**HCV Therapy Follow-up Fractionation (CTF2) by Intra-PBMC Nested RNA PCR Recognizes Early Virologic Response and Relapse**

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**Abstract**

**Background and Aims:** Sustained virologic response is evaluated by single-step reverse transcription (SRT) PCR assay, which assesses hepatitis C virus (HCV) clearance from plasma but not from tissues such as peripheral blood mononuclear cells (PBMCs). Persistence of HCV RNA in PBMCs beyond end of treatment (EOT) is associated with nonresponse. Our goal was to measure intra-PBMC HCV RNA levels during oral antiviral therapy according to the HCV therapy follow-up fractionation (CTF2) protocol. **Methods:** Compensated chronic HCV patients (n = 278 SRT-PCR positive) were scheduled to receive oral antiviral therapy. Subjects were followed-up by SRT and intra-PBMCs HCV RNA PCR at the end of the 2nd, 6th, 10th, 14th, 18th and 24th weeks to evaluate virus clearance from plasma and PBMCs, respectively. The CTF2 protocol evaluated SRT and PBMC PCR status at each follow-up point for determining therapy continuation or interruption to address cost effectiveness. **Results:** All patients tested negative by SRT PCR after therapy for 2 weeks. Application of the CTF2 protocol revealed: a) increasing HCV clearance rate from 75.9% at the end of 10th week to 90.3% at the end of 24th week (p < 0.0001); b) faster clearance of HCV from plasma compared to PBMCs at each point of follow-up until the 18th week (p < 0.05); c) higher viral elimination rates diagnosed by PBMC HCV RNA PCR(−) compared to PBMC HCV RNA PCR(+) from the 6th to 24th week of treatment (p < 0.0001); d) higher over-time increase curve of combined plasma and PBMC HCV RNA determined negativity compared to the decline in positivity curves by PBMC PCR at the 6th–18th week compared to the 24th week (p < 0.01)—these results validated treatment continuation; and e) solitary evaluation of EOT sustained HCV infection and relapses by PBMC HCV RNA (p < 0.001). **Conclusions:** Early elimination of serum and tissue (PBMC) HCV infection by oral antiviral therapy can be achieved and evaluated during a cost-effective CTF2 protocol application.

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**Introduction**

Worldwide, the highest prevalence of hepatitis C virus (HCV) genotype 4 infection was reported among the Egyptian population. According to the Egyptian Demographic Health Survey (2015), around 15% of the survey responders had anti-HCV IgG antibodies, whereas only 10% were found to have active infection. HCV patients are at risk for developing end-stage liver disease because of complications like hepatocellular carcinoma (HCC) and decompensated liver cirrhosis that may mandate liver transplantation. Chronic HCV infection is associated with an elevated risk for liver-related mortality. The relationship between the severity of HCV-induced liver cirrhosis and host genetics has been predicted by calculation of the cirrhosis risk score (CRS) that reflects variation in nucleotide sequences in a seven-gene signature. In previous studies, the association of increased prevalence of liver cirrhosis in peripheral blood mononuclear cell (PBMC) HCV infection were reported in nonviremic patients.

Tracing efficacy of the current anti-HCV medications is now based solely on the sustained clearance of HCV RNA from sera for up to a minimum of 12 weeks. Despite high probabilities of cure rates in experienced HCV RNA seronegative patients after direct-acting antivirals (DAAs)-therapy, positive intra-cellular HCV RNA remains a good predictor of serologic relapse in posttreatment secondary experienced occult HCV infections. Furthermore, diagnosis and treatment of naive patients who present with nonviremic intracellular HCV infections have not been included in the guidelines. These patients are liable to undergo HCV RNA seroconversion and have subsequent development of active liver cirrhosis. In addition, PBMC PCR was reportedly positive in 61% of cryptogenic HCV infections that presented with chronic hepatocellular dysfunction for more than 6 months and without any serologic evidence of viral infection. Because of the above-mentioned
Aims of the work

a. Evaluating response to oral antiviral therapy (OAT) by both PBMCs and plasma; and, c) address early non-responders to antiviral demands a reliable clinically applied protocol that presents the following advantages: a) eliminating unnecessary extra doses of anti-HCV therapeutic agents; b) reducing medication side effects by shortening the duration of exposure to antivirals; c) recognizing HCV patients who have positive PBMC HCV RNA by PCR at EOT; d) identification of patients who need to extend DAAs therapy beyond the scheduled therapeutic course, based upon laboratory evidence during OAT and without interruption of the original therapeutic course.

c. Evaluation of the need to extended HCV treatment beyond the scheduled original therapeutic course, based upon viral clearance from plasma and PBMCs; and, c) address early non-responders to antiviral medications.

Methods

Study subjects

All study patients 

Table 1. Primer sequences

| Primer name | Sequence (5’-3’) |
|-------------|-----------------|
| 1CH         | ggtgcacggtccagagaccc |
| 2CH         | aactcattgcttcacgcaaa |
| P2          | tgcctgttgtagctctga |
| P3          | cttcgcagcaaaacactac |
| P4          | agagcctatgtctgcgg |
end of 14th and 18th weeks. OAT had to be terminated at the end of the 24th week as recommended by current guidelines.\textsuperscript{12}

### Statistical analysis

All tests performed were two-sided and statistical significance was considered at a \( p \)-value of 0.05. Methods of statistical analysis of the current dataset were carried out as previously illustrated in Abd Alla et al.\textsuperscript{5}

### Results

#### HCV RNA elimination by OAT as determined by SRT and PBMC PCR at each point of follow-up time

All studied patients (\( N = 278 \)) who started OAT for chronic HCV infection had negative SRT PCR after treatment for 2 weeks. Results of CTF2 protocol application were evaluated by testing serum and PBMCs for HCV RNA infection by PCR. As shown in Table 2, application of the CTF2 protocol started at the 2nd week, with assays at 4-week intervals thereafter, up to 24 weeks (the 2nd, 6th, 10th, 14th, 18th and 24th week). Serum HCV RNA elimination, as estimated by SRT PCR was significantly higher than posttreatment viral elimination from PBMC, as determined by PBMC PCR, at the end of the 2nd (217 vs. 61), 10th (67 vs. 47), 14th (49 vs. 18) and 18th (45 vs. 4) weeks (\( p < 0.05 \)). Patients who had PBMC HCV RNA but undetectable plasma viral RNA after the 10th week (\( n = 67 \)) continued the treatment course without interruption and were evaluated by plasma and PBMCs PCR every 4 weeks. The number of PBMC-positive patients gradually decreased at the end of 14th (18 out of 67 vs. 4 out of 49), 18th (4 out of 49 vs. 18 out of 45), and 24th (18 out of 45 vs. 0.0 out of 27) weeks (\( p < 0.001 \)).

### Table 2. Noncumulative individual HCV therapy follow-up fractionation (CTF2) at each point of time by PCR every 4 weeks and at the end of 24th week

| PBMCs-PCR results | CTF2 at each point of time by plasma and PBMC HCV PCRs, \( N = 278 \) |
|-------------------|----------------------------------------------------------------------------------|
|                   | 2\textsuperscript{nd} week, \( n (\%) \) | 6\textsuperscript{th} week, \( n (\%) \) | 10\textsuperscript{th} week, \( n (\%) \) | 14\textsuperscript{th} week, \( n (\%) \) | 18\textsuperscript{th} week, \( n (\%) \) | 24\textsuperscript{th} week, \( n (\%) \) | EOTSCI, \( n (\%) \) | Relapsers, \( n (\%) \) |
| −ve cellular in −ve SRT | 61 (21.9) | 103 (37.1) | 47 (16.9) | 18 (6.5) | 4 (1.40) | 18 (6.5) | 0.0 (0.0) | 0.0 (0.0) |
| +ve cellular in −ve SRT | 217 (78.1) | 114 (41.0) | 67 (24.1) | 49 (17.63) | 45 (16.19) | 27 (9.71) | 15 (5.4) | 11 (3.96) |
| \( p \)-value | <0.00001 | 0.384 | 0.045 | 0.00007 | <0.00001 | 0.214 | 0.00003 | 0.0003 |

All patients (\( N = 278 \)) had negative serum HCV PCR after 2 weeks of OAT. HCV cure rates as estimated by negative serum PCR are significantly higher than those recognized by cellular PCR at the 2\textsuperscript{nd}, 10\textsuperscript{th}, 14\textsuperscript{th} and 18\textsuperscript{th} week (\( p < 0.05 \)). EOT HCV-positive patients and relapers were addressed by PBMCs PCR but not the serum one (\( p < 0.001 \)) at the end of the 24\textsuperscript{th} week. Total relapers include 11 (3.96\%) positive PBMCs PCR plus positive 1 (0.36\%) positive SRT and PBMCs PCRs.

**Abbreviations:** EOT, end of treatment; −ve, negative PCR; +ve, positive PCR; EOTSCI, end of treatment sustained HCV infection; OAT, oral antiviral therapy.

**Fig. 1.** HCV therapy follow-up fractionation (CTF2) results as evaluated by testing of plasma and PBMC HCV RNA by PCR. Fractionation started at the 2\textsuperscript{nd} week, continued every 4 weeks, and ended at 24 weeks (2, 6, 10, 14, 18 and 24 weeks). Forty additional cases (14.4\%) showed negative cellular PCR and raised the total HCV RNA(−) rate from 75.9\% (A) at the end of the 10\textsuperscript{th} week to 90.3\% (B) at the end of the 24\textsuperscript{th} weeks (\( p < 0.00001 \)). Abbreviation: EOTSCI, end of treatment sustained HCV infection.
PBMCs PCR succeeded in recognizing 67 out of 278 and for PBMC HCV RNA between the ends of the 14th and 24th weeks that 40 out of 278 additional cases (14.4%) tested negative in patients who tested positive for PBMC HCV RNA. It shows results of the extended therapy after the scheduled 12 weeks both SRT and PBMC PCRs. So, at the end of the 10th week, PBMCs PCR succeeded in recognizing 67 out of 278 and failed to detect 12 out of 211 (p < 0.0001).

All non-responders were addressed by positive PBMC HCV RNA PCR (p < 0.001), and one case tested positive by both SRT and PBMC HCV RNA PCR. Fig. 1 demonstrates the results of the extended therapy after the scheduled 12 weeks in patients who tested positive for PBMC HCV RNA. It shows that 40 out of 278 additional cases (14.4%) tested negative for PBMC HCV RNA between the ends of the 14th and 24th weeks compared to 211 out of 278 (75.9%) patients who did not receive prolonged treatment. This resulted in improving the total cure rate from 75.9% (Fig. 1A) at the end of the 10th week to 90.3% (Fig. 1B) at the end of 24th week (p < 0.00001).

**Table 3. Results of sequential cumulative negative PBMC HCV RNA PCR results at each follow-up point**

| PBMCs PCR results | CTF2 protocol application results at each point of time by HCV RNA, N = 278 |
|-------------------|----------------------------------------------------------------------------------|
|                   | 2<sup>nd</sup> week, n (%) | 6<sup>th</sup> week, n (%) | 10<sup>th</sup> week, n (%) | 14<sup>th</sup> week, n (%) | 18<sup>th</sup> week, n (%) | 24<sup>th</sup> week, n (%) | EOTSCI + Relapse, n (%) |
| −ve cellular in −ve-SRT | 61 (21.9) | 164 (59.0) | 211 (75.9) | 229 (82.37) | 233 (83.81) | 251 (90.29) | 0 (0.0) |
| +ve cellular in −ve-SRT | 217 (88.1) | 114 (41.0) | 67 (24.1) | 49 (17.63) | 45 (16.19) | 27 (9.71) | 26 (9.36) |
| p-value | <0.00001 | 0.00003 | <0.00001 | <0.00001 | <0.00001 | <0.00001 | <0.00001 |

After 2 weeks of OAT administration, all patients (N = 278) had negative HCV SRT PCR. At the end of the 2<sup>nd</sup> week, patients were subdivided into PBMC HCV RNA(−) (n = 61) and PBMC HCV RNA(+) (n = 217) as diagnosed by PBMCs PCR (p < 0.00001). Starting from the end of the 6<sup>th</sup> week of OAT until the end of the 24<sup>th</sup> week, PBMC HCV RNA(−) rates are significantly higher than the PBMC(+) ones (p < 0.0001). The non-responders that include both EOT patients with positive HCV infection and relappers are significantly recognized by PBMC HCV RNA (p < 0.0001). Total non-responders include 26 (9.36%) PBMC HCV RNA(+) plus positive 1 (0.36%) for both SRT and PBMCs HCV RNA.

Abbreviation: EOTSCI, end of treatment sustained HCV infection.

**Cumulative elimination of plasma and intracellular HCV infections by OAT during application of the CTF2 protocol**

The cumulative cure rates as diagnosed by PBMC HCV RNA(+) and PBMC HCV RNA(−) after treatment with OAT are illustrated in Table 3. All the patients (n = 278) showed negative SRT HCV PCR results after 2 weeks of receiving OAT. According to the PBMCs results, the patients were divided into PBMC HCV RNA(−) responders (n = 61) and PBMC RCV RNA(−) non-responders (n = 217) (p < 0.001) at the end of the 2<sup>nd</sup> week. Starting from the end of the 6<sup>th</sup> week until the end of the 24<sup>th</sup> week of the CTF2 protocol application, PBMC HCV RNA(−) (164 at the 6<sup>th</sup> week, 211 at the 10<sup>th</sup> week, 229 at the 14<sup>th</sup> week, 233 at the 18<sup>th</sup> week, and 251 at the 24<sup>th</sup> week) were significantly increased compared to the PBMC HCV RNA(+) (114 at the 6<sup>th</sup> week, 67 at the 10<sup>th</sup> week, 49 at the 14<sup>th</sup> week, 45 at the 18<sup>th</sup> week, and 27 at the 24<sup>th</sup> week) (p < 0.0001). The same table also shows both HCV-relappers and EOTSCI non-responders.

All non-responders were identified only by PBMC HCV RNA PCR compared to plasma HCV RNA PCR (p < 0.0001). The relationship between the gradual increase in PBMC HCV RNA(−) responders and gradual decrease in PBMC HCV RNA(+) non-responders upon applying the CTF2 protocol is illustrated in Fig. 2. There were significant rises in the number of PBMC HCV RNA(−) and decreases in the number of PBMC HCV RNA(+) upon comparison at the end of the 2<sup>nd</sup> week with the 4<sup>th</sup> week (p < 0.00001), 6<sup>th</sup> week with 10<sup>th</sup> week (p < 0.001), and 18<sup>th</sup> week with the 24<sup>th</sup> week (p < 0.05) but

**Table 4. Role of PBMC HCV RNA in identification of EOTSCI as diagnosed by intracellular HCV RNA after OAT**

| PCR Results | HCV RNA (−) | HCV RNA (+) |
|-------------|-------------|-------------|
| SRT PCR, n = 266 | 266/266 (100%) | 0.0 (0.0%) |
| PBMC HCV RNA, n = 251 | 251/266 | 15/266 |
| p-value | 0.000049 |

Responses to OAT in patients who presented with EOTSCI and had positive PBMC HCV RNA infection (n = 15) significantly reduced the total EOT-response rate from 266 (detected by SRT PCR) to 251 (detected by PBMCs PCR) (p < 0.0001).
insignificant changes when comparing the 10th week with the 14th week (p = 0.07), and the 14th week with the 18th week (p = 0.73). Significant rises in frequencies of PBMC HCV RNA(−) response compared to the decreases in PBMC HCV RNA(+) response by PBMC HCV RNA PCR were noted upon comparing both the 10th and 14th weeks with the 24th week (p < 0.00001 and <0.01 respectively).

**Identification of non-responders (EOT HCV RNA-positive and relapers) as tested by SRT and PBMC HCV RNA PCR**

Table 4 presents the results of non-responders who remained positive for PBMC RNA up to the EOT. Although 266 patients had negative PCR results in their plasma at EOT with OAT, only 251 (94.36%) achieved undetectable HCV RNA in PBMC. The residual 15 cases (5.64%) remained positive for intra-PBMCs HCV RNA. These 15 patients who were diagnosed by PBMC PCR lowered the EOT response rate from 266 to 251 (p < 0.0001). Fig. 3 illustrates the significance of detecting HCV RNA in PBMCs over plasma HCV RNA, where 27 out of 278 (9.71%) were non-responders to OAT at 12-week SVR. The total number of SVR after OAT was significantly higher when diagnosed by plasma SRT PCR (n = 277/288, 99.64%) compared to PBMC HCV RNA (n = 251/278, 90.29%) (p = 0.000046).

**Algorithmic model of the proposed CTF2 protocol based upon combined testing with SRT and PBMCs-PCR**

Fig. 4 demonstrates a proposed CTF2 protocol that evaluates OAT outcomes. Application of the CTF2 protocol will be subjected to further research before it is validated for clinical practice. Algorithms are self-explanatory and clarify step-by-step the application of the CTF2 protocol over time. The core idea behind the reported six algorithms, each in a single cell, is the quantitative control of antiviral therapy by recognizing the shortest most effective therapeutic durations that lead to potential SVR. As illustrated in each algorithm, SRT and PBMC PCRs were performed after the 14th tablet of each DAAs medicine and gave a grace period of 2 weeks to evaluate patient cellular and SRT-PCRs results during OAT. When both SRT and intra-PBMC PCRs became negative, the patient could stop treatment at the end of the grace period. Contrarily, patients who still have positive PCR during grace periods will continue OAT without interruption until the end of 24-week course of treatment.

**Discussion**

Early elimination of HCV RNA from plasma in 100% of the studied cases after 2 weeks of therapy was reported in the current study. However, the actual clearance of viral RNA from both plasma and PBMCs at the same time was observed only in 21.9%. Despite the recorded disparity between serum and PBMC HCV RNA results in the current dataset, the authors followed treatment guidelines in all cases, including those who achieved early cellular clearance of viral infection (21.9%) and, therefore, completed the scheduled antiviral therapy for at least 12 weeks. This finding would explain the occasional reporting of early occurrence of SVR after anti-HCV OAT for a few weeks.

As during practice in infectious disease clinics, sporadic cases occasionally presented with side effects of anti-HCV medicines that mandated premature discontinuation of medications after 3 weeks or less. In a long-term perspective, those cases demonstrated virus-free plasma and PBMC that did not need reintroduction of anti-HCV OAT. These selected cases had variable grades of liver cirrhosis, but liver functions were compensated without encephalopathy or HCC manifestations (unpublished data). This early SVR associated with the currently used OAT may be considered as either dramatic or rapid response, a term that needs further evaluation. Based on the current findings, the term ‘HCV therapy follow-up fractionation’ (the CTF2) protocol is proposed. The proposed CTF2 protocol allows for evaluation of HCV eradication after the first 2 weeks, and then every 4 weeks by plasma and cellular PCR. The expected advantages of the currently proposed CTF2 protocol included reducing medication side effects by shortening the duration of exposure to anti-HCV OAT, eliminating unnecessary extra doses of anti-HCV OAT agents (cost-effectiveness), recognizing the non-responders.

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![Fig. 3. Comparison of PBMCs and SRT PCRs yields after OAT at the end of the CTF2 protocol application including 12-week SVR. The total number of HCV RNA PCR(+) after OAT was significantly higher on using PBMCs PCR (n = 27) compared to SRT PCR (n = 1) (p < 0.0001).](image-url)
Fig. 4. Proposed algorithms for HCV therapy follow-up fractionation (CTF2) protocol as guided by serum and PBMC HCV PCR during the use of any antiviral therapeutic regimen. Algorithms (1–6) describe the relationship of OAT administration periods with intra-PBMC clearance of HCV RNA strands that indicates a high probability of cure. Abbreviations: +ve, positive PCR; –ve, negative PCR; Con. Dif. Reg., consider different regimen; TTT, treatment.
before anti-HCV EOT course, continuing treatment of non-responders without therapy interruption gaps, and early expectation of HCV serologic relapse.

The current dataset confirmed the occurrence of occasional complete cure (i.e. SVR) in some patients who were exposed to anti-HCV OAT for shorter periods than originally scheduled, before completing therapy. None of the 21.9% cases that showed PBMC viral clearance had HCV RNA in their plasma or PBMCs throughout the treatment course or during evaluation of SVR. These results would encourage researchers to further study PBMC or plasma HCV RNA relapse after interrupting the antiviral therapy at points where HCV RNA disappeared concomitantly from PBMC and plasma without proceeding to the EOT course.

Recognition of intracellular HCV RNA strand infections by PBMCs PCR during or just before EOT with any of the currently used OAT regimens (e.g., sofosbuvir and daclatasvir with ribavirin for 3 months) is disturbing and needs further research. During application of the CTF2 protocol, herein, we did not impose contradictions with the currently approved guidelines for OAT. Anti-HCV OAT continuation for an extra 4 to 12 weeks according to PBMCs PCR results helped reach the SVR without discontinuation gaps that may otherwise deprive a patient of the chance to achieve SVR. Theoretically, changes in therapeutic regimens add the advantage of recognition of non-responders and the PBMC HCV RNA PCR could not recognize them earlier before completing the 12 weeks of OAT. This limitation might be related to the occasionally decreased availability of the infected mononuclear cells in patient circulation for a while after completing the treatment course.

In conclusion, we report marked disparity between SRT and PBMCs PCR during evaluation of oral anti-HCV therapeutic efficacies. The achievement of earlier elimination of plasma and cellular HCV infection by antiviral therapy can be accomplished as identified by application of the CTF2 protocol and is expected to be associated with reducing treatment cost in future. Therapy extension beyond the scheduled treatment course is productive and leads to increasing SVR rates through minimizing the number of non-responders. Finally, application of the CTF2 protocol grants early identification of non-responders and potential relapsers in HCV RNA seronegative cases.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design, patient management, data analysis and writing of the manuscript (MDAA), patient recruitment (SE); writing of the manuscript (RE, GW), performance of the PCR screening (MEA, RD).

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