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Dietary Protective Effects Against Hepatocellular Carcinoma Development in *Mdr2*$^{-/-}$ Knockout Mice

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Abstract. *Background/Aim: The Mdr2*$^{-/-}$ *mouse develops early chronic cholestatic hepatitis and hepatocellularcarcinoma (HCC) when adult. We tested the effects of a restricted-calorie diet on HCC development in Mdr2*$^{-/-}$ *mice. Materials and Methods: Mdr2*$^{-/-}$ *mice (n=40, divided into two groups of 20 mice each) were randomized to receive ad libitum diet or restricted-calorie diet. Two mice from each group were sacrificed at 3 and 6 months, and liver tissue samples were removed for analysis. The remaining mice were fed their respective diets until the age of 30 months, at which time they were euthanized and livers were collected for analysis. Results: The restricted-calorie diet had partial chemopreventive effect on the development of HCC in Mdr2*$^{-/-}$ *mice. Moreover, mice with ad libitum diet had a median survival of 361 days, while the restricted-calorie group had a median survival of 500 days (p=0.0001). Conclusion: A restricted diet might reduce the chance of developing HCC in patients at risk and could increase the protective action of anti-inflammatory agents.*

Hepatocellular carcinoma (HCC) is a significant health problem, and chronic inflammation is a major risk factor for the development of HCC. The hepatitis C virus (HCV) is one of the major etiological HCC agents: it induces chronic liver inflammation and is responsible for the increased incidence of HCC in Western countries (1-3). The inflammatory process is a contributor, if not a cause, of a wide variety of neoplasms and it is calculated that up to 15-20% of all deaths are related to this phenomenon (4-6). Although the association between chronic immune activation and cancer development has been known for a long time, only recently have the mechanisms underlying this phenomenonbeen unraveled (3-8). The commonest causes of hepatic inflammation leading to HCC, besides viral infections, are metabolic disorders including obesity (9, 10).

Mouse models of HCC have been widely used to study the molecular mechanisms of primary liver cancer (11-18), and it was recently shown that most mouse HCCs share many similarities with their human counterpart (11). In this study, we investigated the potential protective role of a low-calorie diet against HCC in *Mdr2*$^{-/-}$ mice, a model of inflammation-associated HCC (11-18). *Mdr2*$^{-/-}$ mice lack both alleles of the ATP-binding cassette subfamily B1 gene. This leads to a lack of liver-specific P-glycoprotein, responsible for phosphatidylcholine transport across the bile canalicular membrane, and induces portal inflammation at an early age (3 months). This is followed by slowly developing HCC (beyond the age of 12 months) (12). More precisely, the absence of phospholipids in bile results in bile regurgitation and portal inflammation, followed by the development of hepatocyte dysplasia and HCC (19, 20).

In this study, we postulated that an hypo-caloric diet would mildly attenuate the oxidative stress produced by leaking bile, or by secondary infiltration by inflammatory cells. To test this hypothesis, *Mdr2*$^{-/-}$ mice were assigned to one of two groups: regular diet or restricted diet. We

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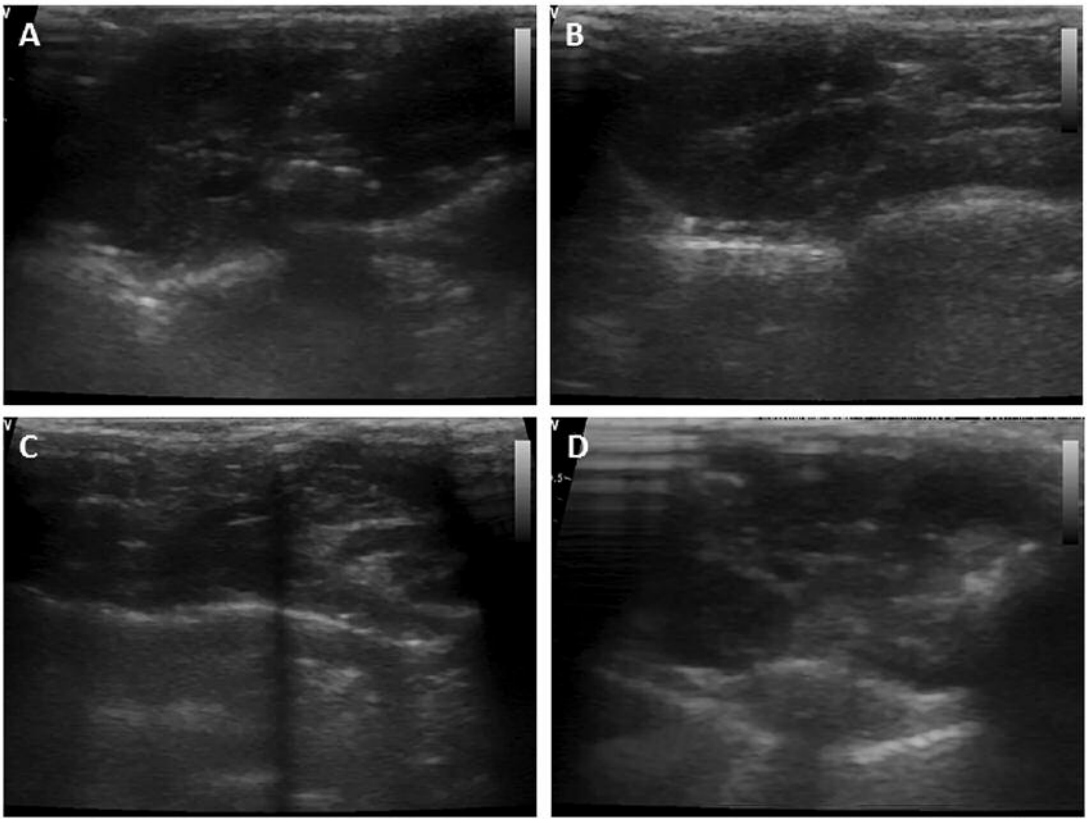

Figure 1. *The ultrasonographic appearance of the livers of Mdr2$^{-/-}$ mice at different time points. A: Normal liver at 3 months in the (hypocaloric diet) group. The liver is seen to have normal echogenicity and architecture. B: Mildly to moderately fibrotic liver in the group fed a normal mouse chow diet. Minimal reduction in volume with irregular margins can be seen. Small hypoechoic areas are suggestive of initial fibrosis. Images taken at 3 months C: Moderately to severe fibrotic liver in the group fed ad libitum diet. Decreased liver volume with more evident signs of fibrosis are apparent. D: Neoplastic liver nodules in liver from a mouse of the group fed ad libitum standard mouse chow diet. Similar appearance to severely fibrotic livers with the addition of multiple poorly echogenic nodular lesions.*

monitored chronic hepatitis inception and its eventual transition to overt HCC development in *Mdr2*$^{-/-}$ mice by ultrasound, and, upon sacrifice, histopathological examination of liver tissues.

Materials and Methods

Mice. All procedures were in accordance with institutional guidelines under the control of the Italian Ministry of Public Health (Italian Law D.lgs 26/2014). The *Mdr2*$^{-/-}$ mice , were previously described (the colony founders were kindly provided by G. Natoli, IEO, Milan, Italy and the colony was expanded at the Regina Elena Cancer Institute Animal Facility) (11, 12). Starting from 8 weeks of age, the mice were housed in individual cages, at a temperature of 22˚C and a photoperiod of 12 hours of light and 12 hours of darkness at the animal facility of the Regina Elena Cancer Institute of Rome. Mice had *ad libitum* access to water and were fed either mouse chow (Mucedola, Italy) *ad libitum* (n=20, control group) or a restricted diet (n=20 experimental group). Calorie restriction was introduced by reducing calories to 52 kcal per week (AIN-93M 40% Restricted Diet No. F05314; Bioserv Flemington, NJ, USA). Mice were weighed weekly and checked with ultrasonography every 2 months starting from the third month of age. Two mice from each group were sacrificed at 3 and 6 months of age, and liver tissue samples were removed for analysis. Throughout the study, mice were sacrificed when showing signs of sufferance such as weight loss (>20%), poor haircoat, decreased activity, decreased food consumption or inability to drink and feed unassisted. At that time, livers were removed and analyzed. The remaining mice were fed their respective diets until they reached the age of 30 months, at which time all the laboratory animals were euthanized and the abdominal organs were collected for analysis.

Ultrasonography. Ultrasonographic examinations were performed with the mice in dorsal recumbency under heavy sedation using a combination of tyletamine-zolazepam and xylazine intramuscularly injected. A General Electric Vividi ultrasonographer (Scill Veterinary Products, Bergamo, Italy) with a 8 L probe was used

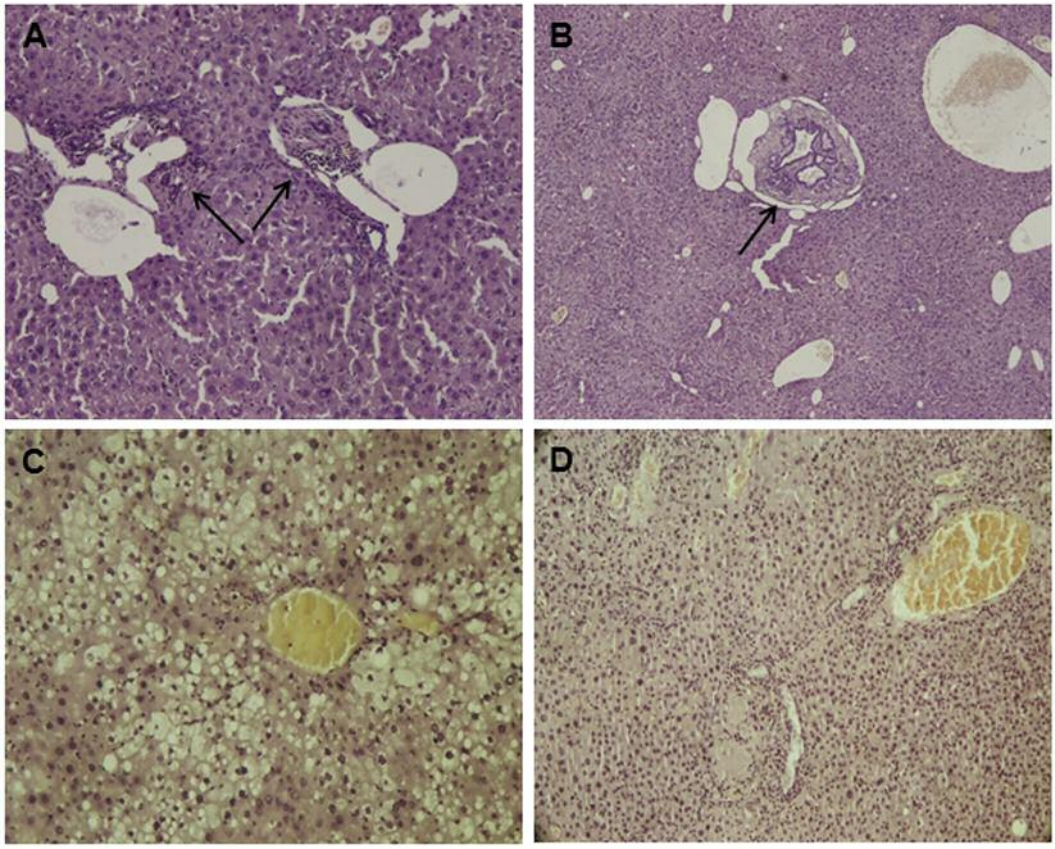

Figure 2. *Typical pathological features seen in the liver of mice fed a restricted-calorie diet (panels A and B) and those fed ad libitum diet (panels C and D) are depicted. A: Lymphoid infiltration limited to the portal tract with no necrosis of the liver cells is shown by arrows (original magnification ×20). B: Ductal proliferation of the biliar tree is shown by an arrow (original magnification ×10). C: Extensive fatty changes visible, with large vacuoles of fat present within hepatocytes (original magnification ×20). D: Hepatocellular carcinoma composed of liver cells with atypical nuclear cytology and abnormal architectural arrangement (original magnification ×10).*

with an ultrasound gel to maximize ultrasound transmission by one of the authors (FM). Ultrasonographic examinations were repeated every 2 months until the completion of the experiments, assessing liver size, number and size of hepatic lesions, and presence of abdominal effusion.

Histology. The excised biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 μm were stained with hematoxylin-eosin, and hematoxylin-vanGieson, evaluating the severity of hepatic damage and the presence and degree of neoplastic transformation.

Statistical analysis. The overall survival (OS) time was calculated as the period from the date of diagnosis of HCC until death from any cause, at which point, data were censored. OS was determined by the Kaplan–Meier product-limit method (21). The difference in terms of OS according to different variables was evaluated by the log-rank test (22).

The cut-off point for survival data was March 2009. SPSS software (version 13.05; SPSS, Chicago, IL, USA) was used for statistical analysis. A *p*-value of less than 0.05 was considered to indicate statistical significance.

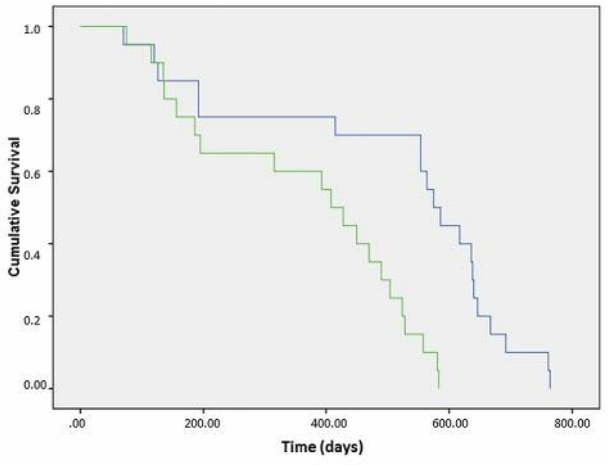

Figure 3. *Kaplan–Meier survival curve for the two mouse cohorts. The median survival time for the cohort fed ad libitum chow was 361 days (gray line), while that for the cohort receiving a restricted-calorie diet was 500 days (black line). This difference was statistically significant (p=0.0001).*

Results

The two groups exhibited a different trend in terms of weight gain, with the control group displaying a tendency towards heavier weights that was more evident at the end of the observational period. At that time, the restricted-diet group had a median weight of 27 g, while the controls had a median weight of 35 g. The sequential monitoring and organ collection revealed progressive hepatitis in both groups that was significantly more pronounced in the group fed standard chow.

The ultrasonographic examination showed much more enlarged organs, with diffuse echogenicity suggestive of liver fibrosis. Significantly less echogenicity was observed in the livers of the group fed the *ad libitum* diet, suggestive of a lower degree of fibrosis. These lesions evolved towards nodular regenerative nodules and multiple neoplastic nodular lesions, characterized by variable diameter, low definition and poor echogenicity. Livers exhibited a tendency towards having a reduced volume, with indistinct margins; the organ architecture was clearly disrupted by the progression of the disease. The patterns of the echogenic appearance of *Mdr2*$^{-/-}$ mice at different times are summarized in Figure 1.

The characteristic pathological features seen in liver of *Mdr2*$^{-/-}$ mice at 3 months of age were: portal inflammation with inflammatory cell infiltration and development of fibrosis, ductal proliferation and fatty change. Later, the mice developed single or multifocal HCC. These pathological features were more prominent in the *ad libitum* diet cohort, and reflected both in number and size of the lesions (Figure 2). Mice began developing HCC at different times in the two groups: the group with unrestricted diet began showing hepatic tumors before 16 months of age as per the current literature, on the other hand, the animals fed a restricted diet developed HCC later in their lives (after 16 months). Moreover, ultrasonography showed a more rapid progression of the disease in the unrestricted-diet cohort. More importantly, besides the delay in the onset and progression of HCC, the restricted-calorie diet resulted in a better overall condition of the mice. This translated into an extended survival time. The Kaplan–Meier statistical examination gave a median survival time for the *ad libitum* diet group of 361 days, while the group on the restricted-calorie diet had a median survival of 500 days (p=0.0001). Figure 3 shows the Kaplan–Meier survival curve for the two experimental groups.

Discussion

Inflammation seems to play a pivotal role in many subsets of HCC (1-7, 20). *Mdr2*$^{-/-}$ mice provide a brilliant model for summarizing the clinical and pathological features of this disease. In fact, this model recapitulates key features of the human disease, comprising chronic inflammation, genomic abnormalities, and fibrosis, with its late developments: cirrhosis and microhepatica (11-19, 23). We adopted this model to investigate the possible protective role of a low-calorie diet on the pathological cascade that starts with hepatic inflammation, ultimately resulting in HCC.

We observed that from the early onset of chronic hepatitis at 3 months of age, the special diet resulted in slower onset and progression of this phenomenon, as shown by physical examination, ultrasonographic examination, and histo-pathology. Interestingly, this protective effect was not only immediate but lasting, as shown by the aforementioned examinations and by the OS time of the mice fed the hypocaloric diet. Additionally, we found a lesser degree of liver fibrosis, cirrhosis and microhepatica. The problem of prevention in the case of HCC, is challenging, since many factors play a role in this disease in humans including: hepatitis C infection, liver inflammation (multiple etiologies), alcohol abuse, fat-rich diet, and exposure to carcinogens (24-29). Several publications pointed out the causative and perpetuating role of fats in several models of murine hepatitis, moreover, it is well known that obesity is a risk factor for multiple diseases in humans, some of them of neoplastic nature (30, 31).

Inflammatory promoters induce production of reactive oxygen species, resulting in oxidation and damage of DNA, and impairment of DNA repair (4-8). In addition, some results suggest a possible oxidative condition in *Mdr2*$^{-/-}$ mice when their inflammation becomes chronic (11, 12, 16, 32). In summary, it is not surprising that chronic inflammation and secondary hepatic fibrosis might be modulated by an appropriate diet (33-35).

In conclusion, we found that dietary restriction throughout the life of *Mdr2*$^{-/-}$ mice reduced the severity of hepatitis and delayed the onset of HCC at the age of 16 months. Although the development of new, reliable drug therapies for the treatment of HCC and arrest of progression to cancer of chronic hepatitis is vital, nevertheless, lifestyle intervention is mandatory in patients at risk of developing HCC. Despite the identification of proper anti-inflammatory therapies in patients with chronic hepatitis at risk of developing HCC, other strategies aimed at the prevention of this type of cancer, and at improving overall mortality, are equally important in the struggle against this insidious disease (36).

Conflicts of Interests

None of the authors have any competing interests in regard to this study.

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