ANSWERING REVIEWERS

30th October, 2013

Dear Editor,

please find enclosed the revised manuscript in Word format (file name: ms 5315 Hepatitis Viruses and Clock gene revised Pazienza V_final WJG2013).

Title: Exploitation of host clock gene machinery by hepatitis viruses B and C
Author: Manlio Vinciguerra, Gianluigi Mazzoccoli, Claudia Piccoli, Tiziana Tataranni, Angelo Andriulli and Valerio Pazienza

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 5315

Reviewer's comments

The authors of the MS "Exploitation of host clock gene machinery by hepatitis viruses B and C" are introducing interesting interactions of immune system, circadian system and viral infection.

Although involvement of the circadian clock in regulation of many aspects of human physiology is obvious and our knowledge about this process is growing, there are still many unclear points and huge space for further experimental work.

Reviews generally possess strong opinion making potential. Therefore the choice of references and synthesis of data from experimental articles is major responsibility of the authors. Recently, many reviews are composed from other reviews (instead of experimental articles) without reasoning based on experimental work.

I see a major deficit of present review in weak referring to experimental evidences. Authors frequently propose idea without supporting evidence.
All statements must be shown to be proved and additional references must be added. Hypothesis and statements (what is believed and what is proved) must be clearly distinguished.

Thank you for careful review of our manuscript entitled "Exploitation of host clock gene machinery by hepatitis viruses B and C". We are pleased to submit a new version, revised according to the reviewer's comments (please see below the comments, in italics, and our point-by-point replies, in red). We hope that the manuscript is now acceptable for publication in World Journal of Gastroenterology, and we look forward to hearing from you at your earliest convenience.
Thank you very much for your time, and best regards.

p. 7
sentence: The clock gene machinery drives the expression of a large array of enzymes involved in lipid metabolism, controls lipogenesis and regulates triglyceride packaging into chylomicrons

(globules that transport dietary lipids) at the level of the intestine, whereas in the liver, clock disruption triggers lipid accumulation [29].
The statement is unsupported. Authors refer to commentary to the Science article (instead of science article alone) and overestimate conclusions of experimental work.
29. Duez H, Staels B. Circadian Control of Epigenetic Modifications Modulates Metabolism. Circulation Research. (2011) 109: 353-355
cited instead of
Feng D, Liu T, Sun Z, Bugge A, Mullican SE, Alenghat T, Liu XS, Lazar MA: A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. Science. 2011 Mar 11;331(6022):1315-9. doi: 10.1126/science.1198125.

We apologize for missing the right reference, we thank the reviewer for the constructive criticisms and we have now replaced the old references with the correct one.

Statement that "clock gene machinery drives expression of a large array of enzymes" must be supported, enzymes activity are in most cases driven by other ways than transcription therefore array can not be used as evidence. I invite authors to name at least 5 enzymes together with experimental work where it was shown that circadian clock drives activity of some enzyme involved in lipid metabolism (please, not coincidence but real regulatory connection).

We thank the reviewer for this comment. We now provide the name of the following enzyme and the appropriate references:
3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), fatty acid synthase (FASN), low density lipoprotein receptor (LDLR), elongation of very long chain fatty acids protein 6 (ELOVL6), fatty acid desaturase (SCD1)
Cretenet G, Le Clech M, Gachon F. (2010). Circadian clock-coordinated 12 Hr period rhythmic activation of the IRE1alpha pathway controls lipid metabolism in mouse liver. Cell Metab. 11(1):47-57.

cholesterol-7alpha-hydroxylase (CYP7A1)
Le Martelot G, Claudel T, Gatfield D, Schaad O, Kornmann B, Sasso GL, Moschetta A, Schibler U. (2009). REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. PLoS Biol. 7(9):e1000181.

- other examples:
p. 2 - abstract
sentence: Dysfunction of the circadian clock molecular circuitry is associated with human health derangements, including neurodegeneration, increased risk of cancer, cardiovascular diseases and the metabolic syndrome.
- evidences to this statement exist but they are not mentioned anywhere in the article. Relationship to the topic is not clear.

We thank the reviewer for this comment. This sentence was reported in the abstract session, for this reason we now provide new references at page 5 supporting the statement about the link between the clock genes and cancer in agreement with the reviewer's comment.

15- Wood PA, Yang X, Hrushesky WJ. Clock genes and cancer. Integr Cancer Ther. 2009 Dec;8(4):303-8. [PMID 20042409 doi: 10.1177/1534735409355292].

16- Fu L, Pelicano H, Liu J, Huang P, Lee C. The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo. *Cell*. 2002;111:41-50. [PMID 12372299 DOI: 10.1016/S0092-8674].

17- Hua H, Wang Y, Wan C, Liu Y, Zhu B, Wang X, Wang Z, Ding JM. Inhibition of tumorigenesis by intratumoral delivery of the circadian gene *mPer2* in C57BL/6 mice. *Cancer Gene Ther*. 2007;14:815-818. [PMID:17589433 DOI:10.1038/sj.cgt.7701061]

p. 3
sentence: It has been already established that alteration of the circadian clock molecular circuitry is involved in carcinogenesis.
- references are missing, unsupported

We preferred to omit the reference as the sentence would be reported in the abstract session. Moreover we provided 3 new references at page 5 as reported in the previous comment.

p. 4
sentence: Among the processes regulated by the clock gene machinery are pathways of cell metabolism and vesicle trafficking, suggesting the potential role for the circadian clock circuitry in the regulation of viral expression/replication [14].
- unsupported statement, reference 14 is not experimental, just theoretical model and/or review

We thank the reviewer for this comment. We introduced a reference (n. 14) dealing with theoretical model as we were referring to a *"suggestion"* from which the experimental models start.

p.5
sentence: ...driven by molecular clockworks ticking through translational-transcriptional feedback loops and operated by a set of genes, called clock genes, encoding circadian proteins.
- I suggest to mention also review of some investigator really involved in describing of feed-back loop together with the reference 4, it is still quit recent discovery.

We thank the reviewer for this constructive criticism: we have now replaced our reference with a very recent review of the one of the most well know scientist in the chronobiology field: Demarque M, Schibler U. Shedding new light on circadian clocks. Elife. 2013 Apr 9;2:e00659. [PMID:23580350 DOI: 10.7554/eLife.00659].

sentence: In the absence of environmental cues, specifically light:dark input, it has been demonstrated that rhythmic food intake influences the hepatic circadian oscillator [21].

- clock gene in the liver synchronize to food even in presence of ligh:dark regimen. This discovery was published in paper: Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U.: Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 2000 Dec 1;14(23):2950-61. cited -758 in scopus
- food is dominant synchronizing cue for the liver even in presence of light:dark input

We thank the reviewer for the constructive criticism. We now add the suggested reference:
Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U.: Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 2000 Dec 1;14(23):2950-61

p. 7
sentence: PPARγ binds eicosanoids deriving from either omega-3 (ω-3) or omega-6 (ω-6) fatty acids and their oxidized counterparts, is rhythmically expressed, its expression is regulated by PER2 and in turn directly regulates BMAL1 transcription [28].
- authors of the article 28 did not show that PPARγ regulates bmal1 transcription, please provide reference
We apologize for the improper reference: we now replaced the reference 28 with the correct one:
Maury E, Ramsey KM, Bass J. Circadian rhythms and metabolic syndrome: from experimental genetics to human disease. Circ Res. 2010 Feb 19;106(3):447-62. [PMID: 20167942 DOI: 10.1161/CIRCRESAHA.109.208355].

sentence: The cross-talk between circadian rhythms and metabolism is operated also by the peroxisome proliferator-activated receptors (PPAR), in particular α and γ.
- unsupported, please provide reference
We apologize for the missing reference. It is now provided the following ref at pag. 7:
Charoensuksai P, Xu W. PPARs in Rhythmic Metabolic Regulation and Implications in Health and Disease. PPAR Res. 2010;2010. pii: 243643. [PMID:20871864 DOI: 10.1155/2010/243643]

p. 12
sentence: Cellular immune rhythms are synchronized by the mammalian central pacemaker located in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus via time dependent changes in the activity of the sympathetic nervous system (SNS), in the release of hormones (growth hormone , prolactin, melatonin, cortisol) and in behavior that is linked to the sleep-wake cycle.
- reference missing, please provide evidence that lesion of SCN causes diminishing of cellular immune rhythms.

We completely agree with the reviewer that there is no direct evidence that lesion of SCN causes diminishing of cellular immune rhythms, but there is evidence of SCN control through autonomic nervous system fibers, hormones and neurotransmitters.

Dimitrov S, Benedict C, Heutling D, Westermann J, Born J, Lange T. (2009). Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. Blood. 113:5134-5143

Dimitrov S, Lange T, Born J. (2010). Selective mobilization of cytotoxic leukocytes by epinephrine. J. Immunol. 184:503-511.
Logan RW, Arjona A, Sarkar DK. (2011). Role of sympathetic nervous system in the entrainment of circadian natural-killer cell function. Brain Behav. Immun. 25:101-109

sentence: During the active period the hypothalamus pituitary adrenal axis becomes activated and cortisol suppresses pro-inflammatory cytokine production, CD4+ T cell numbers and allergic reactions.
- reference missing
We now provide the reference. Lange T, Dimitrov S, Born J. (2010). Effects of sleep and circadian rhythm on the human immune system. Ann. N. Y. Acad. Sci. 1193:48-59
p. 13
sentence: Immune rhythms are influenced by hormone rhythms (e.g. cortisol, melatonin, norepinephrine), and in humans the rhythms of naive, central memory, and effector memory T cell counts are regulated by cortisol, whereas numbers of CD8+ effector T cells follow changes in endogenous epinephrine.
- reference missing
We apologize for the missing references. We now provide the new ones:
Esquifino AI, Alvarez MP, Cano P, Chacon F, Reyes Toso CF, Cardinali DP. (2004). 24-hour pattern of circulating prolactin and growth hormone levels and submaxillary lymph node immune responses in growing male rats subjected to social isolation. Endocrine. 25:41-48.
Esquifino AI, Chacon F, Cano P, Marcos A, Cutrera RA, Cardinali DP. (2004). Twenty-four-hour rhythms of mitogenic responses, lymphocyte subset populations and amino acid content in submaxillary lymph nodes of growing male rats subjected to calorie restriction. J. Neuroimmunol. 156:66-73.
Fauci AS. (1975). Mechanisms of corticosteroid action on lymphocyte subpopulations. I. Redistribution of circulating T and B lymphocytes to the bone marrow. Immunology. 28:669-680.
Dimitrov S, Benedict C, Heutling D, Westermann J, Born J, Lange T. (2009). Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. Blood. 113:5134-5143

sentence: The presence of biological clocks in immune cells and lymphoid organs drives rhythms in the functions of cells within the immune system, but on the other hand immune responses and mediators influence behavioral and molecular circadian rhythms.
- statement that biological clock in immune cells and lymphoid organs drive rhythms in the function is not proved (and cited), are there any data from knockout animals (cells)?

We thank the reviewer for this comment. We have now inserted the new sentences about this topic (at pag 13) re-phrasing as follow: Alterations of the molecular clockwork modify the harmonized expression of NK cell cytolytic factors. In particular, knock-down of Per2 or Bmal1 in rat-derived RNK16 NK cells changes in a diverse way the expression of genes encoding IFN-γ, TNF-α, granzyme B, and perforin (Arjona & Sarkar, 2006). Furthermore, knock-down of Per2 or Bmal1 changes protein levels of granzyme B and perforin, but not of IFN-γ and TNF-α (Arjona & Sarkar, 2008; Liu et al., 2006). In addition, distorted rhythms of granzyme B and perforin as well as altered rhythm and low levels of IFN-γ, together with changes in the rhythm of Bmal1 and Per2, were evidenced in Per2 mutant mice (Arjona & Sarkar, 2006; Logan et al., 2012)

- Arjona A, Sarkar DK. (2006). The circadian gene mPer2 regulates the daily rhythm of IFN-gamma. J. Interferon Cytokine Res. 26:645-649.
- Arjona A, Sarkar DK. (2008). Are circadian rhythms the code of hypothalamic-immune communication? Insights from natural killer cells. Neurochem. Res. 33:708-718.
- Liu J, Malkani G, Shi X, Meyer M, Cunningham-Runddles S, Ma X, Sun ZS. (2006). The circadian clock Period 2 gene regulates gamma interferon production of NK cells in host response to lipopolysaccharide-induced endotoxic shock. Infect. Immun. 74:4750-4756.
- Logan RW, Sarkar DK. (2012). Circadian nature of immune function. Mol. Cell. Endocrinol. 349:82-90.

Moreover, we added the same references at the end of the sentence (Arjona & Sarkar, 2006; Arjona & Sarkar, 2008; Liu et al., 2006 Logan et al, 2012)

sentence: Interestingly, we reported a severe down-regulation of CRY2 in OR6 cells replicating HCV genotype 1b, which could induce increase of cytokine production related to NF-kB signaling pathway.
- reference missing
We apologize for the missing reference. We have now re-cited the right reference in the middle of the sentence and we added a new reference according to the reviewer's comment.
"Interestingly, we reported a severe down-regulation of CRY2 in OR6 cells replicating HCV genotype 1b(*), which could induce increase of cytokine production related to NF-kB signaling pathway (**). "

* Benegiamo G, Mazzoccoli G, Cappello F, Rappa F, Scibetta N, Oben J, Greco A, Williams R, Andriulli A, Vinciguerra M, Pazienza V. Mutual Antagonism between Circadian Protein Period 2 and Hepatitis C Virus Replication in Hepatocytes. PLoS One. 2013 Apr 8;8(4):e60527. [PMID: 23593233 DOI: 10.1371/journal.pone.0060527]

** Narasimamurthy R, Hatori M, Nayak SK, Liu F, Panda S, Verma IM. Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. Proc Natl Acad Sci U S A. 2012 Jul 31;109(31):12662-7. [PMID:22778400 DOI: 10.1073/pnas.1209965109]

Comments:
Aryl hydrocarbon receptor nuclear translocator-like is known as ***Arntl****,* ***Bmal1****, or Mop3. Authors use abbreviations ARNTL and BMAL1, they should unify way how they name this key clock component.*
Authors explain abbreviations PER and CRY, they should explain also abbreviation HCC, HCV and ARNTL when used for the first time.
We thank the referee. We have corrected as suggested.

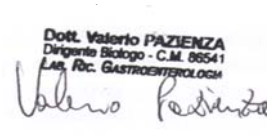
I strongly support effort to introduce chronotherapy into treatment of HCV patients. On the other hand, there is only scarce experimental evidence (and rather indirect) why it should be beneficial. Perhaps it might be useful to search also for some epidemiological studies (if there are some) or perform also this type of research.
Unfortunely, there are no studies dealing with chronotherapy in HCV patients.

I do not quit understand proposed mechanism. It was showed that up-regulation of HBx protein influences clock gene expression but in later chapter authors point out non specific interaction of viral infection and rhythms in immune system function. Finally involvement of NK-kB pathway is suggested. I suggest to provide some scheme of interconnections.

We thank the reviewer for this suggestion. We have added an explanatory figure-scheme.

I strongly support authors in their effort to outline the circadian system and its relationship with immune functions. On the other hand, review in its recent state is slightly overoptimistic (except of conclusions) and miss critical aspect. I am sure that adding of experimental references and scheme will improve factual validity of the review. I believe that revised article will attract a lot to new researchers into this fascinating scientific field and readers of World Journal of Gastroenterology.

All the References and typesetting were corrected.



Dr.Valerio Pazienza
Gastroenterology Unit
Fondazione I.R.C.C.S. "Casa Sollievo della Sofferenza" Hospital
71013 San Giovanni Rotondo-FG
Tel. +39.0882.416281
Fax. +39-0882.410271