HCV patients.

TABLE 2 - *In vivo treatment outcome, in vitro response, and history of previous treatment.*

Patient	*In vivo treatment outcome*	*In vitro response before treatment*	*Previous in vivo treatment*
2	SVR[a]	ivR[b]	Naive
3	Untreated	ivR	Naive
4	SVR	ivR	Naive
6	Untreated	ivNR[c]	Naïve
7	Untreated	ivNR	Naive
8	Untreated	ivNR	Naive
10	Untreated	ivR	Naive
11	Untreated	ivNR	Naive
12	Untreated	ivR	Naive
13	Untreated	ivR	Naive
14	SVR	ivR	Naive
16	Relapser	ivNR	Relapser
17	Relapser	ivNR	NR[d]
18	Relapser	ivNR	Naive
19	Relapser	ivNR	Breakthrough
20	Breakthrough	ivNR	Naïve
21	Breakthrough	ivNR	Breakthrough
22	NR	ivNR	Naïve
23	NR	ivNR	Naive
24	NR	ivNR	Naïve
25	NR	ivNR	NR
26	NR	ivNR	NR
27	NR	ivNR	NR

[a]Sustained Virological Response; [b]in vitro responder; [c]in vitro non responder; [d]Non Responder.

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Competing interests. None declared

Ethical approval: The ethical Committee of the "L.Spallanzani" National Institute for Infectious Diseases (Rome, Italy) approved the study.

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