

Format for ANSWERING REVIEWERS

October 6, 2014

Dear Editor,



Please find enclosed the edited manuscript in Word format (file name: 13347-Review.doc).

Title: Hepatic glycogenosis: an underdiagnosed complication of diabetes mellitus?

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Name of Journal: *World Journal of Diabetes*

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The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Thank you very much for your comments about the manuscript

- 1) It is true that "HG is only diagnosed by liver biopsy" as indicated on page 7, third paragraph. It should be mentioned in this context, however that after conventional tissue preparation (fixation by formaldehyde-solution, staining with H&E) the glycogen is usually eluted from the hepatocytes. Under these conditions, the glycogenotic cells show only an increase in size and a pale or "clear" cytoplasm. It is, thus, mandatory for an appropriate diagnosis of HG that the biopsies are fixed by alcoholic fixatives (such as Carnoy's solution) or shock frozen in advance of the treatment with the periodic acid Schiff-reaction or Best's carmine for the demonstration of the glycogen. In any case, it would be desirable for such a review that examples of the changes described would be documented by figures (preferably with and without demonstration of the glycogen)

We do agree with the reviewer that this is an important issue, which was not addressed in the present review. For this reason, we have incorporated the opinion of an expert pathologist. According to the suggestion of the reviewer, we have included several histological figures to document the changes we described in the text. One figure is a haematoxylin and eosin staining to show the swollen hepatocytes with pale cytoplasm and other features. The other one is a PAS

staining with diastase to demonstrate the presence of glycogen. As the reviewer suggests, we have included the sentence: *“After conventional tissue preparation (fixation by formaldehyde-solution and staining with haematoxylin and eosin) the glycogen is usually removed from the hepatocytes”*.

(See Diagnosis and Histological findings Section, page X, lines 5-24).

- 2) Figure 2 is not acceptable as a documentation of HG for two reasons. First of all, the electron microscopic demonstration of an excessive storage of glycogen is not at all mentioned in the text of the manuscript. Although electron microscopic demonstration of glycogen is possible, it is difficult to verify an increase of the glycogen at the ultrastructural level without additional observations in serial sections (e.g. in the tissue blocks used for the preparation of the ultrathin sections) under the light microscope. The second problem with Fig. 2 is that the glycogen has apparently not been contrasted by appropriate procedures (e.g. “staining” by lead hydroxide or lead citrate). Consequently, instead of defined glycogen particles only “empty” spaces are visible in this figure, in both the cytoplasm and the nucleus. If the authors wish to use an electron micrograph for the demonstration of the glycogen the methods used have to be described in detail in the text and in the legend to the figure. The few mitochondria shown in the insufficient Fig.2 are small, and do not represent “giant mitochondria” as described in the legend to this figure

The presence of increase glycogen accumulation in the cytoplasm and nuclei can be confirmed on electron microscopic (Torbenso M et al. *Am J Surg Pathol* 2006;30:508-513; Chatila R, West AB. *Medicine (Baltimore)* 1996;75:327-33). Because our ultrastructural examination (Figure 2) was obtained from paraffin embedded tissue, an excessive storage of glycogen is not well appreciated. So, after reviewing and taking into account the referee’s comments, we have eliminated the electron microscopic figure. To demonstrate a pathological excessive glycogen accumulation we have added an image in which periodic acid-Schiff staining with diastase in suitably fixed tissue was used (Figure 3A and 3B).

- 3) In the list of diseases which have to be considered in the differential diagnosis of HG (page 7, second paragraph) one important entity is missing: the focal, but sometimes also diffuse, hepatic glycogenosis known for decades to precede the appearance of hepatocellular adenomas and carcinomas (HCC) induced by chemicals, viruses, radiation or oncogenic transgenes in various animal species (see *Rec Res Cancer Res* Vol 19, Springer, Heidelberg 1968; *Biochim Biophys Acta* 605, 217-245, 1980; *Lab Invest* 80, 1399-1411, 2000; *World J Gastroenterol* 18, 6701-6708, 2012). According to an ever increasing number of observations since 1971 (Virchows *Arch A Pathol Anat* 352,157-164, 1971, in German; *Hepatogastroenterol* 34, 10-15, 1987; *Pathol Res Practice* 190, 513-577, 1994; *Hepatology* 28, 347-359, 1998; *Human Pathol* 31, 874-876, 2000; *World J Gastroenterol* as quoted above; *J Hepatol* 58, 1147-1156, 2013) this also holds true for human HCC as demonstrated particularly in more than 150 explanted livers from patients suffering from chronic liver diseases

prone to develop HCC (Virchows Arch 431, 391-406, 1997). The authors may also be interested to learn that insulin-like effects of the oncogenic agents have been suggested to be responsible for the preneoplastic hepatic glycogenosis (J Bioenerg Biomembr 29, 303-313, 1997; Lab Invest as quoted above; World J Gastroenterol as quoted above). In this context, it is noteworthy that a number of epidemiologic studies have shown that, in addition to inborn hepatic glycogen storage disease (glycogenosis), diabetes mellitus is a risk factor for the development of HCC. The intriguing question whether hepatic glycogenosis related to diabetes mellitus might be involved in the evolution of HCC in diabetes mellitus.

We do agree with the reviewer that this is a very relevant question. We have included in the text some of the comments raised by the reviewer and we have added the following sentence: *“On the other hand, there is increasing evidence that focal, but sometimes also diffuse, HG is a potential preneoplastic lesion (Bannasch et al. Biochim Biophys Acta 1980; 605:217-245; Bannasch P et al. Adv Enzyme Regul 1984; 22:97-121; Terasaki S et al. Gastroenterology 1998; 115:1216-1222. Investigations in animal models of chemical, viral and hormonal hepatocarcinogenesis and some observations in humans suggest that focal HG, represents a critical early event in the pathogenesis of benign and malignant hepatocellular neoplasm (Babbasch P. Prog Liver Dis 1996; 14:161-197; Bannasch P. World J Gastroenterol 2012; 18:6701-6708). Although the exact mechanism remains elusive, recent data suggest that oncogenic agents have an early insulin-like effect (Nehrbass D et al. Am J Pathol 1998; 152:341-345; Aleem E et al. Toxicol Pathol 2011; 396:524-243; Bannasch P. World J Gastroenterol 2012;18:6701-6708). It is noteworthy that a number of epidemiological studies have shown that diabetes mellitus is a risk factor for the development of hepatocellular carcinoma (Davila JA et al. Gut 2005; 54:533-539; Vigneri P et al. Endocr Relat Cancer 2009;16:1103-1123). However, no relationship has been described between diabetes-related hepatic glycogenosis and hepatocellular neoplasms, but further studies are warranted in order to clarify this point.*

3 References and typesetting were corrected

Thank you again for all the comments raised that without doubt, have contributed to enhance our manuscript. We hope that now the paper will be suitable for publication in the *World Journal of Diabetes*.

Sincerely yours,

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