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HCV-RNA quantification in liver bioptic samples and extrahepatic compartments, using the abbott Real*Time* HCV assay

FrancescoPaolo Antonucci[a], Valeria Cento[a], Maria Chiara Sorbo[a], Matteo Ciancio Manuelli[b], Ilaria Lenci[c], Daniele Sforza[b], Domenico Di Carlo[a], Martina Milana[c], Tommaso Maria Manzia[b], Mario Angelico[c], Giuseppe Tisone[b], Carlo Federico Perno[a], Francesca Ceccherini-Silberstein[a,*]

[a] *Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Rome, Italy*
[b] *Liver Unit, Polyclinic Tor Vergata Foundation, University of Rome Tor Vergata, Rome, Italy*
[c] *Hepatology Unit, Polyclinic Tor Vergata Foundation, University of Rome Tor Vergata, Rome, Italy*

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ABSTRACT

Background & aims: We evaluated the performance of a rapid method to quantify HCV-RNA in the hepatic and extrahepatic compartments, by using for the first time the Abbott Real*Time* HCV-assay.
Methods: Non-tumoral (NT), tumoral (TT) liver samples, lymph nodes and ascitic fluid from patients undergoing orthotopic-liver-transplantation (N = 18) or liver resection (N = 4) were used for the HCV-RNA quantification; 5/22 patients were tested after or during direct acting antivirals (DAA) treatment. Total RNA and DNA quantification from tissue-biopsies allowed normalization of HCV-RNA concentrations in IU/μg of total RNA and IU/10^6 liver-cells, respectively.
Results: HCV-RNA was successfully quantified with high reliability in liver biopsies, lymph nodes and ascitic fluid samples. Among the 17 untreated patients, a positive and significant HCV-RNA correlation between serum and NT liver-samples was observed (Pearson: rho = 0.544, p = 0.024). Three DAA-treated patients were HCV-RNA "undetectable" in serum, but still "detectable" in all tested liver-tissues. Differently, only one DAA-treated patient, tested after sustained-virological-response, showed HCV-RNA "undetectability" in liver-tissue.
Conclusions: HCV-RNA was successfully quantified with high reliability in liver bioptic samples and extrahepatic compartments, even when HCV-RNA was "undetectable" in serum. Abbott Real*Time* HCV-assay is a good diagnostic tool for HCV quantification in intra- and extra-hepatic compartments, whenever a bioptic sample is available

1. Introduction

Advanced liver disease caused by hepatitis C virus (HCV) infection is the leading indication for orthotopic liver transplantation (OLT) worldwide (Saab et al., 2003).

The post-OLT detection of HCV-RNA in the serum or graft is extremely common, occurring in more than 95% of cases (Berenguer, 2002). During post-OLT follow up, elevated liver enzymes warrant liver biopsies for accurate diagnosis and treatment and it can be difficult to differentiate between acute rejection and recurrence of hepatitis C (Song et al., 2015). HCV treatment before OLT may reduce or eliminate the risk of post-OLT recurrence. In a pilot study, Sofosbuvir and ribavirin treatment before OLT successfully prevented HCV recurrence in 70% of patients with chronic HCV infection and liver cancer who achieved an HCV-RNA level less than 25 IU/ml before transplantation (Curry et al., 2015). The recurrence of HCV infection after OLT in the remaining 30% of patients may be the consequence of a low-level residual viremia, not detectable by current commercial assays in serum. The liver is the primary site of HCV replication, and a direct correlation between liver and serum viral loads has been already established (McGuinness et al., 1996; De Moliner et al., 1998). The quantification of HCV-RNA in both serum and liver might provide helpful and more accurate information regarding the HCV burden and eventual risk of recurrence of HCV infection.

Several home-made methods, based on reverse transcription-polymerase chain reaction (RT-PCR), transcription-mediated amplification, or branched-chain DNA, are available for the detection of HCV-RNA in both serum and liver (Sakamoto et al., 1994; Dailey et al., 1999; Maudar et al., 2012; Ramirez et al., 2009; Harouaka et al., 2016; Hedegaard et al., 2017; Gambato et al., 2016). However, a commercial

Abbreviations: OLT, orthotopic-liver-transplantation; DAA, direct acting antivirals; NT, non-tumoral tissue; TT, tumoral tissue; HCC, hepatocellular carcinoma

* Corresponding author at: Department of Experimental Medicine and Surgery, University of Rome Tor Vergata, Via Montpellier 1, Rome 00133, Italy.
E-mail address: ceccherini@med.uniroma2.it (F. Ceccherini-Silberstein).

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Table 1
Patients Characteristics. PT: Patient; M: Male; F: Female; HCC: Hepatocellular Carcinoma; OLT: Orthotopic Liver Transplantation; LR: Liver Resection; HCV: Hepatitis C Virus; IU/ml: International Unit per milliliter; TND: target not detected (HCV-RNA < 12 IU/ml). Four patients were receiving a treatment with direct acting antivirals agents (DAA) before OLT.

ID	Age (Years)	Gender	Liver disease	Surgical Operation	HCV Genotype	Serum HCV-RNA (IU/ml)
PT1	58	M	HCC	OLT	3a	6643
PT2	71	M	HCC	LR	1b	695503
PT5	64	M	HCC	OLT	1b	1000
PT6	57	M	HCC	OLT	3a	490
PT22	74	M	HCC	LR	1b	54503
PT77	53	F	HCC	OLT	1b	24
PT15	59	M	Cirrhosis, HCC	OLT	1a	64092
PT19	48	M	Cirrhosis, HCC	OLT	1a	2317
PT27	63	M	Cirrhosis, HCC	OLT	1b	TND
PT28	53	M	Cirrhosis, HCC	OLT	3a	6747
PT37	64	F	Cirrhosis, HCC	OLT	1b	TND
PT40	61	M	Cirrhosis, HCC	OLT	3a	2089296
PT43	62	M	Cirrhosis, HCC	OLT	1b	1376956
PT44	71	M	Cirrhosis, HCC	LR	1b	710075
PT49	60	M	Cirrhosis, HCC	OLT	1b	75157
PT57	66	M	Cirrhosis, HCC	OLT	1b	57216
PT62	63	F	Cirrhosis, HCC	OLT	2	TND
PT63	55	M	Cirrhosis, HCC	OLT	3a	TND
PT64	55	M	Cirrhosis, HCC	LR	4d	1000451
PT18	59	M	Cirrhosis	OLT	1b	171419
PT20	46	M	Cirrhosis	OLT	1a	166694
PT53	46	M	Cirrhosis	OLT	1a	387

validated assay for the HCV RNA quantification in the liver tissue is still missing.

For the first time, we evaluated the performance of a rapid method to quantify the HCV-RNA in liver tissue, lymph nodes and ascitic fluids by using the Abbott Real*Time* HCV assay.

2. Materials and methods

2.1. Study population

We collected liver biopsies, on both non-tumoral (NT) and tumoral (TT) tissues, from 22 patients (19 men and 3 women, mean age 59 years, range: 46–74) with HCV infection by genotype 1a (n = 4), 1b (n = 11), 2 (n = 1), 3a (n = 5) and 4d (n = 1). Lymph nodes and ascitic fluids were also collected from 3 and 2 patients, respectively.

Most of the patients underwent liver surgery due to the presence of Hepatocellular Carcinoma (HCC) (N = 19) (Table 1). 18/22 underwent OLT and 4/22 liver resection. Five out of 22 patients (22.7%) were receiving a treatment with direct acting antivirals (DAA) before OLT. All samples were obtained after written consent, which was approved by the local ethics commission.

2.2. HCV-RNA quantification in liver biopsies by Abbott RealTime HCV assay

The Abbott Real*Time* HCV assay was used for the quantification of HCV-RNA in liver, lymph nodes, ascitic fluids and sera samples (Abbott Laboratories, Abbott Park, Illinois, U.S.A.). For the HCV-RNA quantification in the liver, the protocol was applied in more than one patient's liver section to test the reliability of the HCV-RNA values.

Liver biopsies were obtained during OLT or liver resection. At the time of biopsy, tissue samples were promptly submerged in 1 ml of RNAlater RNA Stabilization Reagent (Qiagen, Valencia, CA) and maintained over night at 4 °C, and then stored at −20 °C. Where it was available, lymph nodes biopsies and ascitic fluids were collected as well: the lymph nodes were stored in the same way as the liver biopsies; instead, the ascitic fluids were stored directly at −20 °C like a normal serum sample.

Table 2
RNA and DNA measurements. PT: Patient; NT: Non Tumoral; TT: Tumoral Tissue; LN: Lymph node; n.a.: Sample Not Available; ng/µl: nanogram per microliter.

ID	Bioptic Sample	Total RNA (ng/µl)	260/280	260/230	Total DNA (ng/µl)	260/280	260/230
PT1	NT	96.7	2.14	1.91	n.a.	n.a.	n.a.
	TT	28.4	1.85	1.61	n.a.	n.a.	n.a.
PT2	NT	24.3	1.96	1.94	129.8	1.88	1.99
	TT	112.2	2.34	1.96	84.3	1.87	1.66
PT5	NT	81.5	2.02	1.85	n.a.	n.a.	n.a.
	TT	400.9	2.09	1.90	n.a.	n.a.	n.a.
PT6	NT	252.3	2.34	1.83	n.a.	n.a.	n.a.
	TT	23.1	1.91	1.45	n.a.	n.a.	n.a.
PT22	NT	182.9	2.04	1.99	128.7	1.86	1.60
	TT	53.7	2.05	1.91	81.6	1.90	1.98
PT77	NT	470	1.98	1.84	n.a.	n.a.	n.a.
	TT	397.9	2.08	1.93	n.a.	n.a.	n.a.
PT15	NT	277.6	2.07	1.98	176.7	1.90	2.09
	TT	326.4	2.06	1.83	178.9	1.90	2.15
PT19	NT	46.4	2.04	1.77	139.2	1.87	1.74
	TT	88.9	2.05	2.07	127.3	1.90	1.94
PT27	NT	30.2	2.07	1.96	125.6	1.86	1.21
	TT	169.3	2.07	2.02	98.8	1.90	2.01
	LN	4.7	2.13	1.86	n.a.	n.a.	n.a.
PT28	NT	26.8	2.08	1.74	41.5	1.93	1.18
	TT	86.4	2.40	1.32	228.7	1.88	1.90
PT37	NT	314	2.13	2.07	n.a.	n.a.	n.a.
	TT	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	LN	3.6	2.31	1.92	n.a.	n.a.	n.a.
PT40	NT	446.3	2.06	1.90	114.9	1.89	1.74
	TT	1.3	2.54	2.02	7,0	2.38	0.21
PT43	NT	266.2	2.13	1.96	n.a.	n.a.	n.a.
	TT	248.6	2.05	1.79	n.a.	n.a.	n.a.
	LN	239.1	1.97	1.89	n.a.	n.a.	n.a.
PT44	NT	364.3	1.87	1.65	n.a.	n.a.	n.a.
	TT	214	1.93	1.83	n.a.	n.a.	n.a.
PT49	NT	718.7	2.05	2.00	142.5	1.86	2.08
	TT	7.2	1.92	1.75	71.1	1.94	1.76
PT57	NT	463.3	2.09	1.98	n.a.	n.a.	n.a.
	TT	301.6	2.14	1.77	n.a.	n.a.	n.a.
PT62	NT	135	2.23	1.88	n.a.	n.a.	n.a.
	TT	170	1.99	1.81	n.a.	n.a.	n.a.
PT63	NT	229.9	2.10	1.99	n.a.	n.a.	n.a.
	TT	328.3	1.93	1.91	n.a.	n.a.	n.a.
PT64	NT	1115.1	2.41	1.99	n.a.	n.a.	n.a.
	TT	96.8	1.86	1.65	n.a.	n.a.	n.a.
PT18	NT	142.9	2.04	1.86	n.a.	n.a.	n.a.
	TT	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PT20	NT	215	1.86	1.76	n.a.	n.a.	n.a.
	TT	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PT53	NT	114.6	2.35	1.94	n.a.	n.a.	n.a.
	TT	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

According to samples availability, HCV-RNA quantification was performed by using 13 mg of tissue section (5 mm of thickness in both liver and lymph node bioptic samples). A total of 56 NT liver sections obtained from 22 patients were used for HCV-RNA quantification. The number of NT liver sections obtained from each patient varied as follows: 3–4 different NT liver sections obtained from 14 patients, 2 different NT liver sections obtained from 5 patients, 1 NT liver section obtained from 3 patients. In addition, a total of 44 TT liver sections from 18/19 patients with HCC were assessed for HCV-RNA concentration. The breakdown of available TT liver sections per patient was as follows: 3 different TT liver sections obtained from 11 patients, 2 different TT liver sections obtained from 4 patients, 1 TT liver section obtained from 3 patients. No replicates were applied for the HCV-RNA quantification in lymph nodes and ascitic fluids because of the reduced availability of these samples.

The liver, or lymph node, bioptic sample was homogenized in 600 µl of Buffer RLT (supplied by RNeasy Mini Kit, Qiagen, Valencia, CA) + β-

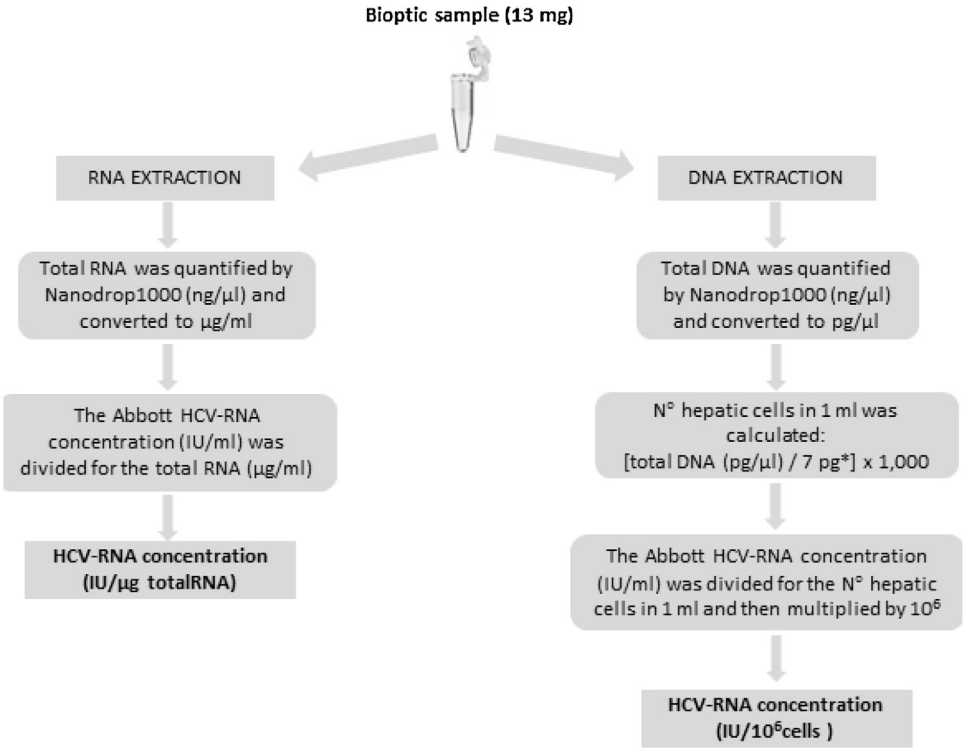

Fig. 1. Normalization procedures. Extracted RNA and DNA from liver and lymph node specimens were used to obtain HCV-RNA concentrations expressed in: IU/µg totalRNA and IU/10^6 cells.*Total DNA amount per cell: 7 pg (Serth et al., 2000).

Mercaptoethanol by TissueRuptor (Qiagen, Valencia, CA) homogenizer; the homogenized sample was centrifuged for 3 min at full speed. Carefully, 333 µl supernatant was removed and transferred into a new microcentrifuge tube containing 667 µl of RNase-free water. This dilution (1:3) was made considering the requirement of 1 ml of sample volume for the Abbott Real*Time* HCV assay, although the extraction volume used is 0.5 ml. HCV-RNA was then extracted automatically by the Abbott *m*2000*sp* system. Amplification and detection was performed, always automatically, by the Abbott *m*2000*rt*, which amplify the 5′ UTR region of the HCV genome. At each cycle, the amplified products were detected by the Abbott m2000rt through the HCV probe fluorescent signal, which is proportional to the log of HCV-RNA concentration present in the original sample.

The concentrations of HCV-RNA samples and controls were calculated from the stored calibration curved; the results were automatically reported on the Abbott m2000rt workstation and multiplied by the dilution factors to obtain the normalized Abbott HCV-RNA result (IU/ml). The Abbott RealTime HCV Negative Control (−), Low Positive Control (+), and High Positive Control (++) were included in each run to verify run validity.

Afterwards, depending on the samples availability, total RNA and DNA extractions from 2 liver bioptic samples, 1 NT and 1 TT, for each patient were performed by using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA); subsequently, the extracted samples were quantified by Nanodrop1000 (Thermo Fisher Scientific Co., Ltd, Shanghai, China) to have both qualitative and quantitative measurements (Table 2). These measurements were used to normalize the Abbott HCV-RNA values (IU/ml) to obtain concentrations expressed as: IU/µg of total RNA and IU/10^6 cells (Fig. 1). When Abbott HCV-RNA values were < 12 IU/ml, the value of 12 was used for the normalization.

Since every liver sample was already used for the quantification of the HCV-RNA and for the extraction of the total RNA, the HCV-RNA IU/10^6 cells normalization was performed in 8/22 patients, where both NT and NT samples were still available to allow extra DNA quantification.

For the HCV RNA quantification in lymph nodes, values were normalized only by using the µg of total RNA. For the ascitic fluid, the same extraction protocol was used as above; in fact, the sample was directly diluted (1:3), and automatically extracted and quantified by the Abbott *m*2000*sp* and Abbott *m*2000*rt*, respectively. The concentration was expressed in IU/ml.

2.3. Statistical analysis

All patients were coded for anonymity and for each patient, virological and clinical data were collected in an anonymous database. Results are expressed as median values and interquartile range (IQR). Values were compared using the Mann-Whitney *U* test, the Pearson or the Spearman correlation test. All the analyses were performed using SPSS software package (version 19.0) for Windows (SPSS Inc., Chicago, Illinois).

3. Results

3.1. HCV-RNA liver quantification and normalization

Considering the Nanodrop1000 measurements, the extracted RNA and DNA from hepatic specimens were of good quality (average of the RNA and DNA absorbances at 260/280 nm: 2.09 ± 0.16 SD and 1.92 ± 0.12 SD, respectively; average of the RNA and DNA absorbances at 260/230 nm: 1.86 ± 0.15 SD and 1.70 ± 0.47 SD, respectively) (Table 2).

HCV-RNA was quantified successfully in all patients, by Abbott Real*Time* HCV assay, in both liver bioptic sections: NT (22 patients) and TT (18 patients with HCC).

The normalization procedures (IU/µg RNA and/or IU/10^6 cells) (Fig. 1) were applied to each liver sample; afterwards a mean HCV-RNA concentration was calculated per patient (Tables 3 and 4).

The obtained results, starting from both NT and TT samples, showed little variation in the triplicate quantifications performed in almost every patient, where it was available. In only one patient (PT28), the standard deviation among the 3 TT measurements was higher than the

Table 3
HCV-RNA serum and hepatic quantifications in non treated patients. PT: Patient; NT: Non Tumoral; TT: Tumoral Tissue; SD: Standard Deviation; n.a.: Sample Not Available; IU/ml: International Unit per milliliter; IU/μgRNA: International Unit per μg of total RNA; IU/10^6cell: International Unit per 1,000.000 of liver cells.

ID	Serum HCV-RNA (IU/ml)	Tissue NT HCV-RNA (IU/ml)	Tissue NT HCV-RNA (IU/μgRNA) Mean ± SD	Tissue NT HCV-RNA (IU/10^6cells) Mean ± SD	Tissue TT HCV-RNA (IU/ml)	Tissue TT HCV-RNA (IU/μgRNA) Mean ± SD	Tissue TT HCV-RNA (IU/10^6cells) Mean ± SD
PT1	6643	56000 63392 169470 176656	1204 ± 587	n.a.	720	25.4	n.a.
PT2	695503	1763640 2265876 2218698	85709 ± 9319	112320 ± 12213	202941 1664208 2234313	12185 ± 7625	113524 ± 71037
PT5	1000	452268 274194 534366	5157 ± 1332	n.a.	729 1386 558	2 ± 0.9	n.a.
PT6	490	799686	3170	n.a.	1973943 1933209 8858295	184206 ± 140907	n.a.
PT22	54503	25285725 23728878 29955087	143889 ± 14461	1431722 ± 143894	2212812 5019435 5740074	80523 ± 28336	370941 ± 130531
PT15	64092	4062609 8246214 6118479	22127 ± 6153	243334 ± 67664	2851542 5325489 4700061	13151 ± 3218	167952 ± 41097
PT19	2317	1017 1206 567	20 ± 5.8	47 ± 13	2097 621 1197	15 ± 6.8	72 ± 33
PT28	6747	7195230 7999110 8228250	291326 ± 16528	1316933 ± 74715	2925 58302 4041	252 ± 299	666 ± 791
PT40	2089296	13128246 16854588 19230399	36756 ± 5627	999398 ± 153004	18873 29421 25254	18858 ± 3337	24516 ± 4338
PT43	1376956	13023081 12752748 11644677	46858 ± 2240	n.a.	1278	5.1	n.a.
PT44	710075	3149397 1893102	6921 ± 1724	n.a.	32490	152	n.a.
PT49	75157	894762 1156734 663894	1259 ± 280	44463 ± 9890	1332 152433 67671	10252 ± 8589	7267 ± 6088
PT57	57216	1122396 2248299	3638 ± 1215	n.a.	437421 1137492	2612 ± 1161	n.a.
PT64	1000451	3022068 2880009	2646 ± 64	n.a.	21357 75192	499 ± 278	n.a.
PT18	171419	553266	3872	n.a.	n.a.	n.a.	n.a.
PT20	166694	11297979	52549	n.a.	n.a.	n.a.	n.a.
PT53	387	306 387	3 ± 0.4	n.a.	n.a.	n.a.	n.a.

mean value, but this unique variation might be correlated with different distribution of HCV-RNA in the 3 liver sections, or also by simply a methodological error.

When HCV-RNA concentration was expressed in IU/μg of total RNA, quantification of HCV-RNA among NT samples varied from TND to 291,326 IU/μgRNA, with a median (IQR) value of 3404 (7–36,756) IU/μgRNA. In TT samples, HCV-RNA varied from TND to 184,206 IU/μgRNA, with a median (IQR) value of 202 (5.1-12,185) IU/μgRNA (Tables 3 and 4).

Similarly, when HCV-RNA was expressed in IU/10^6 cells, its concentration varied in NT samples from 11 to 1,431,722 IU/10^6cells, with a median (IQR) value of 177,827 (22,255–1,158,166) IU/10^6cells. In TT samples, HCV-RNA varied from 72 to 370,941 IU/10^6cells, with a median (IQR) value of 15,892 (639–140,738) IU/10^6cells (Tables 3 and 4).

3.2. Comparison of HCV-RNA concentrations between serum and liver samples of non-treated patients

Among the 17 untreated patients, a positive and significant HCV-RNA correlation between serum and NT liver samples was observed (Pearson: rho = 0.544, p = 0.024) only after the normalization with RNA, when HCV-RNA was expressed in IU/μgRNA, but not when expressed in IU/10^6cells (Panels A and B, Fig. 2). Differently, no correlation was observed between serum and TT (14/17 untreated patients) with both normalization methods (Panels C and D, Fig. 2).

In patients with HCC (14/17), the median (IQR) HCV-RNA concentration was generally higher in NT samples compared to TT samples, although this difference was not statistically significant. In fact, when tissue HCV-RNA was expressed in logIU/μgRNA, the median (IQR) HCV-RNA in NT was 3.8 (3.4–4.7) logIU/μgRNA, *vs.* 3.1 (1.4–4.1) logIU/μgRNA in TT (Mann-Whitney, p = 0.114). Similarly, when tissue HCV-RNA was expressed in logIU/10^6 cells, the median (IQR) HCV-RNA in NT samples was 5.4 (4.6–6.1) logIU/10^6 cells, *vs.* 4.4 (2.8–5.2) logIU/10^6 cells in TT samples (Mann-Whitney, p = 0.209).

By plotting TT and NT HCV-RNA results obtained with the 2 different normalization methods, the correlation between the 2 variables was positive by Pearson correlation test, yet not statistically significant (Panels E and F, Fig. 2). Notably, the normalization of HCV-RNA values in IU/10^6 cells, where it was available, led to a more

Table 4
HCV-RNA serum and hepatic quantifications in patients treated with direct acting antiviral agents (DAAs). When Abbott HCV-RNA values were < 12 IU/ml, the value of 12 was used for the normalization. PT: Patient; W: week; DAA: Direct Acting Antiviral Agents; OLT: Orthotopic Liver Transplantation; NT: Non Tumoral; TT: Tumoral Tissue; IU/ml: International Unit per milliliter; IU/μgRNA: International Unit per μg of total RNA; IU/10^6cells: International Unit per 1,000.000 of liver cells; TND: target not detected (HCV-RNA < 12 IU/ml); n.a.: Sample Not Available; SD: Standard Deviation; SVR, sustained virological response.

ID	Duration of DAA treatment pre-OLT	Serum HCV-RNA (IU/ml)	Tissue NT HCV-RNA (IU/ml)	Tissue NT HCV-RNA (IU/μgRNA) Mean ± SD	Tissue NT HCV-RNA (IU/10^6cells) Mean ± SD	Tissue TT HCV-RNA (IU/ml)	Tissue TT HCV-RNA (IU/μgRNA) Mean ± SD	Tissue TT HCV-RNA (IU/10^6cells) Mean ± SD
PT27	6 w	TND	297 153 153	7 ± 2.2	11 ± 4	7902 8910 9108	51 ± 3.1	612 ± 37
PT37	19 w	TND	< 12 < 12 < 12	0.1 ± 0	n.a.	n.a.	n.a.	n.a.
PT62	5 w	TND	117 < 12 < 12	0.5 ± 0.3	n.a.	< 12 < 12 < 12	0.2 ± 0	n.a.
PT63	SVR12	TND	TND TND TND	TND	n.a.	TND TND	TND	n.a.
PT77	RELAPSER: 12 w before OLT	24	1977 972	3.1 ± 1.1	n.a.	< 12 < 12	0.1 ± 0	n.a.

marked linear correlation between TT and NT quantifications (rho = 0.666, p-value = 0.103 with HCV-RNA expressed in IU/10^6 cells, *vs*. rho = 0.316, p-value = 0.271 with HCV-RNA expressed in IU/μgRNA).

3.3. Comparison of HCV-RNA concentrations between serum and liver samples of treated patients

Five patients started a DAA treatment before OLT. At the time of OLT, 3 patients were still in treatment, while 2 patients had completed therapy. One of these 2 patients, PT63, showed undetectable HCV-RNA at week-12 of follow-up (sustained virological response, SVR) (Table 4) while another was a relapser with low HCV RNA (PT77); indeed, the only patient who had undetectable HCV-RNA in both serum and all liver specimens was PT63.

Notably, at the moment of OLT, all the other 3 patients still in treatment had undetectable HCV-RNA in the serum, but had detectable HCV-RNA in liver tissues, confirmed also by the triplicate quantifications (Table 4). In fact, any liver tissue sample of these cases had HCV-RNA "undetectable". Interestingly, one patient, who started sofosbuvir + ribavirin 6 weeks before OLT (PT27), showed still a consistent amount of HCV-RNA in both liver tissues, higher in TT than NT tissue (mean ± SD: 51 ± 3.1 vs 7 ± 2.2 IU/μgRNA, respectively, and 612 ± 37 vs 11 ± 4 IU/10^6 liver cells, respectively).

3.4. HCV-RNA quantification in extrahepatic tissue

HCV-RNA was quantified successfully also in the few extrahepatic samples (2 ascitic fluids and 3 lymph nodes) by the Abbott Real*Time* HCV assay (Table 5). When patients were HCV-viremic, ascitic fluid and/or lymph nodes provided positive and quantified HCV-RNA values, differently, when patients had in the serum HCV-RNA as TND, also ascitic fluid and/or lymph nodes showed TND results.

4. Discussion

In this study, we have adapted a validated and highly sensitive technique, the Abbott Real*Time* HCV assay used for HCV-RNA measurement in serum, to quantify HCV-RNA in liver, lymph nodes and ascitic fluid samples collected from HCV patients undergoing OLT or liver resection.

The purpose of our study was to evaluate the reliability of a rapid and commercial method to quantify the HCV-RNA in intra- and extrahepatic compartments of untreated and DAA treated patients.

To our knowledge, the Abbott Real*Time* HCV assay was used for the first time to quantify HCV-RNA in liver, lymph nodes and ascitic fluids, starting directly from a homogenized tissue sample. In addition, after total RNA and DNA quantifications using the same amount of bioptic sample (13 mg), two different normalization procedures were applied to obtain HCV-RNA concentrations expressed in IU/μg of total RNA and/or IU/10^6 liver cells, respectively. This procedure has been used for the first time in our study (Ramirez et al., 2009; Harouaka et al., 2016; Hedegaard et al., 2017; Gambato et al., 2016), and even if the availability of liver samples didn't allow the IU/10^6 liver cells normalization for all patients, it seems a good alternative option. A possible limitation for this type of normalization is the absence of a significant correlation between HCV-RNA in serum and in NT or TT liver samples, probably related to the few cases normalized on cells number in this study.

The results of HCV-RNA quantification in both NT and TT samples showed overall a very small variation proved by the triplicate analysis, where it was available. The reliability of this test was particularly evident among the three measurements of the only treated patient who reached SVR before OLT (PT63), in which HCV-RNA was "undetectable" in all the NT and TT samples.

Notably, the result of "undetectable" HCV-RNA reported in the serum of the other 3 treated patients receiving DAA treatment was not confirmed in NT and TT samples, while it was confirmed in ascetic fluid and/or lymph nodes. This indicates that the Abbott Real*Time* HCV assay is highly sensitive in identifying potential residual HCV-RNA in the liver during treatment, even in the absence of circulating virus in the serum and in other compartments. This result may explain why some patients relapse after the end of treatment, due to the presence of residual HCV RNA in the liver, normally not analyzed.

Interestingly, all our patients, still on treatment during OLT, achieved SVR, independently from the duration of undetectable serum HCV-RNA before OLT, with the exception of PT37, who died immediately after OLT. Our results, though based on a very limited number of patients, are in line with recent data showing that patients with chronic HCV infection and liver cancer receiving sofosbuvir and ribavirin before OLT had no recurrence of HCV infection in 70% of cases when HCV-RNA concentrations were less than 25 IU/mL before transplantation (Curry et al., 2015).

The positive correlation between serum viremia and HCV-RNA in NT tissue from untreated patients may indicate equilibrium between these two compartments: the viral particles released from infected

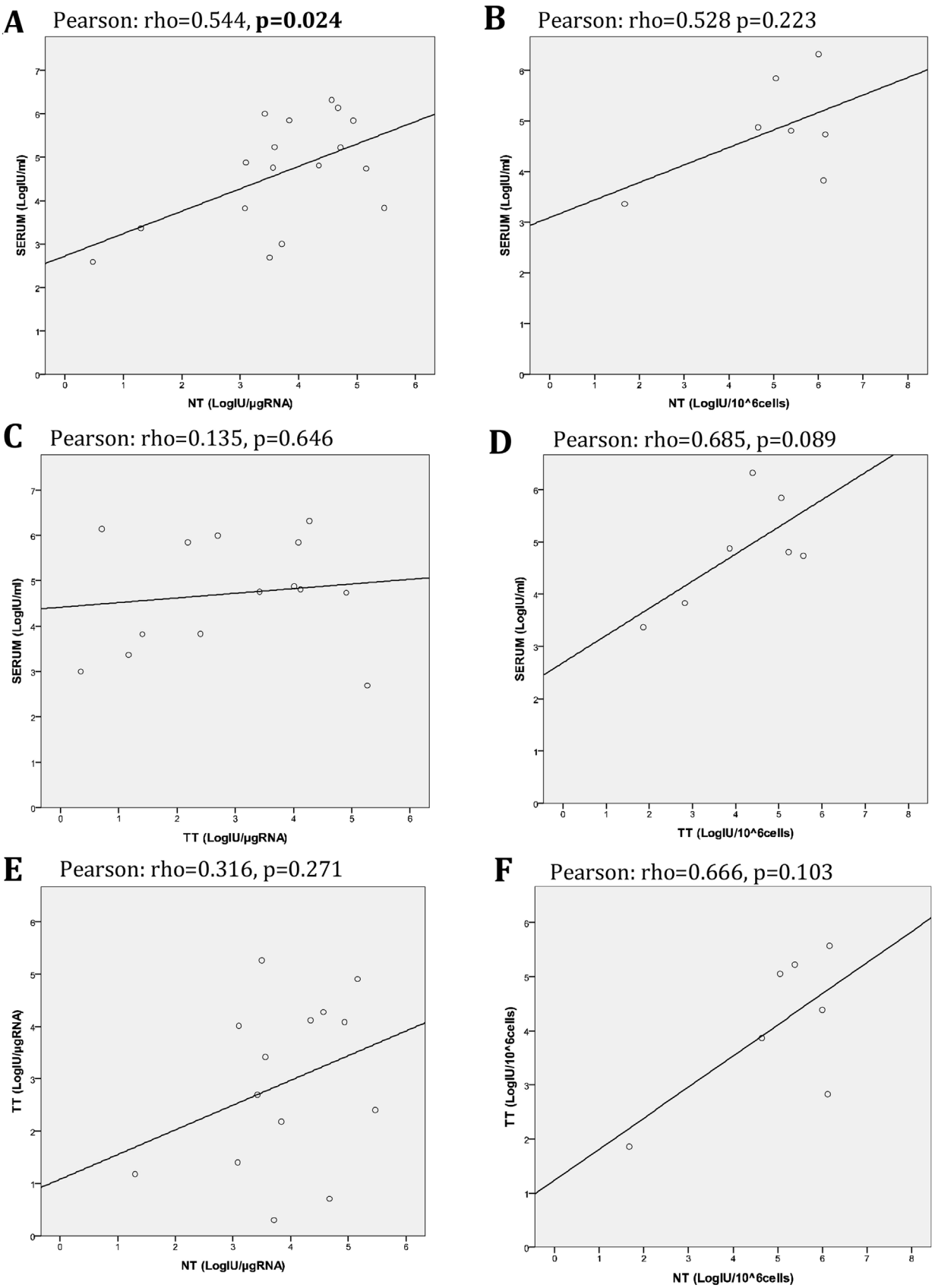

Fig. 2. Correlation between serum, Non Tumoral (NT) and Tumoral Tissue (TT) HCV-RNA in untreated patients. HCV-RNA correlation between serum and NT liver tissue was performed with both Units of Measurement: logIU/µg of total RNA (17 patients) and logIU/10^6 cells (7 patients) **(A, B)**. In **Panel C** and **D**, HCV-RNA correlation between serum and TT liver tissue was performed with both Units of Measurement: logIU/µg of total RNA (14 patients) and logIU/10^6 cells (7 patients). In **Panel E** and **F**, HCV-RNA correlation between NT and TT liver tissues was performed with both Units of Measurement: logIU/µg of total RNA (14 patients) and logIU/10^6 cells (7 patients). Significant P values are shown in bold.

Table 5
HCV-RNA serum and extrahepatic quantifications in patients treated* and not treated with direct acting antiviral agents. PT: Patient; AF: Ascitic Fluid; LN: Lymph node; IU/ml: International Unit per milliliter; IU/μgRNA: International Unit per μg of total RNA; TND: target not detected (HCV-RNA < 12 IU/ml); n.a.: Sample Not Available.

ID	Serum HCV-RNA (IU/ml)	AF HCV-RNA (IU/ml)	LN HCV-RNA (IU/ml)	LN HCV-RNA (IU/μgRNA)
PT27*	TND	TND	TND	TND
PT28	6747	711	n.a.	n.a.
PT37*	TND	n.a.	TND	TND
PT43	1376956	n.a.	22875	95.7

hepatocyte are "efficiently" vehiculated into portal circulation thanks to appropriate vascularization of non-tumoral tissue. On the contrary, HCC, which arises in the setting of cirrhosis, and fibrinogenesis, that results from liver injury and cirrhosis, lead to reduction in vascularization (Wu et al., 2007). The distorted vascularization of tumoral tissue, in association with the altered functionality of tumoral cells, may thus account for the weaker correlation among serum viremia and TT tissue HCV-RNA.

One limit of this study is that we analyzed bioptic liver samples obtained from surgical operations (mainly during OLT), with a general weight of 13 mg of tissue. Future analysis will be required in order to validate the same quantification procedure in smaller bioptic samples obtained by needle aspiration. Furthermore, another limit is that the viral RNA for the quantification and total RNA/DNA were extracted by different procedures with unknown, but probably different, efficiency, as well as by using different liver samples. The quantification of total RNA/DNA from the same liver sample and by applying the same procedure would seem more appropriate, but in our case it was not possible due to the automatic Abbott system.

Interestingly, as a proof of concept for HCV-RNA quantification in extrahepatic compartments, the Abbott Real*Time* HCV assay was also used in a skin biopsy of a patient with cryoglobulinemia and itchy rashes, and the test confirmed the presence of HCV-RNA in the biopsy, with a concentration of 188.6 IU/μgRNA.

In conclusion, our study shows that the Abbott Real*Time* HCV assay is a fast and reliable methodology for the quantification of HCV-RNA in tissues, whenever a liver (or extrahepatic) biopsy is available from a HCV infected patient.

Contributors

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Competing interests

None declared.

Patient consent

Obtained.

Ethics approval

The research study has been approved by the local ethics committee, from the Polyclinic Tor Vergata Foundation.

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