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Research Paper

Increased genomic instability following treatment with direct acting anti-hepatitis C virus drugs

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ABSTRACT

Mixed Cryoglobulinemic Vasculitis (MCV) is a prominent extra-hepatic manifestation of Hepatitis C virus (HCV) infection. HCV has been reported to cause B-cell disorders and genomic instability. Here, we investigated B-cell activation and genome stability in HCV-MCV patients receiving the direct antiviral agent, Sofosbuvir, at multiple centers in Egypt. Clinical manifestations in HCV-MCV patients were improved at the end of treatment (EOT), such as purpura (100%), articular manifestations (75%) and neuropathy (68%). Eighteen patients (56%) showed vasculitis relapse after EOT. BAFF and APRIL were higher at EOT and continued to increase one year following treatment onset. Chromosomal breaks were elevated at EOT compared to baseline levels and were sustained at 3 and 6 months post treatment. We report increased expression of DNA genome stability transcripts such as topoisomerase 1 and TDP1 in HCV-MCV patients after treatment, which continued to increase at 12 months from treatment onset. This data suggest that B-cell activation and DNA damage are important determinants of HCV-MCV treatment outcomes.

1. Introduction

Hepatitis C virus (HCV) is infecting approximately 180 million people worldwide. Risks of HCV infection do not only involve cirrhosis and hepatocellular carcinoma but also extra-hepatic manifestations [1, 2]. Mixed Cryoglobulinemic Vasculitis (MCV) is an important extra-hepatic manifestation of HCV. Circulating mixed cryoglobulins complexes are detected in 40–50% of HCV patients [3]. MCV is a complex immune disease that involves small and medium vessels of the skin, kidneys, peripheral nerves and other tissues [4]. HCV induced MCV (HCV-MCV) is a B-cell proliferative disorder that results from monoclonal B-cell expansion [5]. B-cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL) levels are elevated in several autoimmune disease such as systemic lupus erythematosus (SLE), rheumatoid arthritis, and Sjögren syndrome [6–12]. BAFF plays an important role in activation of B lymphocytes and is increased in HCV-MCV patients [13]. The role of APRIL in autoimmunity is not well identified and is yet to be investigated in HCV-MCV patients. Direct-acting antivirals (DAAs) are proven to provide a high sustained virological response (SVR) with minimal side effects. So far, several researchers have studied the immunological and clinical outcomes in HCV-MCV patients [14–18].

HCV infection can induce double-strand breaks (DSBs) and is able to escape DNA repair mechanisms leading to cancer predisposition and immune dysfunction [19]. HCV core proteins interfere with the normal

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Research in context

Approximately 180 million individuals are infected with HCV. Mixed Cryoglobulinemic Vasculitis (MCV) is an important extrahepatic manifestation of HCV; circulating mixed cryoglobulins are detected in almost half of HCV patients. HCV-MCV involves several organs and tissues in the body and results in high mortality and morbidity. The introduction of treatments that directly targets HCV has greatly improved outcome of antiviral therapy. HCV-MCV is a B-cell disorder that is linked to B-cell activation. B-cell activating factors showed elevated levels in patients with autoimmune diseases, including MCV and correlated with poor IFN-treatment response. HCV patients were reported to exhibit impaired DNA repair capacity and elevated DNA damage has also been reported in patients with HCV extrahepatic manifestations.

Here, we hypothesized that there is an interplay between HCV treatment, B cell activation and DNA damage response for the determination of MCV outcomes as an autoimmune disease and for the manifestation of HCV. We consequently report, *for the first time*, an increased B-cell activation, DNA damage and expression of genome stability markers. We also show that viral clearance is not associated with sustained clinical outcome of HCV. Our study highlights a potential role of B cell and DNA damage response in driving patients' inflammation and determining treatment outcomes. We suggest that inhibition of B-cell activating and manipulating DNA repair capacity could be exploited as novel approaches to improve the treatment outcomes of autoimmune diseases.

activity of many DNA repair proteins such as ATM, NSB1, Chk-2, NEIL1 and P53 leading to inhibition of different DNA repair pathways and impairment of cellular repair capacity [20]. HCV patients were reported to have increased DNA damage levels [21] and patients-derived peripheral blood mononuclear cells (PBMCs) were impaired for base excision repair (BER) activity which was restored with antiviral therapy [22]. Although HCV-associated manifestations such as cirrhosis and hepatocellular carcinoma seem to correlate with increased genomic damage [23], accumulation of DNA damage in HCV-MCV patients has not been investigated so far. In the current study we set out to investigate the roles of DNA damage response and B-cell activation in determining treatment outcomes and long term efficacy of DAAs in HCV-MCV patients.

2. Patients and methods

2.1. Patients

This long term follow-up study initially included 34 Egyptian patients with HCV-MCV diagnosed according to the validated 2014 classification criteria of MCV [24] but ended up with 32 patients. Two patients were excluded from the study; the first patient developed hyperviscosity syndrome diagnosed as Waldenstrom Macroglobulinemia and the second developed an active nephritis which required a high dose of immunosuppression. Thirteen HCV patients without MCV and eight healthy controls were included in the study. The study took place between 2014 and 2017 in several centers in Egypt and only patients who were DAAs treatment naïve were included in the study. Patients with other viral infection such as HIV or HBV, or other autoimmune diseases were excluded from the study. The study was approved by the local institutional research board and the institutional review board for human subject research at National Hepatology and Tropical Medicine Research Institute (NHTMRI) and was performed in compliance with the Declaration of Helsinki. Informed consents were obtained from all patients.

2.2. Antiviral therapy

Different DAAs antiviral therapy combinations were used for patients treatment, according to Egyptian ministry of health HCV treatment protocols. We used three different protocols 1) Sofosbuvir (SOF) plus Ribavirin (RBV) and pegylated interferon (p-IFN) for 3 months ($n = 8$), 2) SOF plus RBV for 6 months ($n = 13$), 3) SOF plus Daclatasvir (DACLA) for 3 months ($n = 11$). Oral prednisolone at a maximum dose of 30 mg/day was allowed while a washout period of at least 4 weeks was needed for higher doses of steroids and other immunosuppressive drugs except rituximab (6 months). After EOT, all patients were followed up at 6 and 12 months from treatment start to study clinical and/or laboratory treatment outcomes. Relapses were studied in 23 out of 32 (72%) patients, who were able to complete the one year follow up. Disease activity was evaluated using the Birmingham Vasculitis Activity Score version 3 (BVAS.v3) [25].

Clinical response was reported as complete response defined by absence of any clinical manifestations (purpura, articular, peripheral neuropathy, constitutional manifestations) at EOT, or partial response: partial improvement (^{3} 50% decrease, compared to baseline) of the clinical manifestations, while no response was reported for patients who did not show any improvement compared to their pretreatment status [26].

Immunological response was reported as complete response (CR) defined by normalized serum levels of rheumatoid factor (RF) and complement (C4) and disappearance of circulating Cryoglobulins (CGs) [26], partial response (PR) is a ^{3} 50% decrease "compared with baseline", and no response (NR) was used when patients did not show any improvement compared to their pretreatment status. Owing to absence of specific renal response criteria for MCV in the literature, we adopted the American College of Rheumatology (ACR) response criteria for proliferative and membranous renal disease in SLE clinical trials to assess renal response at EOT on our patients [27].

2.3. Detection of immunological markers

Immunological markers including RF and C4 were assayed by Nephelometry following established procedures (RF is positive if >15 IU/ml, C4 is consumed if <10 mg/dl), using BN ProSpec; Siemens, Germany. The CGs were obtained by cold precipitation (4 °C for one week) (considered positive if >1%). Briefly, blood samples were incubated at 37 °C and centrifuged at a warm temp., obtained serum was then incubated at 4 °C for 7 days until a whitish floccules or precipitate is visually observed. To obtain cryocrit, serum samples were incubated at 4 °C for 7 days, whitish floccules or precipitates could be observed, then Wintrobe tubes were centrifuged at 4 °C, 2000 rpm for 10 min. To confirm that the observed precipitates are Cryoglobulins, Wimtrobe tubes were incubated at 37 °C water bath for 30 min, the precipitates became translucent and upon refrigeration for 30 min the whitish precipitates formed again [28–31] . HCV-RNA was measured by real time polymerase chain reaction (PCR) and anti-HCV antibodies were detected by enzyme-linked immunosorbent assay (ELISA).

2.4. Quantification of DSBs in patients-derived PBMCs

PBMCs were isolated from fresh whole blood samples using Ficoll-Hypaque density gradient centrifugation method. Cell viability was determined by the trypan blue exclusion method. Double strand breaks were quantified by neutral comet assay as described in [32]. Briefly, freshly isolated PBMCs were suspended at a concentration of 2×10^5 in pre-chilled phosphate buffered saline (PBS) and mixed with an equal volume of 1.3% low-gelling-temperature agarose (Sigma, Type VII), the suspension was then immediately casted on pre-chilled frosted

glass slides (Fisher) pre-coated with 0.6% agarose and were allowed to set under cover slips on an ice-packs. After solidification, the slides were incubated at 4 °C in the dark for 1–2 h in the lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mMTris–base, pH 10), immediately before use 1% Triton X-100 and 10% dimethylsulphoxide (DMSO) were added to the buffer and mixed for 20 min. After lysis, the slides were washed three times with prechilled distilled water and incubated for 30 min in prechilled electrophoresis buffer (300 mM sodium acetate, 100 mMTris–HCl, pH 8.3). Electrophoresis was conducted at 1 V/cm for 30 min, followed by neutralization in 400 mMTris- HCl (pH 7.0). For strand breaks quantification, DNA was stained with SybrGreen I nucleic (Sigma) (1:10000, in PBS) for 30 min. Average tail moments from 100 cells/sample were measured using Comet Assay IV software (Perceptive Instruments, UK) [33].

2.5. RNA isolation

RNA was extracted from patients PBMCs using QIAamp RNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer instructions. Briefly, 500 to 1500 μl of fresh whole blood were washed with EL buffer with a single incubation on ice for 10–15 min, followed by centrifugation at 400 xg for 10 min at 4 °C. Pelleted leukocytes were resuspended in 600 μl RLT buffer (supplemented with β-Mercaptoethanol). Samples were vortexed shortly and loaded directly to QIAshredder column and centrifuged. An equal amount of ethanol was added to the flowthrough and was run through the spin column followed by washing. RNA was eluted in 30 μl RNAae/DNAae free water. RNA purity was assessed (A260/A280) using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Massachusetts, USA). RNA was stored at −80 °C.

2.6. Reverse transcription

cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (ABI), with random priming. A concentration of 100–200 ng RNA/μl was used for cDNA preparation and the reaction was performed according to the manufacturer instructions to a final reaction volume of 100 μl. A simultaneous reverse transcription negative control reaction (without the reverse transcription enzyme) was prepared in parallel to the samples. cDNA was stored at −20 °C until use.

2.7. Quantification of gene expression assays using taqman probe assays

The cDNA product from the RT reaction was used for TaqMan PCR quantification in a final reaction volume of 20 μl, using the SensiFAST Probe low-ROX master mix (BIOLINE, UK). A 20× mix of primers and FAM-labeled probe for the human TOP1, TOP2B, TDP1, TDP2, PARP1, XRCC1, APRIL and BAFF gene expression assays were purchased through ABI's Gene Expression Assay-on-Demand (Assay ID: Hs00243257_m1, Hs00172259_m1, Hs00217832_m1, Hs01099017_m1, Hs00242302_m1, Hs00959834_m1, Hs00601664_g1, Hs00198106_m1, Hs02758991_g1, respectively). Each assay contains 20× mix of primers and FAM-labeled probe. The housekeeping gene GAPDH was used for normalization (Assay ID: Hs02758991_g1). TaqMan qRT-PCR gene expression assays were conducted in 0.1 ml fast tubes (Applied Biosystems) according to the manufacturer instructions, in a final volume of 20 μL. All samples were run on Quantstudio 12KFlex (Applied Biosystems) RT-PCR system using standard settings (thermal profile included 10 min incubation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min). Each sample was run in triplicates; Cycle Threshold (Ct) values were collected automatically using the QuantStudio 12 k Flex software (Applied Biosystems). gene expression values were calculated using comparative delta delta CT method, as reported previously [34].

2.8. Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 24. Data were summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (%) for categorical data. For comparison of serial measurements of each patient the non-parametric Wilcoxon signed rank test was used [35]. For comparing categorical data, Chi square ([2] test was performed. Exact-test was used instead when the expected frequency was <5 [36]. Correlations between quantitative variables used Spearman correlation coefficient [37]. *P*-values<.05 were considered statistically significant. Pretreatment and follow-up values were analyzed and compared using paired samples *t*-test, at 95% confidence intervals were calculated using the exact formula. All analyses and graphs were performed with Graph Pad Prism version 7 (GraphPad Software, La Jolla California USA).

3. Results

3.1. DAAs antiviral therapy improves Cryoglobulin levels after therapy but not on the long term

Our study included 32 patients, 25 females (78.1%) and 7 males (21.9%) with a mean age of (54.9 ± 9.7) years. Fourteen patients had liver cirrhosis (43.8%), all of whom were Child A class. All patients (100%) showed negative HCV-RNA as detected by RT-PCR after one month of treatment and throughout the follow up period. We observed an overall significant improvement in most of the clinical and laboratory parameters of MCV including purpura, articular, peripheral neuropathy and constitutional manifestations such as BVAS, C4, RF and Cryocrit at EOT. Serum levels of creatinine, creatinine clearance, and 24 h urinary proteins were also improved, although non-significantly. All the observed improvements were independent of treatment protocols (Table 1).

Overall, HCV-MCV patients showed an initial improvement in clinical and laboratory responses, 87.4% and 81.6%, respectively, at EOT and BVAS also improved by >50% in 87.5% of the patients. CGs levels were measured in all patients at four points; baseline (before treatment), 3 months, 6 months and 12 months from treatment start. Baseline levels of CGs were (8.4% ± 1.6) and decreased to (3.19% ± 1.1), (1.58% ± 0.32) and, (1.82% ± 0.5) by wk. 12, 24 and 48, respectively. Overall, CG levels improved in 16/32 patient (50%) at EOT and 7/32 patients (22%) relapsed after completing the antiviral therapy, either at 3

Table 1
Clinical and laboratory improvement at EOT

Clinical and laboratory data	Before treatment	End of treatment	*P* value
Purpura N (%) (Number)	31(96.9%)	0(0%)	<**0.001**
Articular manifestations N (%)	29(90.6%)	5 (15.6%)	<**0.001**
Peripheral neuropathy N (%)	27(84.4%)	5(15.6%)	<**0.001**
Constitutional manifestations N (%)	32(100.0%)	5(15.6%)	<**0.001**
Serum creatinine (mg/dl)	1.69 ± 0.72	1.31 ± 0.91	0.176
Creatinine clearance (mg/ml/min)	66.71 ± 40.08	92.29 ± 50.67	0.204
24 h urinary protein (mg/day)	1528.20 ± 1665.15	367.60 ± 271.29	0.08
BVAS	13.47 ± 4.56	4.97 ± 2.79	<**0.001**
Serum RF (IU/ml)	382.45 ± 940.01	727.99 ± 3699.97	<**0.001**
Serum C4 (mg/dl)	7.85 ± 7.49	11.18 ± 8.06	**0.011**
Serum Cryoglobulins (%)	8.41 ± 9.06	2.94 ± 5.59	< **0.001**

EOT, end of treatment; BVAS, Birmingham vasculitis activity score; RF, rheumatoid factor; C4, Complement 4; N, Number of cases; mg/dl, milli- grams per deciliter; mg/ml/min, milli- grams per milli-liter per minute; IU/ml, International unit per milli-liter; %, percent. Bold denotes statistical significance with p value < 0.01.

Table 2
Relapses of clinical and laboratory parameters of HCV-MCV

Relapses at 1 year follow up	EOT			P value
	Total N	Deterioration		
		Count	%	
Purpura	22	1	4.5%	–
Articular manifestations	17	5	29.4%	**0.009**
Peripheral neuropathy	15	6	40.0%	1
Constitutional manifestations	19	5	26.3%	**0.006**
Serum RF (IU/ml)	18	5	27.8%	1
Serum C4 (mg/dl)	17	2	11.8%	0.270
Serum Cryoglobulins (%)	20	5	25.0%	**0.002**

HCV-MCV, Hepatitis C virus induced Mixed Cryoglobulinemic Vasculitis; EOT, end of therapy; RF, rheumatoid factor; C4, Complement 4; %, percent.
Bold denotes statistical significance with p value < 0.01.

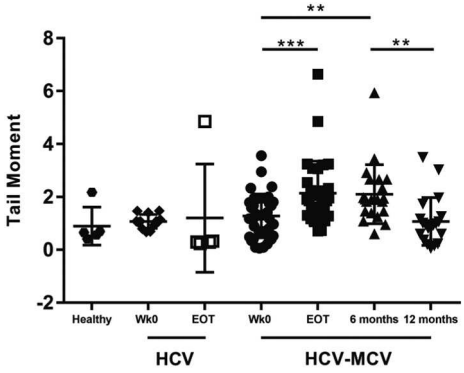

Fig. 2. DNA damage level, measured using single cell electrophoresis (comet assay),in fresh PBMCs isolated from controls (healthy individuals) ($n = 9$) and HCV-MCV patients at pretreatment (n = 32), EOT (end of treatment) (n = 32), month 6 (n = 32) and 12 months ($n = 23$). Data is reported as mean ± SEM. * = $p < .05$, ** = $p < .005$. HCV, hepatitis C virus; EOT, end of treatment; HCV-MCV, Hepatitis C virus induced Mixed Cryoglobulinemic Vasculitis; N, Number of cases.

months or 6 months. At end of follow up period (one year after EOT), five patients (29.4%) relapsed for articular manifestation ($P < 0.005$), and five patients (26.3%) also relapsed for constitutional manifestations ($P < 0.005$), and CGs levels showed a significant relapse in 5 patients (25.0%, $P < 0.002$) (Table 2).

3.2. B cells are hyperactive after receiving DAAs antiviral therapy

We measured the expression of two main B cell factors, BAFF and APRIL in HCV patients receiving different SOF treatment combinations with and without MCV at different time points, before, at EOT, and at two points after treatment (6 and 12 months). HCV patients showed increased BAFF expression levels from 3.29 ± 1.1 (before treatment) to 7.3 ± 5.9 (at EOT) ($p > 0.05$). HCV-MCV patients showed a mild increase in BAFF levels at EOT from 1 (normalization point) to 1.22 ± 0.37, $p > 0.05$. At 6 and 12 months follow up points, BAFF levels spiked to 1.91 ± 0.7, $p < 0.05$ and 6.08 ± 2.7, $p < 0.005$, as compared to their baseline levels (Fig. 1A). The other B cell marker, APRIL, displayed an increased expression at end of treatment 7.03 ± 3.3 compared to baseline levels of 5.93 ± 1.8 in HCV patients, $p > 0.05$. In HCV-MCV patients, APRIL expression increased at EOT to 2.58 ± 0.7, which is significantly higher than its pretreatment level ($p < 0.05$). APRIL expression continued to increase at 6 month (2.83 ± 1.4) and 12 months (3.77 ± 1.5), $P < 0.005$ (Fig. 1B). The increased expression of those two markers indicates a continuous activation of B cells even after completing antiviral therapy and achieving viral eradication.

3.3. Increased chromosomal breaks in patients receiving Sofosbuvir based treatments

We employed the single-cell gel electrophoresis (comet assays) to evaluate DNA damage levels in PBMCs derived from HCV-MCV patients. DNA damage levels were also measured in the control groups, HCV patients without MCV and apparently healthy individuals. DNA damage levels were assessed at pretreatment (wk0), EOT, 6 and 12 months. Healthy individuals and HCV patients without MCV showed similar damage levels. We did not find a difference in baseline damage levels between HCV patients with and without MCV ($P > 0.05$). HCV-MCV patients showed significantly increased damage levels at EOT (2.15 ± 0.2), compared to pretreatment levels (1.28 ± 0.1; $P < 0.0005$). Importantly, the DNA damage levels at six months were maintained at similar levels compared to those at EOT (2.10 ± 0.2). However, at 12 months, DNA damage levels showed a remarkable reduction (1.07 ± 0.2) and returned back to a baseline levels observed before antiviral therapy (Fig. 2).

3.4. Increased activity of genome instability markers after DAAs therapy

We measured the expression levels of six DNA damage repair genes. *TOP1, TOP2, TDP1, TDP2, XRCC1 and PARP1*, which play key roles in a variety of DNA metabolism and repair processes. Consistent with the increased DNA damage levels, HCV-MCV patients showed reduced expression for all measured repair genes compared to HCV patients without MCV, at both points (before and at EOT). The expression of

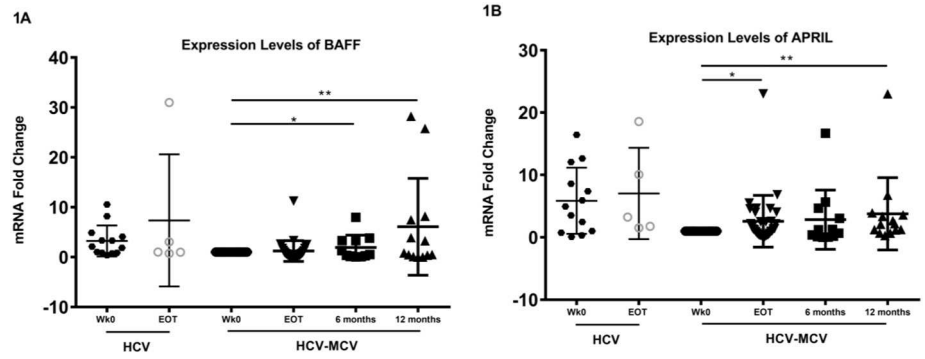

Fig. 1. B cell markers BAFF and APRIL expression levels in HCV patients with and without cryoglobulinemia. Gene expression was carried out as described in the last figure. BAFF and APRIL levels were higher in cryoglobulinemia and HCV patients compared to patients without cryoglobulinemia. After treatment BAFF and APRIL levels continued to increase until 12 months, in ($n = 32$), at the end of treatment (EOT) ($n = 32$), 6 months ($n = 19$) and 12 months ($n = 23$). Data is reported as mean ± SEM, * = $p < .05$, ** = $p < .005$. BAFF, B-cell activating factor; APRIL, A proliferation-inducing ligand.

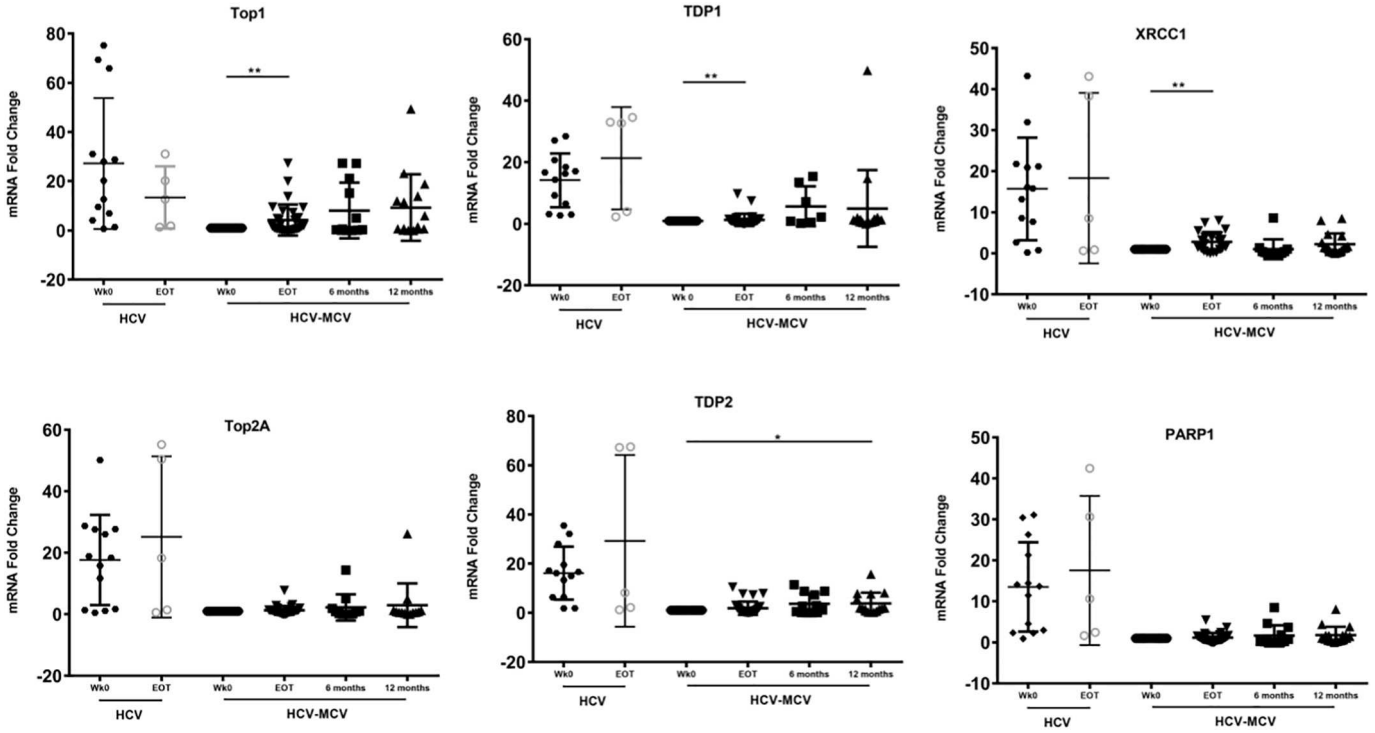

Fig. 3. Increased expression of major DNA instability markers in HCV-MCV after receiving DAAs therapy. At baseline, HCV-MCV patients are showing reduced expression of DNA repair proteins but at EOT their activity starts to improve. At 3 and 6 months from treatment start the activity of repair proteins increases to reach its peak at 12 months. RNA was isolated from patients leucocytes and expression levels of six repair genes was measured at the indicated points using taqman probe assays and GAPDH was used a house keeping gene, HCV mRNA levels were normalized to health individuals and patients post treatment levels were normalized to their pretreatment levels. Repair genes expression levels were calculated using delta deltact method in, before treatment (n = 32), at the end of treatment (EOT) (n = 32), 6 months ($n = 19$) and 12 months (n = 23). Data is reported as mean ± SEM, * = $p < .05$, ** = $p < .005$. HCV-MCV, Hepatitis C virus induced Mixed Cryoglobulinemic Vasculitis; TDP1, Tyrosyl- DNA phosphodiesterase 1; TOP1, Topoisomerase 1; PARP1, poly (ADP-ribose) polymerase 1; XRCC1, X-ray repair cross-complementing protein 1; N, Number of cases.

DNA repair genes showed a high level at EOT, after viral eradication, and subsequently their expression continued to increase at 6 and 12 months. Furthermore, DNA single-strand break repair markers such as TOP1, TDP1 and XRCC1 showed higher expression levels at EOT, 6 and 12 months follow up, compared to pre-treatment levels (Fig. 3).

4. Discussion

The introduction of DAAs had greatly altered HCV therapy in the past few years [38]. Most of the available studies included patients receiving SOF-based treatment combinations [14, 15, 17] but there is a lack of follow up studies that can rule out the efficacy and safety of DAAs treatment regimen. HCV-MCV results from B-cell expansion and B cell activating factors such as BAFF and APRIL, which are predicted to play a role in disease progression and treatment outcomes [6–11]. HCV has been reported by several studies to interfere with DNA damage response [19, 20, 39–41]; also increased DNA damage levels were reported in patients with HCV-related manifestations such as cirrhosis and carcinoma [23]. Here we report, *for the first time*, the effect of SOF based treatment combinations on B cell activation, DNA damage accumulation and genome stability markers in the landscape of HCV-MCV patients. We also evaluated their long term efficacy in reducing MCV-related immunological burden in HCV-MCV patients.

Our data show that constitutional manifestations (100%), purpura (96.9%), articular manifestations (90.6%), peripheral neuropathy (84.4%) and renal manifestations (21.9%) to be the most frequent clinical manifestations of HCV-MCV. This is consistent with rates reported by other groups [18, 42]. Since HCV-MCV is an antigen driven disease, viral eradication is expected to be the most effective therapy [43]. After initial improvement at EOT, as shown by the significant reduction in BVAS score (From 13.47 ± 4.56 to 4.97 ± 2.79, $P < 0.001$) and SVR through the one year follow up, MCV relapses were significantly evident in our patients, as shown by the articular and constitutional manifestations, and elevated serum cryoglobulins (Table 2). The observed variation in the clinical response to antiviral therapy showed by our patients (Table 1) does not seem to be dependent on viral clearance only. This variation could be attributed to the difference in viral genotypes, genetic and environmental factors. Another explanation could be the difference in duration between viral infection and the onset of viral infection or MCV manifestations, especially that the efficacy of IFNα was attributed to its anti-proliferative and immune-modulatory properties in addition to its antiviral activity [44]. Moreover, the high rates of DAAs SVR, >90%, were argued not to achieve equivalent rates in HCV-MCV patients, maybe due to their inability to suppress the immune-mediated process once it has been triggered [45]. Despite the previous findings, patients who received IFN treatment regimens did not show an improved immunological response or lower relapse rates compared to patients who received interferon free regimens ($P > 0.05$). Overall, 12.6% of the patients were immunological non-responders and suffered a relapse rate of 24.6% at one year follow up, which sheds more light into the importance of long follow-ups after completing antiviral therapy.

BAFF and APRIL activity is under a tight cellular control in order to maintain B-cell homeostasis [46]. Dysregulated BAFF and APRIL expression is related to different autoimmune diseases such as SLE and Sjogren's syndrome [6, 10, 47, 48]. In mice, ectopic expression of BAFF caused excessive B-cell expansion, BAFF transgenic mice suffered dysregulated immunity and exhibited circulating autoantibodies [49, 50]. At EOT, BAFF levels were higher in HCV-MCV patients compared to patients with HCV infection only; decreased BAFF levels are associated with better IFN treatment outcome [51, 52]. Our results showed an increased expression of BAFF and APRIL in HCV-MCV patients at EOT compared to their pre-treatment levels. BAFF and APRIL levels continued to increase throughout the follow up points (Fig. 1). These high levels of BAFF and APRIL seemed to stimulate B cell survival and could be an

explanation to the recurrence or relapse of cryoglobulinemia, which indicates that despite the observed initial improvement in cryoglobulinemia and viral clearance self-reactive B cells remained active. Blocking BAFF signaling was suggested to attenuate SLE in disease-prone mice and enhance mice survival [10, 53] and our results recommend using BAFF and APRIL blocking agents to improve MCV treatment outcome after antiviral therapy.

Although the interplay between HCV infection and genomic stability has been reported by few studies, the role of DNA repair in HCV-MCV patients receiving SOF treatment remains largely unknown. HCV replication induces oxidative stress resulting in DNA damage [39, 40], which is thought to be playing a pathogenic role in HCV infection [54]. HCV has also been reported to impair multiple DNA repair activities, leading to chromosomal rearrangements and deletions, which results in cancer predisposition and immune dysfunction [19]. In addition, HCV core proteins inhibit the repair function of p53 in HCV related carcinoma [41]. Increased DNA breaks were observed in HCV infected HepG2 cells as shown by high γH2AX [55]. In the light of this, we expected HCV-MCV patients to display a higher DNA damage levels than patients without MCV. However, we did not find a difference in baseline DNA damage levels between both groups ($P > 0.05$). In contrast, and to our surprise, we found an elevated DNA breaks at EOT, in HCV-MCV patients compared to pretreatment levels (2.15 ± 0.21 and 1.28 ± 0.13, $P < 0.005$). This increased DNA damage levels persisted for 6 months and returned to normal levels only after 12 months. These results are consistent with previous reports showing high rate of tumor recurrence in patients treated with DAAs and failure of DAAs to reduce hepatocellular carcinoma (HCC) in HCV patients [56–58]. It is worth noting that another study did not find higher carcinoma risk among patients receiving DAAs [59]. Compared to HCV patients, pretreatment and EOT levels of DNA repair genes were reduced suggesting an inhibition of DNA repair in HCV-MCV patients. After EOT, repair genes expression increased to show its highest level at 12 months, the point at which most of HCV-MCV showed high relapse rate. In this regard, our findings of reduced baseline topoisomerases 1 and 2 (TOPs) expression in MCV patients are consistent with other studies of decreased TOP1 activity in patients with autoimmune diseases [60]. Unfaithful TOPs activity is involved in developing several human diseases [61–63]. Furthermore, TOP1 inhibition was suggested to suppress microbial activated genes [64] and thus our data suggest a role for TOPs in driving host inflammation in the context of HCV-MCV.

PARP1 is a DNA damage sensor that regulates transcription in several immune cells and affects their stimulatory ability and antibody production [65]. PARP1 deficient cells are compromised for the genome master kinase caretaker, ataxia telangiectasia mutated (ATM) activity and shows reduced repair in response to irradiation [66]. Insufficient ATM activity leads to DNA damage accumulation and T-cells death [67]. PARP1 inhibition decreases pro-inflammatory cytokines secretions and enhances autoimmune disease outcomes [68]. Consistent with this, our results suggest that PARP1 is implicated in another autoimmune disease, MCV. TDPs (TDP1 and TDP2) function to release trapped TOPs from the DNA [69, 70]. Interestingly, simultaneous inhibition of TOP1 and TDP1 was suggested as a promising approach for SLE treatment [71]. Although they are playing an important role in removing TOPs covalent complexes, which are implicated in several immune diseases, TDPs role remains poorly studied in autoimmune diseases and viral infections. Our results suggest that TDPs could be valuable tools to determine the clinical outcome of MCV after antiviral therapy. XRCC1 functions as a scaffold for several repair proteins [72]. XRCC1 also participates along with TDP1 in repairing TOP1-DNA covalent complexes [73]. Polymorphisms in XRCC1 gene have been linked to SLE susceptibility and clinical symptoms [74, 75]. Overall, the high expression of DNA repair genes that are involved in driving host inflammation could be implicated in long- term cryoglobulinemia relapses. Our data is consistent with work by *Obata* et al., *2017* showing complete remission of MCV after receiving DAA therapy without immunosuppressant, which was followed by cryoglobulinemia recurrence after 17 months from treatment initiation [76].

In conclusion, this work reinforces the utility of DAAs as promising treatments for HCV-MCV with proven efficacy and safety. Notably, our patients displayed cryoglobulinemia relapses despite viral clearance. This work suggests that increased B cell activation and DNA damage are important determinants of treatment outcome in HCV-MCV patients. The elevated B cell activation and reduced DNA repair capacity following DAAs treatment highlight the need for comprehensive studies that could better address the long-term safety of DAAs therapy, especially with the increasing reports of increased hepatocellular carcinoma rates in HCV patients following DAAs therapy. Our data show that the cellular DNA damage response is an important determinant of not only the outcome of HCV infection but also its related pathogenesis. We suggest that pharmacologic manipulation of the DNA repair proteins could be a promising approach for improving treatment outcomes of HCV and MCV.

Declarations of interests

The authors have declared that there are no conflicts of interest.

Author contribution

M.T.H. recruited the patients, conducted clinical examination, collected the samples and managed all clinical data. W.R.A. processed the clinical samples and conducted the DNA repair and B-cell experiments. N.Z. supervised the serological and immunological examinations. W.R.A., M.T.H, M.A.H., G.R. and S.F.*E*-K wrote the manuscript. All authors contributed to statistical analyses, data interpretation and manuscript writing. S.F.E-K and G.R. conceived the study, coordinated and managed the project.

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