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# Elevated serum activity of MBL and ficolin-2 as biomarkers for progression to hepatocellular carcinoma in chronic HCV infection

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ABSTRACT

Hepatocellular carcinoma (HCC) is an uncommon but significant outcome of chronic hepatitis C virus (HCV) infection. A serum biomarker for predicting progression to HCC would have a major impact on patient monitoring and clinical management. We explored circulating liver-expressed lectins, ficolin-2, ficolin-3 and mannose binding lectin (MBL), as potential biomarkers for the development of HCC. The activity of these three lectins were analysed in HCV positive patients who developed HCC (n = 31) with comparable HCV-positive HCC-negative patients (n = 106) and healthy controls (n = 79). Serum binding activity of ficolin-2 and MBL were elevated compared to controls. Analysis of pre-HCC onset samples revealed that MBL levels were significantly elevated up to 3 years, and ficolin-2 was elevated up to 1 year, prior to diagnosis of HCC over controls. This preliminary study identifies MBL and ficolin-2 as potential biomarkers for the development of HCC in chronic HCV infection.

## 1. Introduction

Infection with hepatitis c virus (HCV) is an important cause of hepatocellular cancer (HCC) and it is currently estimated that 71 million people are chronically infected with HCV (Collaborators POH, 2017). Infection is associated with progressive fibrosis and loss of liver function. Cirrhosis develops in approximately 20–30% of infections and in these individuals there is a 1–7% annual risk of developing HCC (Fattovich et al., 2004). The mechanisms underlying this progression to severe disease are still incompletely understood, but many molecular mechanisms have been proposed. Viral factors including HCV genotype and viral load influence progression (Kanwal et al., 2014). Co-infection with hepatitis B virus (HBV) (Zampino et al., 2015) and environmental factors such as alcohol consumption increase the risk of developing HCC (Donato et al., 2002). Host genetic factors may also influence progression. A recent systematic review (Walker et al., 2018) indicated significant associations between HCC and seventeen host genes in HCV infections, however only weak associations could be made between individual single nucleotide polymorphisms (SNPs) and HCC.

Fibrosis and development of HCC are closely linked to altered innate immune signalling during chronic HCV infection (Arzumanyan et al., 2013). Chronic inflammation is known to trigger activation of liver-resident stellate cells (reviewed in (Weiskirchen and Tacke, 2014)), differentiating into collagen-producing myofibroblasts. Activation by protease cleavage of PAR 1 results in production of extracellular matrix (ECM) and initiation of fibrosis (Fiorucci et al., 2004). One of the major triggers of stellate cell activation is the action of mannose binding lectin (MBL)-associated serine proteases (MASPs) (Saeed et al., 2013), enzymes associated with the pattern recognition receptors (PRRs) MBL, CL-11, ficolin-2 and ficolin-3. Binding of these PRRs to viruses is well described (Brown et al., 2010; Hamed et al., 2014; Hansen et al., 2010; Pan et al., 2012; Verma et al., 2012) and increased activity of MASPs is associated with development of liver fibrosis (Brown et al., 2007). MBL, ficolin-2 and ficolin-3 are predominantly expressed in the liver but regulation of their expression is still not completely understood, although they are believed to be expressed constitutively, with acute-

*Abbreviations:* HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MBL, mannose binding lectin; PRRs, pattern recognition receptors; HBV, hepatitis B virus; SNPs, single nucleotide polymorphisms; ECM, extracellular matrix; MASPs, MBL-associated serine proteases; gt, genotype; InDel, internal deletion; MAF, minor allele frequency

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phase responses observed in only few individuals (Dean et al., 2005). The contribution of MBL to disease progression in HCV infection has been investigated, and MBL polymorphisms have been associated with fibrosis (Alves Pedroso et al., 2008; Koutsounaki et al., 2008). The impact of genetic polymorphisms in the collagen domain of MBL with progression to HCC is still unclear (Eurich et al., 2011; Segat et al., 2008). However, no studies have compared the expression of both MBL and ficolins in patients with different outcomes disease progression.

Chronic HCV infection induces liver damage, which may disrupt its ability to synthesise these lectins, thus potentially impairing the antiviral immune response in later stages of infection. In chronic HBV infection, HCC has been associated with reduced ficolin-2 expression (Chen et al., 2015; Hoang et al., 2011). To investigate the hypothesis that altered PRR activity is associated with development of HCC in chronic HCV infection we studied the activity of MBL, ficolin-2 and ficolin-3 in individuals infected with HCV who experienced different disease endpoints. Assays measuring binding of MBL, ficolin-2 or ficolin-3 to reference ligands were used to determine PRR activity in different individuals. Given that it is clinically important to understand which HCV-infected individuals are most likely to progress to developing hepatocellular carcinoma, we also investigated the possibility that serum PRRs are elevated prior to diagnosis of cancer.

## 2. Methods

### 2.1. Samples

Archived HCV-positive serum samples were retrieved from the Trent HCV clinical cohort (Mohsen, 2001). Ethical approval for the Trent study was granted by the Northern & Yorkshire Multicentre Research Ethics Committee (MREC/98/3/55) and patients provided biological samples with informed consent. Samples represented chronic HCV infections and patients with HCV infection who had developed HCC. Healthy HCV-negative controls were obtained commercially from UK HCV-negative blood donor surplus material. Sample IDs were blinded and samples tested in a randomised order. PRR activity was assessed in ELISA using serum diluted 1/5 in an inactivating buffer (TBS, 0.1% Triton X100). Each sample was analysed in duplicate and the mean value was used for subsequent analysis. Positive controls and standards were included on each assay plate to enable direct comparison of datasets.

### 2.2. Ficolin binding assays

Ficolin activity was determined by an in-house ELISA (Hamed et al., 2014). Briefly, MaxiSorp® ELISA plates (SLS) were coated overnight at 4 °C with 1 mg $mL^{-1}$ acetylated-bovine serum albumin (BSA - Sigma) in PBS. Wells were blocked with PBS with 0.05% (v/v) tween 20 (PBS-Tween) and 5% (w/v) milk powder for 2hrs at room temperature (RT), followed by washing (three washes with Wash Buffer [10 mM Tris–HCl, 140 mM NaCl, pH 7.4, containing 0.05% Tween 20 and 5 mM $CaCl_2$]). Serum, including a serially-diluted reference serum (a kind gift from Anders Krarup, University of Zurich) ((Hamed et al., 2014)) was added and incubated overnight at 4 °C followed by washing. Wells were incubated for 2 h at RT with 0.5 μg $mL^{-1}$ anti-human ficolin-2 antibody (clone GN5, Hycult Biotech) or anti-human ficolin-3 purified goat IgG (AF2367, R&DSystems) followed by washing. Wells were then incubated with anti-mouse IgG (FC-specific) alkaline phosphatase antibody (A1418, Sigma) or anti-goat/sheep IgG alkaline phosphatase antibody (A8062, Sigma) for 1 h followed by washing. Assays were developed with Sigma-Fast ρ-Nitrophenyl Phosphate substrate (pNPP, N-2770, Sigma), according to the manufacturer's instructions and absorbance measured at 405 nm after 20 min incubation at RT. Ficolin activity was reported as the proportion of binding compared to a standard curve generated using serial dilutions of the standard reference serum of known ficolin concentration.

### 2.3. MBL binding assay

MBL binding was determined by ELISA as previously described (Brown et al., 2007) with some modifications. Briefly, MaxiSorp® plates (SLS) were coated overnight at 4 °C with 1 μg $mL^{-1}$ mannan in coating buffer [0.1 M $NaHCO_3$; pH 9.5]. Wells were incubated for 2 h at room temperature (RT) with blocking buffer [Phosphate Buffered Saline (PBS) and 0.1% (v/v) Tween-20] followed by washing (three times with Wash buffer [10 mM HEPES; 1 M NaCl; 5 mM $CaCl_2$; 0.1% (v/v) Tween 20; pH 7.4]). Serum samples, initially diluted 1:5 in inactivating buffer, were diluted to a final dilution of 1:50 in wash buffer. A serial dilution of purified MBL (expressed in vitro) was used as a control. Samples were incubated overnight at 4 °C, followed by washing. Rabbit polyclonal antiserum to recombinant MBL (Arnold et al., 2004), diluted 1:700 in Wash Buffer, was added to each well and incubated for 2hrs at RT followed by washing. Anti-rabbit IgG-alkaline phosphatase (AP) (A-2556, Sigma, 1: 2000) was added and incubated for 1 h at RT followed by washing. The assay was developed using Sigma Fast pNPP substrate as above. MBL activity was quantified by comparing to the standard curve generated using serial dilutions of the reference MBL, with activity reported as binding proportional to the wild-type protein.

### 2.4. Direct PCR and sequencing

The promoter region of the MBL2 gene was directly amplified from the serum. A Phusion Blood Direct PCR kit (F-547S, Thermo) was used according to the manufacturer's instructions and all primers used in this study were as described by Thio et al. Thio et al.,al. (Thio et al., 2005). *MBL2* promoter was amplified using MBLP550F [ACTCTGCCAGGGCC AACGTA] and MBLP221R [TGATGAGCAGTGGGGATCCTA] primers. 20 μl PCRs included 0.5 μl inactivated serum sample, 5 pmol of each primer, Phusion Blood PCR buffer, 2.0 mM EDTA, 1.5 mM $MgCl_2$ and 0.4 μl Phusion Blood II DNA polymerase. Thermocycler parameters were 98 °C for 5 min followed by 35 cycles of: 98 °C for 15 s, 63 °C for 5 s and 72 °C for 15 s, with a final step of 72 °C for 2 min. PCR products were Sanger sequenced using the PCR amplification primers and two additional internal primers: MBLP550R [CAGCTGATTCCCCTCCAG GAC] and MBLP221F [GGGATTCAGGTGGCAGATGG] and analysed using MEGA7 (Kumar et al., 2016).

### 2.5. Statistical analysis

Statistical analysis was performed using Graph Pad prism 7. Comparison of lectin activity between healthy controls and chronic HCV patients with and without HCC was performed by Kruskal-Wallis test utilising Dunn's Multiple Comparisons test. Serum level linkage analysis was performed using standard linear regression and Pearson correlation coefficients, two-tailed P value and 95% CI. Mann-Whitney U tests were used for evaluating differences in lectin levels between pre-HCC and non-HCC samples at different sampling time points.

## 3. Results

### 3.1. Serum lectin activity alters with age in healthy individuals

HCC occurs in HCV infections only after prolonged infection (usually > 20 years after infection). To first determine if age at time of sampling influenced the observed PRR expression, initially the binding of all 3 lectins to their ligands were determined using the sera isolated from healthy controls, stratified by age and gender (Fig. 1). Sera were obtained from 72 males and 72 females ranging from 17 to 70 years of age (at time of sampling). Lectin binding was determined by ELISA, using a reference wild-type ligand for each lectin. Relative binding provides an indication of the activity of these proteins in serum, combining the quantity and binding avidity of each lectin.

Ficolin-2 and -3 activities were generally low across all age groups

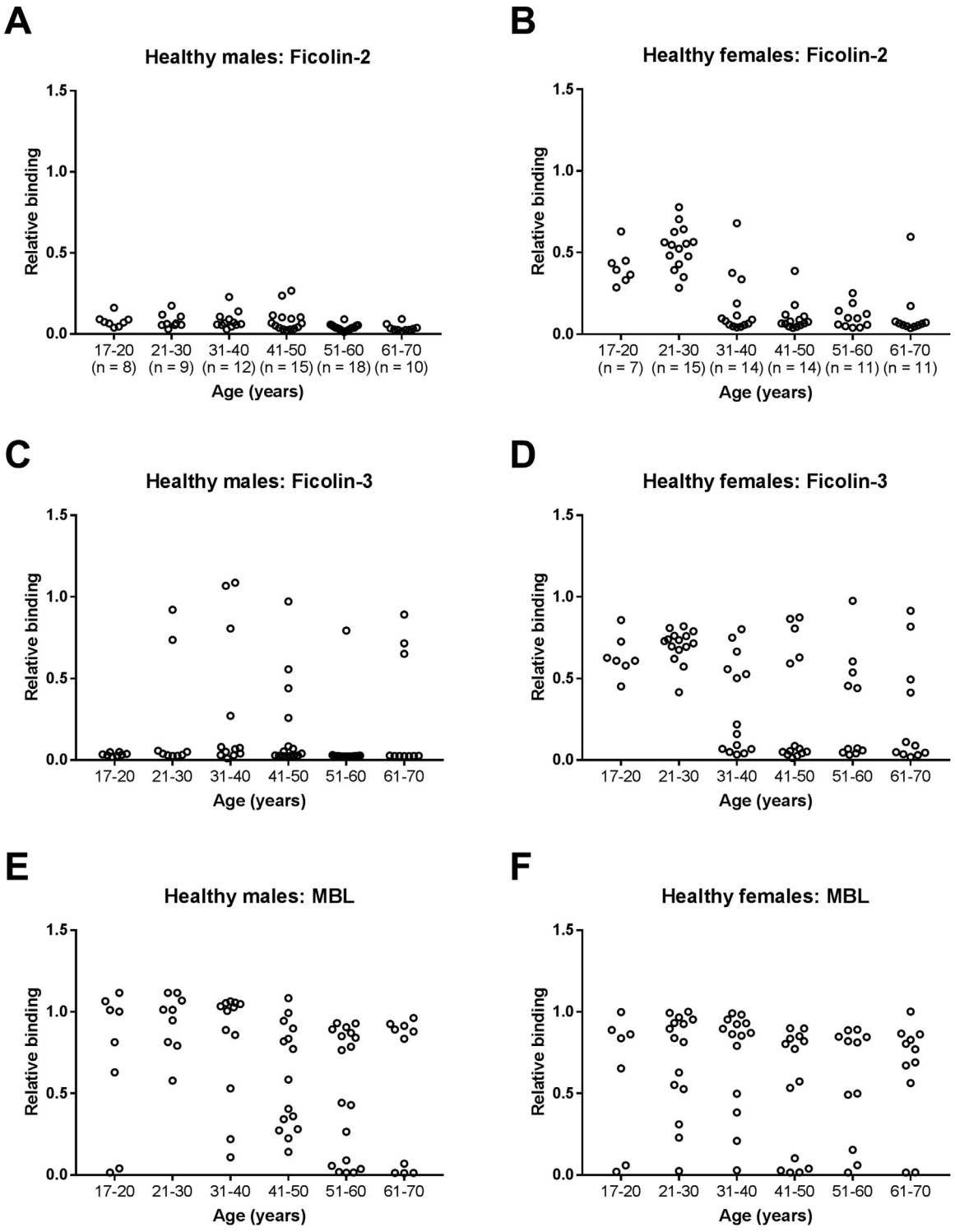


**Fig. 1.** Serum binding activity in healthy donors are influenced by gender and age. Serum binding activities of MBL, ficolin-2 and ficolin-3 of healthy donors (n = 144) were determined by ELISA. Binding is reported relative to a reference, wild-type sample of known concentration. Samples were separated by gender and age at time of sample donation (17 − 20, 21 − 30, 31 − 40, 41 − 50, 51 − 60, 61 − 70). Samples were tested in duplicate and the mean value reported (open circles). The median for each age group is shown (black lines).

in men (Figs. 1A and C). The binding signal for MBL was lower in older men, however the data appeared to become bimodal (Fig. 1E). In women; the mean ficolin-2 and ficolin-3 binding levels were higher in younger females (17–30 yrs) compared with older females (Figs. 1B and D) whereas MBL binding signal was comparable across the age groups (Fig. 1F). The ficolin-3 data in women over 30 also appeared to become bimodal.

### 3.2. HCV-infected sample groups

Using retrospective, cross-sectional sampling we identified 31 patients with HCC for whom samples were available, both prior to, and at the time of, HCC diagnosis from the UK Trent HCV cohort. A group of age-matched individuals with persistent HCV infection and without evidence of HCC were selected as controls. The sample closest to time of

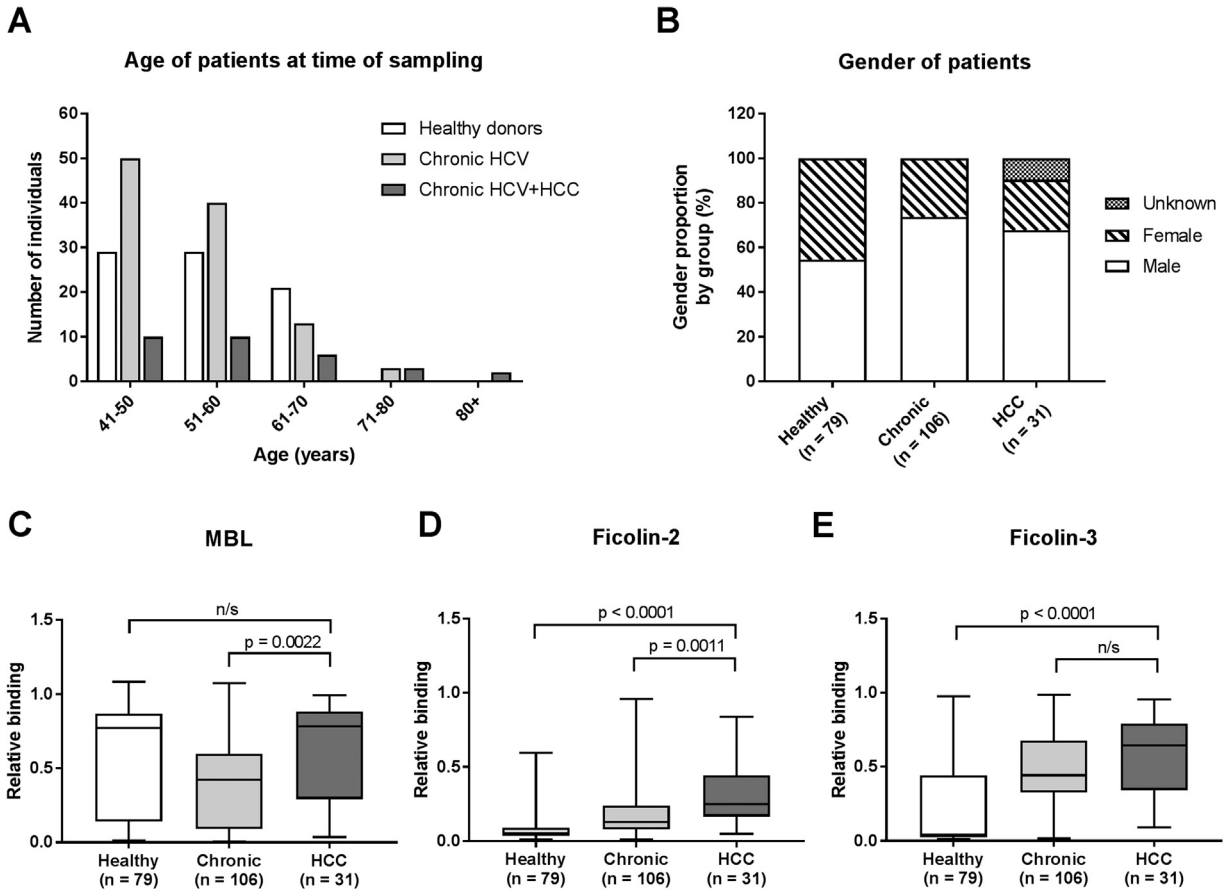


**Fig. 2.** Patients with chronic HCV and diagnosed HCC have significantly elevated Ficolin-2 and MBL binding activities compared to control group. Serum samples from HCV-positive patients diagnosed with HCC were obtained from the Trent HCV biobank. A) Patient age at time of sampling closest to diagnosis was noted (ranging from 41 to 81 years of age) and used to select comparable samples from healthy donors and HCV-positive patients with no diagnosis of HCC. B) Gender distribution within each group as proportion of group. Records for three patients within the HCC group did not include gender so noted as gender 'unknown'. The number of patients within each group is noted below. Serum ficolin-2 (C), ficolin-3 (D) and MBL (E) binding activities were determined by ELISA from the sample taken closest to the date of HCC diagnosis and compared with control samples. Lectin levels were compared to healthy and Chronic HCV control groups by Kruskal Wallis test with adjustment for multiple comparisons test with p values for each comparison are noted above. n/s, not significant.

HCC diagnosis (less than six months from diagnosis) was tested for the HCC-positive patients. The age of the HCC-positive patients at time of sampling closest to HCC diagnosis ranged from 43 to 83 yrs. Each HCV-positive, HCC-negative patient (n = 106) (Fig. 2A) had repeated samples taken over periods of > 10 years, and as such their age at time of sampling fell into several age brackets. To avoid biased or repeat sampling and to achieve an 'age at time of sampling' profile comparable to the HCC positive patients, the most recent time point with known date of sample collection was used for these patients. All healthy controls were selected to cover the age range of the HCC-positive patients (40 + years of age). Only a single sample was available for each of the healthy controls (n = 79).

Of the 31 patients identified with chronic HCV and diagnosed with HCC, 21 were identified as male (67%), seven were female and for three individuals this information was not available (Fig. 2B). A similar gender bias was seen for the chronic HCV group (73% male). For the healthy control cohort 54% were male and 46% were female.

Although small fluctuations in lectin-binding signal were observed among healthy individuals, the data were comparable between men and women older than 40 years of age (the lowest age limit of our HCC group) (Fig. 1). This observation, combined with comparable gender demographics of the HCV positive patients – where differences in biomarker levels was specifically relevant, led us to exclude gender as a discriminating factor in our subsequent analysis.

### 3.3. Serum lectin-binding activities are elevated in HCV-positive patients with HCC compared to HCV-positive, HCC-negative patients

The serum activity of all three lectins varied between the three sample groups. MBL levels in patients with HCC were comparable to healthy controls yet significantly elevated over HCV positive patients without HCC (Fig. 2C). Ficolin-2 activity in patients with HCC was elevated above both control groups (Fig. 2D) and ficolin-3 levels were lower in healthy controls compared to HCV positive patients, regardless of HCC status (Fig. 2E).

To determine if the elevated binding activities of serum lectins in patients with HCC was due to a global increase in PRR activity, the binding activity of each PRR was compared to the other PRRs in the same sample. There was no correlation between any of the lectin levels for HCV-positive, HCC-negative patients (Fig. 3A, B and C). The only correlation between lectin-binding activity was found in HCC-positive individuals and only between ficolin-3 and MBL (Fig. 3F).

### 3.4. Serum lectin-binding activity as a biomarker for progression to HCC

To determine if the levels of these PRRs were persistently elevated in individuals who develop HCC compared to those who do not, we identified those patients with HCC where a sample was collected within 6 months of diagnosis and for whom earlier samples were also available. We compared lectin activity to those in age-matched samples taken from HCV-infected individuals with no liver cancer. MBL levels

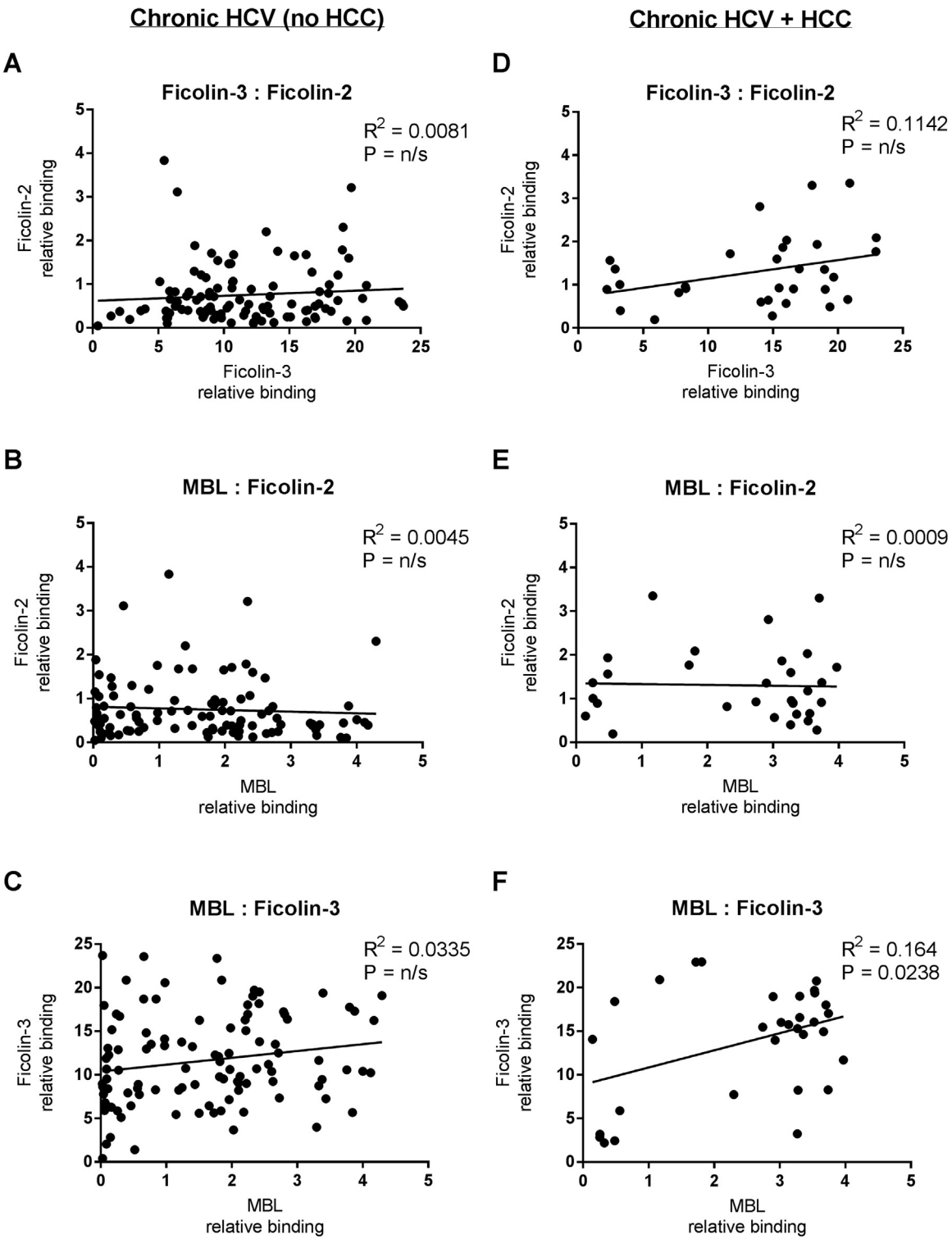


**Fig. 3.** Ficolin-3 levels correlate with elevated ficolin-2 and MBL levels in patients with HCC. Linear regression analysis of paired lectin activity data for HCV-positive patients with (**A**, **B** and **C**) and without HCC (**D**, **E** and **F**). R squared and P values are shown for each correlation. n/s, not significant.

were consistently elevated in patients who progressed to cancer, at least 3 years prior to diagnosis of HCC, while Ficolin-2 activity was only observed to increase closer to the time of HCC diagnosis (Fig. 4). There was also some evidence that ficolin-3 activity was elevated at a time point 3 years before HCC diagnosis. Due to the nature of the available samples it was not possible to extend analysis of lectin levels beyond 3 years prior to diagnosis.

### *3.5. Differences in MBL binding activity are not influenced by infecting genotype (gt), ethnicity or MBL promoter polymorphisms*

As elevated MBL binding activity was associated with HCC development, other factors influencing MBL activity were investigated. As HCV genotype and patient ethnicity could play a role in the progression to HCC and influence lectin activity, respectively, we investigated these factors in our HCV-positive groups. Individuals with HCC were mostly

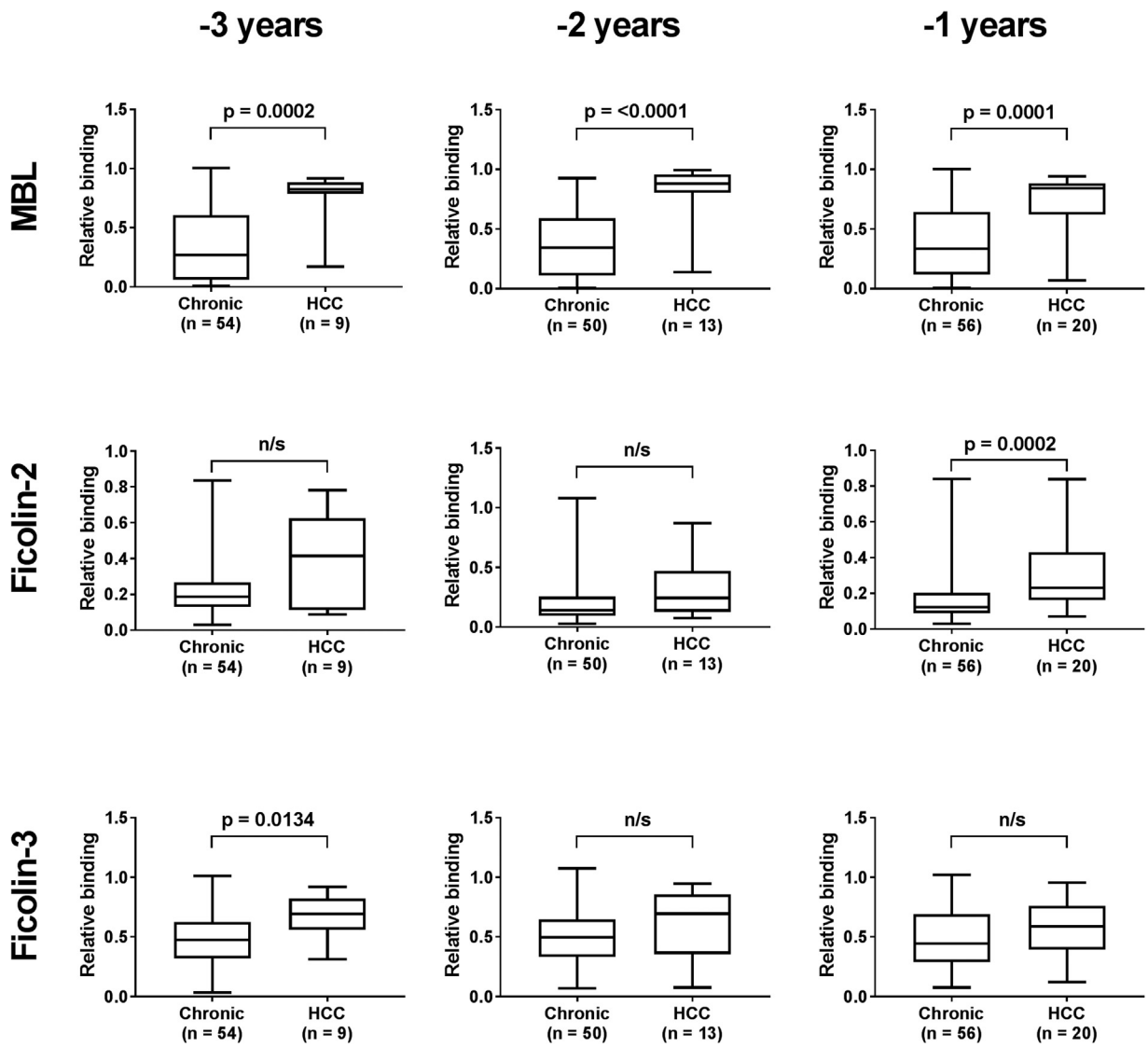


**Fig. 4.** MBL and ficolin-3 binding are elevated up to 3 years prior to diagnosis of HCC. The sample taken closest to time of HCC diagnosis was set as year '0'. Each analysis was restricted to patients where appropriately spaced samples existed, with 20, 13 and 9 patients eligible for comparison analysis for 1 year, 2 years and 3 years pre-diagnosis, respectively. Mann Whitney tests were used to compare lectin activity at time of sampling with time-matched samples taken from patients who did not have HCC, and for whom samples were available at the defined intervals. P values are noted above each comparison. n/s, not significant.

**Table 1**
Ethnicity and HCV genotypes of patients with diagnosed HCC (n = 31).

| | | Genotype | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | n/a | total |
| Ethnicity | Caucasian | 8 | 1 | 12 | 1 | 2 | 24 |
| | South Asian | 0 | 0 | 2 | 0 | 1 | 3 |
| | n/a | 0 | 0 | 0 | 1 | 3 | 4 |
| | Total | 8 | 1 | 14 | 2 | 6 | 31 |

Ethnicity and virus genotype data was not available for 4 and 6 individuals, respectively (n/a).

**Table 2**
Ethnicity and HCV genotypes of patients with chronic HCV without development of HCC (n = 106).

| | | Genotype | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | n/a | total |
| Ethnicity | Caucasian | 30 | 7[†] | 34 | 0 | 4 | 75 |
| | Afro-Caribbean | 1 | 2 | 0 | 1 | 0 | 4 |
| | South Asian | 0 | 0 | 3 | 0 | 0 | 3 |
| | n/a | 9 | 2 | 10 | 2 | 1 | 24 |
| | Total | 40 | 11 | 47 | 3 | 5 | 106 |

Ethnicity and virus genotype data was not available for 24 and 5 individuals, respectively (n/a).

[†] A single patient was noted to be co-infected with a genotype 2 and a genotype 3 virus. This patient was arbitrarily counted as genotype 2.

Caucasian (77%) with gt3 (45%) or gt1 (26%) HCV infections (Table 1). This was similar to the HCV-positive, HCC-negative group, where 71% were Caucasian with gt3 (44%) and gt1 (38%) infections (Table 2). Ethnicity has been linked to differences in lectin levels and the high prevalence of gt3 infections is in line with widely reported observations of gt3 being a risk factor for the development of HCC. However, the comparable ethnic profiles and proportion of gt3 infections in the two HCV-positive groups suggests these are likely not the determining factors in the observed lectin levels.

Two SNPs in the promoter region of the *MBL2* gene, nucleotide positions −550 (H/L variants) and −221 (X/Y variants) upstream of the start codon, are phenotypically linked to altered MBL expression

levels (Madsen et al., 1995). An additional 33 SNPs and an internal deletion (InDel) have also been described for the human *MBL2* gene. Therefore, we investigated the promoter sequences to see if the elevated levels of MBL in patients with HCC as compared to HCV positive, HCC-negative patients was due to a bias in the representation of these SNPs. Although only archival serum samples existed for the HCV-positive patients, we were able to amplify and sequence the *MBL2* promoter region from residual cells for 33 HCV-positive, HCC-negative individuals and 13 of the HCC-positive individuals to determine the haplotypes. Importantly, the MBL levels for this subset displayed similar difference to those of the full cohort, with HCV-positive patients with HCC having significantly elevated serum MBL levels compared to those without HCC (Supplementary Fig. S1). The minor alleles for 5 previously described SNPs (-550, −427, −349, −336 and −221) and the InDel −328–323 were present in our sample set, and were broadly comparable between the two HCV-positive patient groups (Supplementary table S1). The polymorphisms at positions −427, −349, −336 and the InDel at −328–323 appeared to be closely linked, as they were all heterozygous in 23 patients (Supplementary table S1). Only one sample was homozygous for the minor variant of one of these SNPs; −427 (C). The minor allele frequency (MAF) for each of the SNPs represented in our samples was comparable between HCC-positive and -negative groups and to the global MAF (Supplementary table S1).

An additional 4 minor allele SNPs were identified, each in a single sample. These were at position −367 (rs35615810; C/A) and 3 unassigned polymorphisms at −430 (G/C), 406 (T/C) and 384 (G/T). The exon region of the MBL gene contains 3 SNPs (codon 52, 54 and 57) that influence oligomerisation of the MBL trimers, resulting in reduced function (Garred et al., 2006). However, we were only able to obtain unambiguous sequence data for a very small number of samples over this region (data not shown) so it was not possible to speculate on the prevalence, and thus the influence, of these SNPs upon our observations.

## 4. Discussion

The aim of this study was to investigate whether serum lectin-binding activity is associated with development of HCC in HCV-positive individuals. Using ELISA-based assays of lectin-binding activity, we demonstrated that both age and gender influence the circulating binding activity of MBL, ficolin-2 and ficolin-3 in healthy controls. Men consistently displayed lower binding activity of serum ficolin-2 and ficolin-3 than women at ages < 40 years but were comparable in donors greater than 40 years old. The pattern observed for MBL was less clear; while the expression levels did appear to have a small decline in older individuals, the spread of values obtained was much greater, with some donors having an MBL-deficient phenotype. These data are consistent with reports that MBL expression declines in older healthy Chinese individuals (Ip et al., 2004) and that ficolin-2 expression is influenced by pregnancy (Halmos et al., 2012). It is important to note that genetic polymorphisms, several of which are linked to ethnicity, influence PRR expression levels. While complete data on these factors was lacking, it is unlikely that demographic factors biased any one group in our study due to the comparable ethnicities of the three groups. We did attempt to address the impact of polymorphisms on the expression of MBL in our HCV-infected patients. However, due to the nature of the samples collected, we were only able to determine promoter genotypes for a small subset of the cases. The descriptive statistics produced suggested that polymorphisms do not account for the differences in MBL activity observed between HCV-infected patients with, or without, HCC.

The signalling pathways regulating expression of these liver-expressed PRRs is still unclear and requires further definition. Comparison of expression/binding of the three PRRs revealed that lectin activity is generally independent of each other. Only for MBL and ficolin-3 in individuals with HCC was a correlation observed, although the strength of this association was low. This was surprising as ficolin-3 was not significantly elevated compared to the control HCV positive, HCC-negative group. The lack of correlation between ficolin-2 and MBL activity in HCV-infected individuals implies that the increased activity of these lectins seen in the HCC-positive samples is not linked, providing evidence that the expression of these genes is independently controlled.

Together, the data presented here indicated that elevation of MBL expression (at least up to 3 years before HCC onset) might be indicative of progression to HCC. Monitoring for the development of HCC is currently performed on high-risk individuals using ultrasound. Compliance to bi-annual checks is known to be limited and diagnosis of HCC is often made too late for effective intervention. Binding of ficolin-3 to reference ligands was greater in chronic HCV infection, while ficolin-2 and MBL were significantly elevated in those individuals who develop HCC compared to HCV-infected individuals without HCC. Ficolin-2 and MBL were elevated 1 year and 3 years prior to HCC diagnosis, respectively, suggesting they could be used as prognostic serum markers for the development of HCC. As these markers could be tested following a blood sampling in primary care, it might contribute to improved compliance with monitoring.

The observation that HCV-infected patients with HCC had higher levels of MBL and ficolin-2 activity than those without HCC is consistent with a mechanism of fibrosis/disease progression controlled by MASP activation of stellate cells (Saeed et al., 2013). Interestingly, this increase in lectin levels is in contrast to a previous study measuring total lectin concentration (rather than binding activity) that observed reduced ficolin-2 serum concentrations in patients with HCC following HBV infection (Hoang et al., 2011). This suggests that these two chronic hepatotropic viruses may cause liver cancer by distinct mechanisms, affecting liver-expressed PRRs to different extents. Serum concentrations of ficolin-2 and ficolin-3 are increased in ovarian cancer (Szala et al., 2013). However, in all these cancers it remains to be determined whether the altered expression of ficolins is causal, or a result of the growth of tumour tissue.

The assays used in this study measured the functional binding of PRRs to their ligands, compared to reference samples. Non-synonymous polymorphisms in the PRR coding regions may alter binding to ligands. Assessing the activity of serum lectins in this way provides the most accurate comparison of binding activity present in serum. The estimated serum concentrations of the three PRRs were broadly similar to that observed in previous studies (data not shown), with the elevated levels of ficolin-2 being comparable to those observed in patients with ovarian cancer (Szala et al., 2013).

While the present study is limited by the retrospective, cross-sectional nature of the sampling of patients, the strong evidence for an association between elevated MBL binding activity and the development of HCC is supportive for a larger prospective study of these biomarkers in HCV-induced liver cancer.

## Acknowledgements

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## Conflict of interest

The authors note no conflict of interest.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.02.002.

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