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Hepatic mRNA Expression Levels of the Oncogenes Alpha-Fetoprotein and Osteopontin as Diagnostics for Liver Cancer in a Murine Model of Diet-Induced Non-Alcoholic Steatohepatitis

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Abstract: Non-alcoholic steatohepatitis (NASH) is associated with an increased risk of hepatocellular carcinoma (HCC). Expression levels of hepatic oncogenes, alpha-fetoprotein (*afp*) and osteopontin (*opn*)/secreted phosphoprotein 1 (*spp1*), were investigated using a model of diet-induced NASH. Mice were randomized to a standard diet or a fast-food diet (FFD) for 17 months. Livers from the FFD cohort exhibited hallmark characteristics of NASH with liver fibrosis, with a subset of animals exhibiting HCC. Expression levels of hepatic *afp* and *opn/spp1* were elevated ~2.5 and ~5-fold, respectively, in the FFD cohort. Hepatic *opn/spp1* exhibited a direct (r = 0.65) and significant ($p < 0.01$) correlation with liver hydroxyproline content. Receiver operating characteristic (ROC) curve analysis for hepatic *afp*, as a diagnostic for HCC, returned an area under (AU) ROC 0.84, a sensitivity of 87.5%, a specificity of 77% and a threshold of >1.05-fold change in mRNA level. The use of hepatic *opn/ssp1* as a diagnostic for HCC returned an AUROC 0.88, a sensitivity of 83.3%, a specificity of 86.7% and a threshold of >2.4-fold change in mRNA level. These data point to a transformation of NASH to an oncotype with hepatic oncogene levels as a diagnostic for NASH.

Keywords: non-alcoholic steatohepatitis; fibrosis; mRNA; alpha-fetoprotein; osteopontin; hepatocellular carcinoma

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1. Introduction

In the United States, millions of adults present with non-alcoholic fatty liver disease (NAFLD), with this number being higher worldwide [1,2]. Incidence and prevalence of NAFLD is typically higher in diabetics and those with an elevated body mass index [1]. The NAFLD continuum progresses from accumulation of lipids to non-alcoholic steatohepatitis (NASH), scarring, cirrhosis and primary liver cancer or hepatocellular carcinoma (HCC) [3–5]. The paradigm where cirrhosis precedes HCC has been challenged with increasing numbers of reports of HCC in the absence of cirrhosis, a phenomenon termed non-cirrhotic HCC [6–10]. Given the size of the NAFLD epidemic, the risk of NASH-related HCC becomes significant. Early detection of HCC is paramount, as outcomes worsen with advancing disease [11,12]. A NASH patient, unlike a cirrhotic patient, is rarely screened for liver cancer [11,12]. This is unfortunate considering that (a) NASH is emerging as a major cause of primary liver cancer and (b) liver tissue is frequently obtained to diagnose NASH [13] and can presumably also be used to screen for markers of cancer. Nevertheless, it is entirely possible that the core biopsy sample is from a tumor-free site, resulting in misdiagnosis.

Surveillance of HCC in high-risk individuals, i.e., cirrhotics, is commonly performed using ultrasonography, coupled with monitoring levels of serum alpha-fetoprotein (AFP) [13,14].

Osteopontin (OPN), a glycoprotein secreted by macrophages, osteoblasts and T cells, is also highly expressed in a variety of tumors including gastrointestinal tumors [3,15–18]. Serum levels of OPN have been proposed as a companion diagnostic for HCC [3]. Nevertheless, serum levels of AFP and OPN are not specific to tumors originating in liver tissue. The present study utilized a diet-induced model of NASH to evaluate hepatic transcriptomic levels of *afp* and the OPN gene, secreted phosphoprotein 1 (*spp1*).

2. Material and Methods

2.1. Animal Model

Adult male C57BL/6 mice (18–20 g, ~6 weeks old) were used in this study, which was approved (#2019-014) by the Institutional Animal Care and Use Committee (IACUC, D16-00778). Animals had free access to chow and drinking water.

Animals were randomized to a standard diet (5001, LabDiet, St. Louis, MO, USA, $n = 9$, sham cohort) or a fast-food diet (FFD, $n = 15$) containing 40 kcal % fat, 20 kcal % fructose and 2% cholesterol (D09100301, Research Diets, New Brunswick, NJ, USA) for 17 months [3–5]. At the end of the in-life period, animals were anesthetized with ketamine/xylazine (25/5 mg/kg, intraperitoneal) and blood and liver samples were obtained.

2.2. Histopathology

Analysis was conducted by an observer unaware of the identity of the groups. Formalin-fixed, hematoxylin and eosin (H&E)-stained liver sections were scored for steatosis, inflammation and ballooning using the NAFLD activity score (NAS) [3–5]. The extent of picrosirius red (PSR) staining, a marker of matrix deposition, was measured using ImageJ and normalized to the area of the field. Livers were also examined for the presence of tumors with further microscopic characterization in H&E-stained sections as previously reported [5]. To confirm HCC, liver sections were stained with cytokeratin 7 staining (anti-Cytokeratin 7 antibody (RCK105), Abcam, San Francisco, CA, USA) and carbohydrate antigen (CA) 19-9 staining (anti-CA-19-9 antibody, orb27274, Biocompare, South San Francisco, CA, USA) as previously reported [5].

2.3. Liver Function Tests

Liver function tests, i.e., measurements of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, were conducted at Northwell Health (Lake Success, NY, USA).

2.4. Liver Hydroxyproline

Liver hydroxyproline levels were measured, as reported previously [19], by colorimetric (catalog # MAK008, Sigma Aldrich, St. Louis, MO, USA) analysis and expressed as μg hydroxyproline/mg of liver.

2.5. Hepatic afp and opn/spp1

Measurements were conducted in samples obtained from remote (tumor-free) liver tissue. RNA isolation was performed using the RNeasy Mini Kit by Qiagen and all manufacturer protocols were followed. The liver tissue was removed from the storage freezer and was cut into even smaller samples. These samples were placed into 350 μL of RLT buffer and 10 μL of ALI BME. One mm diameter zirconium-oxide beads were then added to the tubes and placed into a Next Advance Bullet Blender Storm 24 bead homogenizer, where the solution was homogenized. The liquid was then poured into a new tube where it was spun inside a centrifuge. The centrifuged tubes were transferred into new tubes so that 350 μL of ethanol could be pipetted into the mix. The tube was centrifuged before the RNA was pipetted onto a Thermo Scientific NanoDrop Lite spectrophotometer, where the quantity and quality (A260/A280) of the RNA were measured. Following the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (catalog # 4368814, Bohemia, New York, NY, USA) manufacturer's protocol, the RNA samples were converted into

cDNA. A quantitative polymerase chain reaction (qPCR) was then performed on the cDNA in triplicate with the Applied Biosystems TaqMan Fast Advanced Master Mix following the manufacturer's protocol. Analysis was performed for *afp* (Thermofisher TaqMan Gene Expression Assay- ID: Mm00431715_m1), and opn/*spp1* (Thermofisher TaqMan Gene Expression Assay- ID: Mm00436767_m1) and data were normalized to the housekeeping gene *gapdh* (Thermofisher TaqMan Gene Expression Assay- ID: Mm99999915_g1). The synthesized cDNA was diluted with water and vortexed, ensuring that the resulting solution was mixed entirely. TaqMan probes (for *afp*, *opn/spp1* and *gapdh*) were added to 520 µL of TaqMan master mix and a multi-channel pipette was used to dispense the mixture into plate wells. Following the dispersion of the probe and master mix, the cDNA and water mixture was added to the master mix wells and mixed into the original mixture by pipetting. Once the master mix and cDNA were added to each plate well, the plate was covered with tape and centrifuged. The plates were then placed in the Applied Biosystems QuantStudio Real-Time PCR system, where a qPCR was run.

2.6. Statistical Analysis

Data are expressed as averages ± the standard error of the mean. Differences between groups were calculated using a T-test, with a p value < 0.05 signaling significance. The p value for the Pearson product moment, r, was calculated using a website [20].

3. Results

Animals on an FFD for 17 months exhibited salient features of NASH, including an accumulation of lipid droplets in the liver, hepatic inflammation and hepatocyte ballooning (Figure 1A,B). Indeed, consistent with this diagnosis, NAS, AST and ALT were each elevated (Figure 1C–E) in animals fed an FFD.

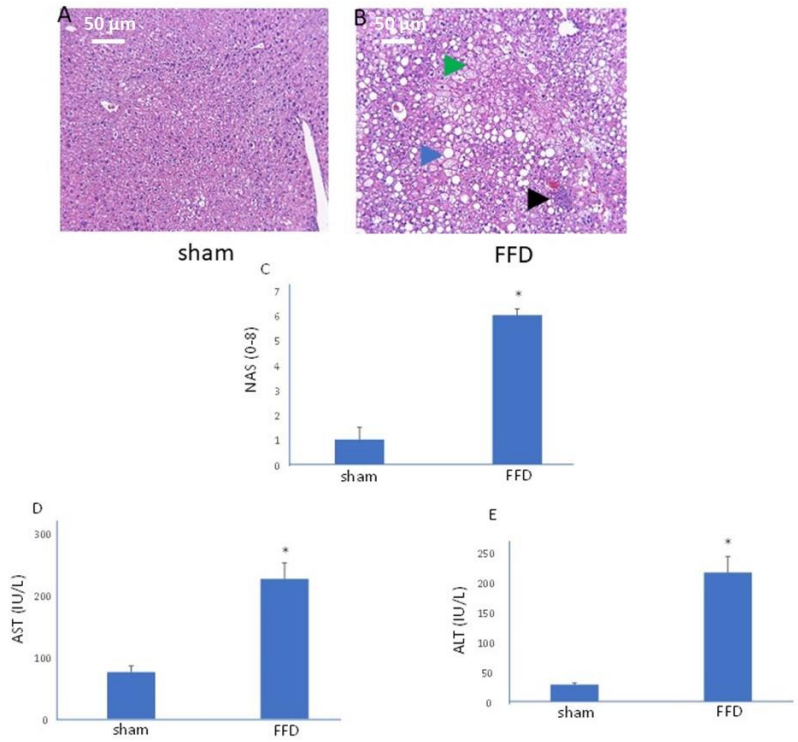

Figure 1. A murine model of diet-induced NASH: (**A**) images of H&E-stained liver sections of mice from the sham cohort; (**B**) Images of H&E-stained liver sections of mice from the FFD cohort. The blue arrow shows lipid droplets, whereas the black arrow highlights inflammation and the green arrow depicts a site of hepatocyte ballooning. (**C**) NAS, (**D**) AST and (**E**) ALT were elevated in the FFD cohort vs. sham. *, $p < 0.01$.

In this model, NASH was accompanied with matrix deposition, evidenced by the network of PSR staining (Figure 2A,B). Semi-quantification of staining revealed a significant increase in matrix deposition in the liver (Figure 2C) and, supporting this notion of increased matrix deposition, liver hydroxyproline content was elevated in the FFD cohort (Figure 2D).

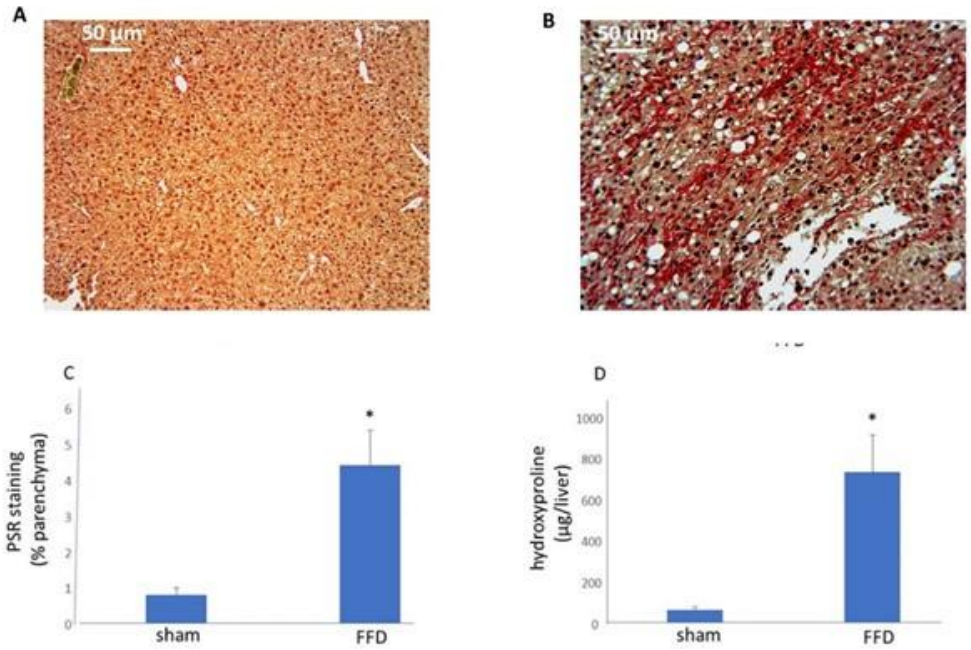

Figure 2. NASH accompanied by liver fibrosis. Representative images of PSR-stained liver sections from mice randomized to a standard diet: (**A)** sham; (**B**) FFD, with increased staining in the latter. (**C**) Matrix deposition had increased in the FFD cohort relative to the sham cohort. (**D**) Livers from the FFD cohort had an elevated level of hydroxyproline. *, $p < 0.01$.

Liver tissue was elevated for levels of the oncogenes *afp* and *opn/spp1*. A ~2.5-fold increase in *afp* and a ~5-fold increase in *opn/spp1* were observed from livers in the FFD cohort (Figure 3A,B). Consistent with the oncological nature of these genes, there was an excellent (r = 0.83) and a highly significant ($p < 0.01$) correlation between hepatic *afp* and *opn/spp1* (Figure 3C).

To determine whether these cancer biomarkers titrate against the degree of liver scarring, correlations were performed against hepatic hydroxyproline content, a continuous variable. Interestingly, while there was no relation (r = 0.36, $p = 0.09$) between *afp* and hydroxyproline, *opn/spp1* exhibited a direct (r = 0.65) and significant ($p < 0.01$) correlation with liver hydroxyproline (Figure 4A,B).

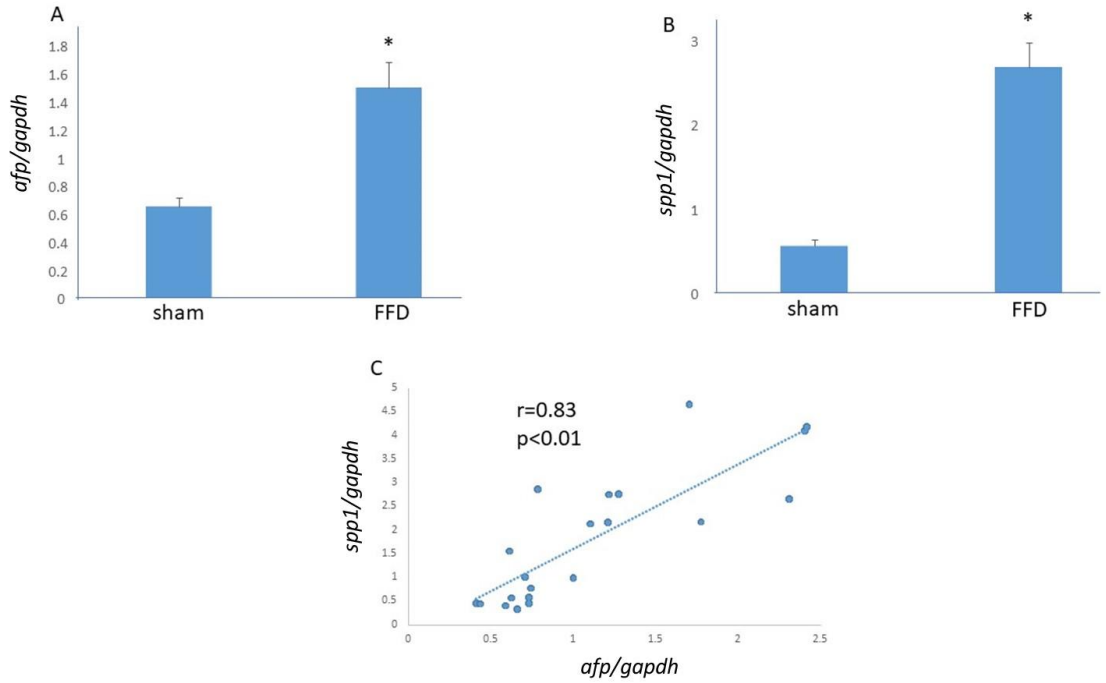

Figure 3. Hepatic oncogenes in NASH with fibrosis: (**A**) an increased expression of *afp* in the FFD cohort. (**B**) An increased expression of *opn/spp1* in the FFD cohort. *, $p < 0.01$. (**C**) The correlation between hepatic *afp* and *opn/ssp1* yielded an r = 0.83, $p < 0.01$.

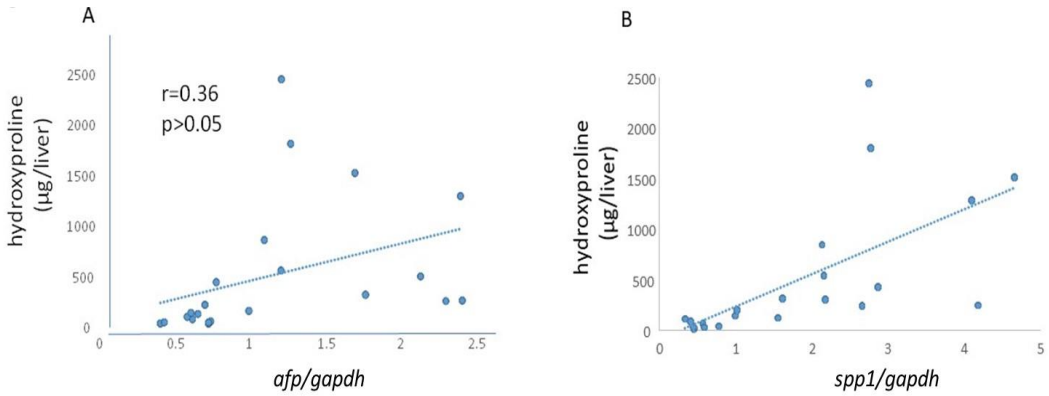

Figure 4. Oncogenes and liver scarring: (**A**) there is no relation between *afp* and hepatic hydroxyproline content in this model of NASH with fibrosis. (**B**) A direct and significant correlation exists between *opn/spp1* and hepatic hydroxyproline content.

To evaluate the use of these hepatic oncogene levels as a diagnostic for HCC, at least in this model of NASH, livers were first examined for the presence of HCC. A subset of livers from the FFD (17 months) cohort exhibited one or more tumors (Figure 5). Microscopic evaluation revealed a trabecular growth pattern of abnormal hepatocytes and clusters of nuclei (Figure 5) with a distinct margin between the cancerous and noncancerous parenchyma.

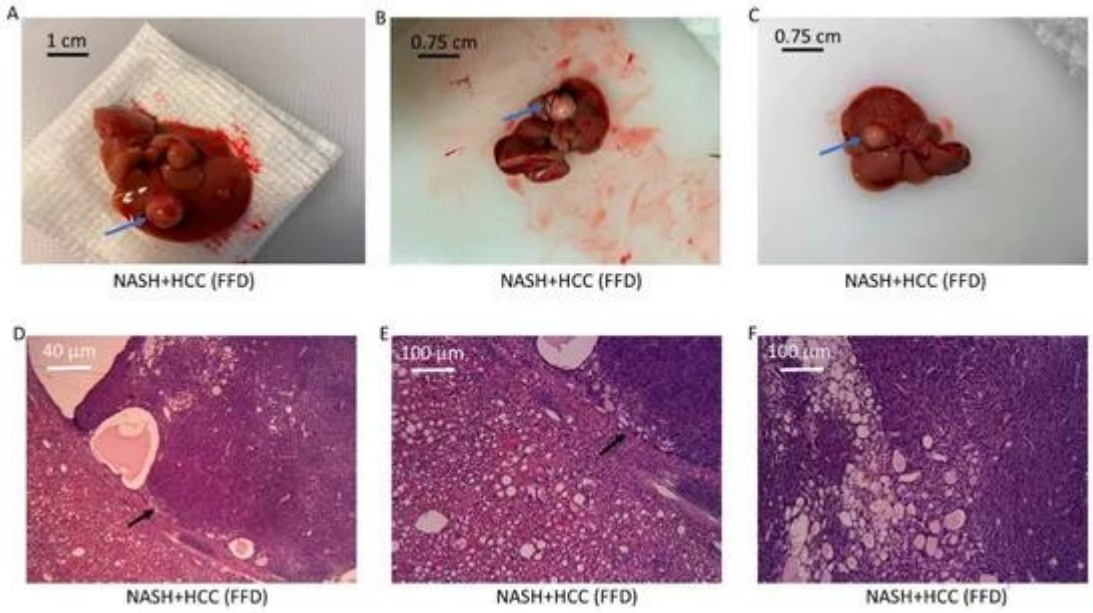

Figure 5. HCC. (**A**–**C**) Several animals within the FFD (17 months) cohort exhibited tumors (blue arrows) on the surface of the liver. (**D**–**F**) Representative H&E-stained section from an FFD (17 months) liver with (**F**) showing abnormal hepatocytes and clusters of nuclei. There is a distinct margin between the noncancerous and cancerous parenchyma (black arrows, (**D**,**E**)).

Enriched CA 19-9 and cytokeratin 7 staining (Figure 6) were only observed in sections of HCC. By contrast, livers that were tumor-free, be it from the sham cohort or the NASH cohort, exhibited little or no CA 19-9 or cytokeratin 7 staining.

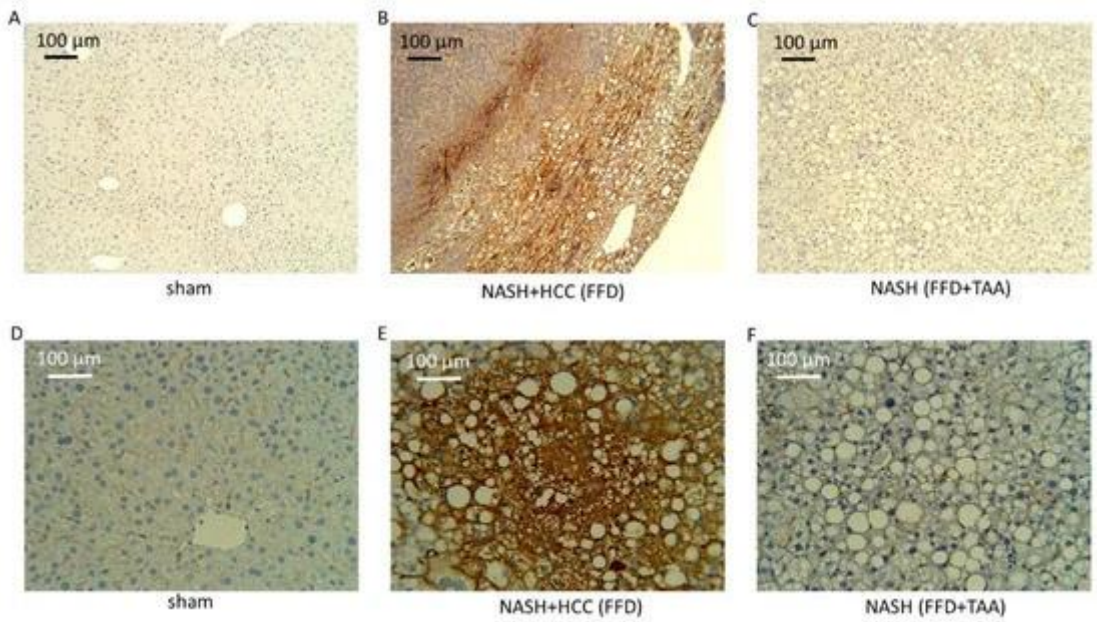

Figure 6. HCC. (**A**–**C**) Representative liver sections (10×) from Ca-19-9-stained sham, FFD and FFD+TAA groups. Staining is enriched in the FFD liver indicative of HCC. This liver bore a large tumor at sacrifice. (**D**–**F**) Representative liver sections (25×) from cytokeratin-7-stained sham, FFD and FFD+TAA groups. Staining is enriched in the FFD liver, indicative of HCC. This liver bore several tumors at sacrifice.

Use of *afp* as a diagnostic for HCC yielded an AUROC of 0.84, a sensitivity of 87.5%, a specificity of 77% and a threshold >1.05-fold increase in hepatic *afp* (over sham/healthy liver, Figure 7). Use of *spp1* as a diagnostic for HCC yielded an AUROC 0.88, a sensitivity

of 83.3%, a specificity of 86.7% and a threshold >2.4-fold increase in hepatic *spp1* (compared to the sham/healthy liver, Figure 7).

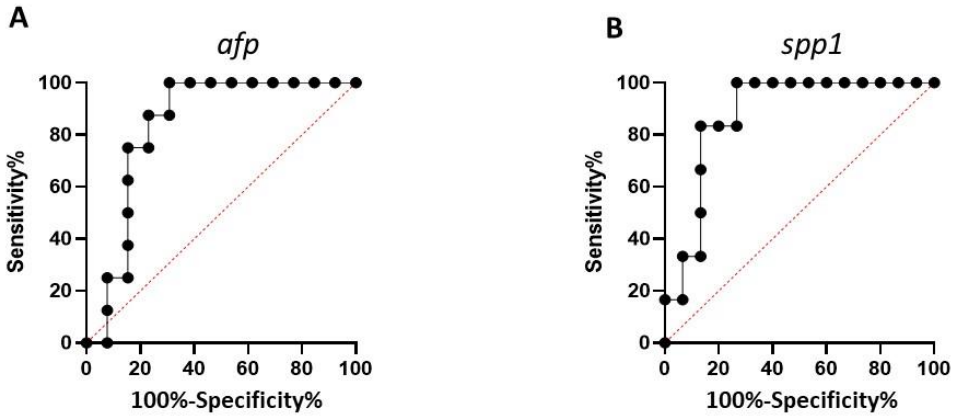

Figure 7. Hepatic oncogenes as diagnostics for HCC: (**A**) ROC for hepatic *afp;* and (**B**) *spp1* as diagnostics for HCC in NASH.

4. Discussion

Using a murine model of diet-induced NASH, we have identified liver scarring and increased hepatic expression of the oncogenes *afp* and *opn/spp1*. Levels of hepatic *opn/spp1* correlated with liver hydroxyproline content. Both hepatic *afp* and *opn/spp* levels in remote, tumor-free tissue were diagnostic for HCC. These data point to a process of transformation of NASH at the transcriptomic level to HCC.

Given the burgeoning diabetes and metabolic syndrome epidemics, the incidence and prevalence of NAFLD are increasing [1,2]. In fact, it is estimated that millions of people in the United States alone have excess liver fat, with subsets of this population exhibiting NASH or NASH with fibrosis [21]. Major risks associated with NAFLD are decompensated liver failure requiring organ transplant and progression to cirrhotic or non-cirrhotic HCC [1]. Historically, cirrhotics are at the highest risk of HCC [3–5]. However, growing evidence suggests that NASH, especially NASH with fibrosis, can lead to HCC, even in the absence of cirrhosis [10]. Outcomes in patients with HCC are intricately linked to early diagnosis [11,12]. Since NASH is a biopsy-proven label [13], screening for HCC at the time of biopsy can potentially save lives.

Imaging, coupled with monitoring serum levels (>400 ng/mL) of AFP, has historically been used to diagnose HCC in cirrhotics [14,15]. Circulating levels of another biomarker, OPN, are also elevated in HCCs [3,16–18]. However, the origin of both these markers is not specific to the liver, with several stromal and gastrointestinal tumors also releasing these proteins [3,16–18]. Second utility of their circulating levels in non-cirrhotic HCC remains to be determined.

In the present study, we tested the hypothesis that NASH with fibrosis is associated with the upregulation of hepatic *afp* and *opn/ssp1*. Consistent with previous reports [3–5], livers from mice randomized to an FFD exhibited hallmark features of NASH, viz., lipid deposition, inflammation, elevated liver function tests, hepatocyte ballooning and matrix deposition, with several livers bearing tumors consistent with HCC. The first novel finding in this study was increased hepatic *afp* and *opn/ssp1* accompanying NASH. Indeed, there was little doubt regarding the source of these mRNA as the livers were analyzed. Furthermore, there was an excellent correlation between these two genes, indicating that this model of NASH with fibrosis is indeed associated with a transformation to an oncotic genotype. Interestingly, levels of one of these genes, viz., *opn/spp1*, exhibited a robust correlation with hepatic hydroxyproline content, a marker of liver matrix deposition. In fact, in contrast to a "liver fibrosis score", which can be influenced by the observer and is a discrete variable, liver hydroxyproline is a continuous variable and is independent of observer bias. Unlike hepatic *opn/spp1*, whose levels are associated with organ fibrosis,

hepatic *afp* levels did not exhibit any such correlation, irrespective of the presence of a trend. While the underlying mechanism for these differences remains to be investigated, it may be that hepatic *opn/ssp1* expression is more sensitive to scarring compared to hepatic *afp* expression. Our findings that NASH with fibrosis is accompanied by the increased hepatic expression of two independent oncogenes, and the expression level of at least one oncogene correlates with the degree of scarring, are consistent with the overarching hypothesis of a continuum starting from NASH with fibrosis to HCC. The second novel finding of this study is that hepatic *afp* and *opn/ssp1* expression, even in samples obtained from remote (non-tumor) liver, were diagnostic for HCC. Both these oncogenes exhibited excellent AUROCs, sensitivity and specificity as diagnostics for HCC. Indeed, to the best of our knowledge, this is the first report of hepatic oncogene levels serving as a diagnostic for HCC in a model of NASH. Consistent with the notion that hepatic *opn/ssp1* expression is more sensitive to changes in the liver, levels of this oncogene exhibited a greater dynamic range with progression to HCC. The totality of these data appears to point to an initial genetic component of this transformation. These data are also consistent with clinical reports. Xu and colleagues [14] reported that subjects with fatty liver disease exhibited higher serum AFP levels compared to the control cohort. Interestingly, results from that study suggested that serum AFP levels may act as a cofactor, but not an independent factor, for fatty liver disease. In a retrospective analysis of patients with NAFLD-related fibrosis, Glass and colleagues [22] reported increased serum OPN levels and hepatic *opn/ssp1* expression; however, no attempt was made to draw a correlation between hepatic oncogene expression and the degree of scarring.

This study does have weaknesses, in that findings might be applicable potentially only to this murine model of NASH and at a single time point. These findings therefore need to be validated in additional models of liver disease. Second, hydroxyproline content is not typically measured in patient liver biopsy samples. The relationship between hepatic oncogene expression and histopathologically obtained fibrosis scores (less objective) remains to be investigated. Nevertheless, the translational potential of these data should not be underestimated, in that core hepatic biopsies can be queried for hepatic oncogene levels as diagnostic for HCC, potentially complementing image-based diagnosis and reducing the need for biopsy followed by histopathology of the actual tumor.

Author Contributions: A.J.P. and S.M. conducted the experimental studies; P.N. conducted data analysis; P.N. designed and supervised the project and prepared the manuscript. I.D.G. provided funding for the study. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Angion (5 May 2019, #2019-014; 5 May 2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available by authors upon request.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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