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IMPLICATIONS FOR THE DEVELOPMENT OF NOVEL THERAPIES**

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COAGULATION STATUS MODULATES MURINE HEPATIC FIBROGENESIS: IMPLICATIONS FOR THE DEVELOPMENT OF NOVEL THERAPIES

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Abstract

Background There is strong evidence demonstrating that coagulation system activation contributes to wound healing and promotes organ fibrosis. Several epidemiological studies have now shown that prothrombotic status, including carriage of the Factor V Leiden mutation (FvL), is associated with rapid progression of hepatic fibrosis.

Objectives To assess the effect of a procoagulant state on progression of hepatic fibrosis in a controlled environment and to test whether anticoagulation could attenuate fibrogenesis.

Methods We investigated the effects of coagulation status on liver fibrosis development in a mouse model of chronic toxic liver injury. Prothrombotic FvL mutant mice, C57BL/6 control animals and anticoagulated mice were studied after chronic exposure to carbon tetrachloride.

Results Carriage of the FvL mutation caused a significant increase in hepatic fibrosis. Anticoagulation with warfarin significantly reduced fibrosis progression in wild-type mice but was less effective against the profibrotic FvL mutation. Changes in the fibrosis scores were mirrored by changes in liver hydroxyproline content and hepatic stellate cell activation detected by α -smooth muscle actin expression.

Conclusions These results demonstrate that coagulation status has a strong influence on hepatic fibrogenesis. It is likely that thrombin signalling through the PAR₁ receptor expressed on hepatic stellate cells is responsible for this relationship. These results represent the first reported use of anticoagulation to slow hepatic fibrogenesis and suggest a potential novel anti-fibrotic therapeutic approach for the future.

Abstract: 223 Words

Introduction

Hepatic fibrosis results from a sustained wound healing response to chronic liver injury[1]. Unchecked, stellate cell mediated collagen accumulation within the hepatic parenchyma proceeds to cirrhosis. Existing therapy is targeted at the aetiology of hepatic fibrosis. If this proves unsuccessful, there are currently no effective therapeutic interventions to slow the rate of fibrogenesis.

Hypercoagulability plays an important but under-recognised role in many aspects of liver disease[2]. Early evidence of a role for coagulation in the pathogenesis of liver disease came from studies examining the effects of murine hepatitis virus infection where microthrombi were demonstrated within the hepatic microvasculature[3;4]. Wanless et al subsequently reported similar findings in patients with cirrhosis[5;6] and epidemiological studies identified an association between host pro-thrombotic status and rapid progression of hepatic fibrosis[7-9]. Under normal physiological conditions, thrombin catalyses the conversion of fibrinogen to fibrin, which then mediates clot formation. Thrombin has a well recognised role in the coagulation cascade and in activating platelet aggregation. In addition, it is also recognised to act as an activator of hepatic stellate cells, the mediators of hepatic fibrogenesis, via proteinase-activated receptor 1 (PAR₁). Increased thrombin generation may thus elevate levels of PAR₁ signalling and promote hepatic fibrogenesis.

As part of a negative feedback loop, thrombin activates Protein C (APC) which in turn cleaves Factor V at the R506 primary cleavage site[10;11]. The Factor V Leiden (FvL) mutation is a single base pair substitution conferring an amino acid change from Arginine to Glutamine in codon 506. This abolishes the primary APC cleavage site, leaves Factor V activity unchecked and hence confers a procoagulant tendency. This common mutation affects 1-8.5% of the Caucasian population and predisposes to thromboembolic disease[12].

Using the carbon tetrachloride induced liver injury model [13], we sought firstly to establish whether carriage of the FvL mutation (as an exemplar of pro-thrombotic tendency) could indeed influence progression of hepatic fibrosis. Secondly, we tested whether anticoagulation

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2 with warfarin (coumarin) could ameliorate the fibrosis progression in the same model. Both
3
4 male and female animals were studied to ascertain whether the gender effect observed in the
5
6 original epidemiological studies persisted in a laboratory environment[14].
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8 **Materials & Methods**

9 *Animals Studied & Carbon tetrachloride Exposure*

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11 Transgenic FvL mice generated by site directed mutagenesis were purchased from the
12 Jackson Laboratory, USA and maintained on a congenic C57BL/6 background (Strain
13 B6.129S2-F5^{tm2Dgj}/J)[15]. The $\Delta R504Q$ mutation carried by these mice is homologous to the
14 human mutation. Homozygous carriage of the FvL mutation was confirmed by
15 genotyping[15]. FvL and wild-type C57BL/6 mice (Harlan, UK) aged 6-8 weeks were used in
16 this study (group sizes in Table 2). Animals were housed under standard conditions. All
17 research was approved by the local ethical review process and carried out in accordance
18 with the Animal (Scientific Procedures) Act 1986 taking care to minimise any distress caused
19 to the animals.
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23 Based on effective induction of fibrosis in pilot studies, mice were treated with carbon
24 tetrachloride (CCl₄) diluted in a corn oil vehicle and administered by intraperitoneal injection
25 on alternate week days. The dose of CCl₄ administered was increased weekly in a stepwise
26 fashion (0.125, 0.25, 0.5, 1ml/kg body weight) with the maximum dose maintained from day
27 21.
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31 Warfarin was administered to anticoagulate mice by adding the drug to the drinking water. A
32 warfarin dose of 1 $\mu\text{g/ml}$ was chosen as pilot studies indicated that this approximately
33 doubles the whole blood clotting time but minimises spontaneous adverse events.
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36 *Histopathology & Digital Image Analysis*

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38 Tissue collected at each time point was fixed in 10% formalin and processed into paraffin
39 wax. Sections were stained with Haematoxylin & Eosin or Chromotrope Aniline Blue (CAB)
40 trichrome to delineate fibrosis. Samples were examined by two histopathologists, blinded to
41 which study group each sample was from, and a joint score agreed using an adapted Ishak
42 Modified Histological Activity Index[16;17] (Table 1). Sections were also stained using the
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collagen specific stain Pico-Sirius Red before undergoing digital image analysis to determine the mean percentage area of fibrosis. An average of 12 fields (10x objective) throughout each section were captured using a Zeiss AxioVert 200M microscope and analysed using AnalySIS software (Soft Imaging System, USA). Tissue from a subset of male animals culled at the 4 week time point was also immunohistochemically stained for a marker of hepatic stellate cell activation, alpha-Smooth Muscle Actin (primary antibody Clone 1A4, Dako)[18].

Total RNA Extraction & Quantification of Relative Gene Expression by rt-PCR

The effects of coagulation status were most marked in male animals culled at the 4 week time point and so this group was selected for further detailed analysis of stellate cell activation, collagen deposition and gene induction.

Total RNA was extracted from snap frozen liver tissue (RNeasy Mini kit, Qiagen), reverse transcribed (RETROscript kit, Ambion) and quantitative PCR analysis performed in triplicate using TaqMan Gene Expression Assay reagents (Applied Biosystems). Primers specific for Pro-collagen type I (Col1a2, Mm00483888_m1) were studied. Data were normalised to the endogenous house-keeping gene Hypoxanthine Guanine Phosphoribosyl Transferase (*hprt*) and fold change differences in expression relative to non-warfarin treated C57BL/6 control animals calculated by the Comparative $\Delta\Delta C_T$ method[19].

Comparative Western Blot Analysis of Alpha-Smooth Muscle Actin (α SMA) Protein

Expression

Expression of α SMA is a sensitive marker of hepatic stellate cell activation to a fibroblast-like phenotype therefore α SMA levels were measured as an indicator of HSC activation. Protein was extracted from snap frozen liver tissue as previously described[20]. Fifty micrograms of protein pooled from each study group was loaded onto a 4-12% NuPage MOPS gel (Invitrogen) and a Western blot performed onto a nitrocellulose membrane. α SMA was labelled with a primary mouse monoclonal antibody (Clone 1A4, Dako) and chromogenic detection performed (Western Breeze, Invitrogen). Gel images were captured and comparative quantification of protein performed using Image-J software (US NIH).

Tissue Hydroxyproline Measurement

Collagen helices deposited within the liver during fibrogenesis are stabilised by hydroxyproline. This amino acid is almost exclusively confined to collagenous connective tissue and so may be used as a surrogate to quantify collagen deposition[21]. Liver hydroxyproline content was determined using a colorimetric technique as previously described[22]. Samples were tested in triplicate compared to standards of known hydroxyproline concentration and results expressed as μg hydroxyproline per gram of liver tissue.

Lipid Peroxidation Malondialdehyde (MDA) Assay

CCl_4 induces tissue damage by release of free radicals and reactive oxygen species as it is metabolised by cytochrome P450[23]. Tissue oxidative stress is reflected by the MDA content[24]. To determine if warfarin interacts with CCl_4 metabolism and thus limits its injurious effect, hepatic MDA content was measured. Liver tissue from cohorts of warfarin treated and non-warfarin treated C57BL/6 mice culled 24 hours after their last CCl_4 exposure ($n = 5$ per group) were analysed using a colorimetric MDA assay (Lipid Peroxidation Assay #437634, Calbiochem, USA).

Statistical Analysis

Statistical analysis performed using SPSSv12 (USA). Normally distributed continuous variables were compared by Student's t-test; results are represented as mean ($\pm\text{SEM}$). Ordinal and non-normally distributed variables were tested using the non-parametric Mann-Whitney U test. Statistical significance was accepted at $p < 0.05$.

Results

At the start of the experiment FvL mice weighed on average 23.5 ± 0.3 g and control mice weighed 21.1 ± 0.2 g, CCl₄ doses were body weight adjusted as described above. No fibrosis was observed in warfarin treated and non-treated animals that had not been not exposed to carbon tetrachloride. In warfarin treated animals whole blood clotting time was 8.5 ± 2 minutes (mean \pm standard deviation) compared to 4.5 ± 0.6 minutes in control animals. Prothrombin time, measured in subgroup of animals during the study, was 24.6 ± 1.5 seconds in warfarin treated vs 11.3 ± 0.15 seconds in non-warfarin treated mice. No differences in warfarin response between mutant and non mutant mice were observed. Early mortality (ill health necessitating cull of animal in accordance with regulatory requirements) was limited to three warfarin treated animals where death was due to haemorrhage soon after intraperitoneal injection.

Lipid Peroxidation Malondialdehyde (MDA) Assay

No significant difference in hepatic MDA content was observed between warfarin treated and untreated C57BL/6 mice 24 hours after exposure to CCl₄ (mean MDA concentration \pm SEM: warfarin treated animals 0.24 ± 0.03 μ moles/mg; control mice 0.26 ± 0.02 μ moles/mg). We therefore conclude that effects of anticoagulation described below are not an artefact due to a reduction in the injurious capacity of CCl₄.

Fibrosis progression in wild type C57BL/6, FvL and anticoagulated mice

Representative histological images are shown in Figure 1. Histopathology and DIA scores are presented in Table 2 with inter-group analysis summarised in Table 3 and Supplementary Figure A. Animals culled at the start of the experiment had no evidence of spontaneous hepatic fibrosis (score = 0, C57BL/6 n=5, FvL n=4).

After 2 weeks of CCl₄ exposure, FvL mice exhibited bridging fibrosis and occasional nodule formation (Figure 1C). In contrast, C57BL/6 control mice showed milder fibrosis with evidence of fibrous expansion of peri-venous areas and minimal bridging (Figure 1B). Warfarin treated mice of both strains had lower fibrosis scores than the corresponding mice

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2 given CCL₄ without warfarin but the differences did not reach statistical significance at this
3 stage (C57BL/6 shown in Figure 1A). Digital analysis (presented in Table 2) supports these
4 observations. Mean fibrosis area was increased by 80% in male FvL mutants compared to
5 C57BL/6 controls (p=0.002) and more than doubled in female mice (p=0.007).
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11 Non-warfarin treated C57BL/6 control mice culled at 4 weeks exhibited greater hepatic
12 fibrosis than those culled at 2 weeks however progression was much more pronounced in
13 FvL mutant mice. After 4 weeks CCl₄ exposure, FvL mutant mice had developed extensive
14 bridging fibrosis with nodule formation and in some cases established cirrhosis (Figures 1F &
15 1I). Numerically this is a 60% increase in mean fibrosis area over C57BL/6 controls for FvL
16 males, p=0.003 and a 74% increase for females, p<0.001. Table 2 also shows that FvL and
17 the non-warfarin treated C57BL/6 male mice have significantly higher mean histology scores
18 than female littermates (C57BL/6 $\Delta \pm$ SEM 0.86 \pm 0.28, p=0.012; FvL 0.71 \pm 0.28, p=0.023).
19 Warfarin administration reversed this gender effect. Warfarin treated male C57BL/6 mice
20 showed only moderate fibrous expansion around the central veins (Figures 1D & 1G) with a
21 mean reduction in histological score of 2.25 \pm 0.38 (SEM) compared with control (p=0.007).
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26 DIA demonstrated that mean fibrosis area was 33% reduced in male warfarin treated
27 C57BL/6 mice (Table 2, p=0.005). There was no significant difference in fibrosis area
28 between warfarin treated C57BL/6 mice at 2 and 4 weeks (Δ 0.44% \pm 0.27, p=0.171) although
29 control animals had a 1.0% \pm 0.22 increase (p=0.01). At 4 weeks, the effects of warfarin were
30 less marked in females with no significant differences from control detected. FvL mice
31 treated with warfarin showed levels of fibrosis similar to FvL mice which had not been
32 anticoagulated (Table 2).
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51 *Tissue Hydroxyproline Content*

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54 Carriage of the FvL mutation in males was associated with a significant 49% increase in tissue
55 hydroxyproline content over control (FvL 215.6 \pm 27.4 μ g/g liver tissue vs C57BL/6
56 146.6 \pm 23.7 μ g/g, p=0.043). Warfarin treatment of C57BL/6 mice was associated with a 32%
57 reduction in hydroxyproline content although this did not reach statistical significance (C57BL/6
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2 + Warfarin $98.4 \pm 18.3 \mu\text{g/g}$, $p=0.243$). Warfarin did not have a significant effect on hydroxyproline
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4 content in FvL mice.
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6 7 *Hepatic Gene Expression*

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9 Expression of genes that reflect hepatic fibrogenic activity were measured using quantitative rt-
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11 PCR. Data are presented in Figure 2 as fold change relative to the C57BL/6 control group and
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13 SEM range. Pro-collagen type 1 expression was significantly increased by 32% in mice carrying
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15 the FvL mutation (1.32, 1.02-1.67, $p=0.027$) and reduced by 40% (0.6, 0.45-0.82, $p=0.001$) in
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17 C57BL/6 animals treated with warfarin.
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19 20 *Relative quantification of alpha-Smooth Muscle Actin Protein Expression*

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22 Western blot analysis demonstrated that the level of αSMA expression in male FvL mice after
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24 4 weeks CCl_4 exposure was approximately twice that of C57BL/6 controls (Figure 3).
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26 Treatment with warfarin reduced αSMA expression in both C57BL/6 and FvL animals by 10-
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28 12% respectively. As illustrated in Figure 4, immunohistochemical staining of a subset of
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30 tissue samples supported these observations. An increase in the number of αSMA positive
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32 cells was seen in FvL mice whilst a reduction was seen in warfarin treated C57BL/6 animals.
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34 These findings are consistent with increased stellate cell activation in response to CCl_4
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36 exposure in animals that carry the FvL homozygote mutation and reduced activation in those
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38 concomitantly treated with warfarin (Figure 4).
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Discussion

The data presented here supports the original observations in chronic viral hepatitis that carriage of procoagulant mutations such as the Factor V Leiden mutation confer an accelerated hepatic fibrosis phenotype during chronic liver injury[7-9]. The effect of mutation carriage was apparent in subjects of either gender after two weeks and became more marked at the four week time point. The histological demonstration of increased fibrous tissue deposition was corroborated by demonstrably greater tissue hydroxyproline content and higher levels of Pro-collagen type 1 gene expression. The association between prothrombotic status and tissue fibrosis is also pertinent to fibrogenesis in other organ systems. Indeed, corroborative data has been reported by investigators studying pulmonary fibrosis where prothrombotic status, and specifically carriage of the FvL mutation, have been shown to lead to more rapid development of fibrosis in mice after Bleomycin inhalation[25]. Reports describing enhanced collagen accumulation leading to the development of fibrotic skin lesions in tissue plasminogen activator deficient mice have also been published[26] and the role of Plasminogen Activator Inhibitor-1 (PAI-1) in tissue fibrogenesis has been described in several pathologies including renal, hepatic, cardiovascular and pulmonary disease[27].

The reduction in fibrosis observed in animals treated with warfarin suggests that anticoagulation retards fibrogenesis. This was most evident in male C57BL/6 mice whereas FvL mice appeared to be resistant to warfarin. The dose of warfarin used in these experiments was limited by risk of haemorrhage and may have been insufficient to overcome the pro-thrombotic and pro-coagulant effect of the homozygous carriage of the FvL mutation. In human populations only the effect of heterozygote FvL carriage has been studied and we aim to study FvL heterozygote mice in the future. Interestingly the direct thrombin inhibitor, Ximelagatran, appears to have been sufficiently potent to overcome the effect of the FvL mutation without observable risk of adverse events in the mice (QA & MT, unpublished data). Whilst this study represents the first reported therapeutic use of coumarin based anticoagulation to slow hepatic fibrogenesis, similar results have been reported with low

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2 molecular weight heparin [28] and dipyridamole[29] in other models of liver damage and with
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4 PAR₁ knockout mice, aerosolized heparin and urokinase in pulmonary fibrosis[30;31]. There
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6 is also precedent for the use of anticoagulation to treat pulmonary fibrosis in humans,
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8 exemplified in a trial of warfarin anticoagulation in patients with idiopathic pulmonary fibrosis
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10 in which mortality was reduced by 50%[32].

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12 The interaction of gender on the rate of fibrosis has previously been observed in human HCV
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14 studies where, as demonstrated here, it is recognised that males progress more
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16 rapidly[33;34]. Evidence suggests that oestrogen acts as an *in vivo* antioxidant, inhibits
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18 stellate cell profibrotic TGF β expression and possibly reduces PDGF-induced cell
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20 proliferation[35;36]. Whilst an effect of anticoagulation on fibrogenesis was hypothesised, the
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22 significant differences in response to anticoagulation between the genders were an
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24 unexpected finding. Whilst a full explanation for this effect is not yet available recently
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26 published data suggests that female sex hormones are able to modulate PAR-1 expression
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28 in certain tissues[37] and so may blunt the contribution of this pathway to stellate cell
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30 activation. Our findings are consistent with previous reports that the association between FvL
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32 mutation carriage and accelerated hepatic fibrosis with HCV infection was most pronounced
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34 in men[7] and may explain in part the failure to identify an effect of FvL carriage on fibrosis
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36 rate in an all female patient cohort[38]. Further, in humans an increased risk of recurrent
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38 thromboembolic events is seen in males[39]. Taken together these findings suggest that
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40 males may be more sensitive to APC resistance and the generation of thrombin[39] and that
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42 the effects of coagulation status may therefore be of greater significance in males. Due to
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44 these phenotypic differences, subsequent detailed study of fibrogenesis was confined to
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46 male animals culled at the 4 week time point.

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48 The mechanism through which coagulation status influences hepatic fibrosis is not fully
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50 elucidated. Some have proposed that micro-thrombi disrupt the flow of blood within the
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52 hepatic parenchyma and so lead to tissue ischaemia, parenchymal extinction and
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54 fibrosis[5;6]. PAR₁ (thrombin) receptor expression has been shown to be upregulated during
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56 acute and chronic tissue injury[40]. PAR₁ receptors are present on hepatic stellate cells
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2 which, when activated, deposit fibrous matrix. Hepatic stellate cells may be activated directly
3 by thrombin[41] or by pro-inflammatory cytokines secreted by macrophages and platelets
4 (which also express PAR₁). In support of PAR₁ mediated fibrosis acceleration, it has been
5 shown that PAR₁ antagonists ameliorate fibrosis in an animal model[42] and that
6 polymorphisms in the PAR₁ gene influence the rate of disease progression in HCV
7 infection[43].
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15 In conclusion, these data provide support for the hypothesis that coagulation status promotes
16 hepatic stellate cell activation and enhances hepatic fibrogenesis. Alongside the clinical
17 response to warfarin therapy in pulmonary fibrosis these results provide a rationale for testing
18 anticoagulants as a treatment for liver fibrosis where the cause of the underlying liver disease
19 cannot be removed.
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32 **Abbreviations**

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34 CCl₄ Carbon tetrachloride; HCV Hepatitis C; IFN α Interferon-Alpha; PAR₁ Proteinase-activated
35 receptor 1; FvL Factor V Leiden; SEM Standard Error of the Mean; α SMA Alpha-Smooth Muscle Actin;
36 CAB Chromotrope Aniline Blue; APC Activated Protein C.
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Figure 1: Representative Histological Images

Liver tissue sections were stained with CAB trichrome to delineate fibrosis.

A, B & C Warfarin treated (**A**) and non-warfarin treated (**B**) C57BL/6 mice after 2 weeks CCl₄ exposure. FvL mutant tissue (**C**) showing evidence of bridging fibrosis and early nodule formation after 2 weeks CCl₄ exposure. Original magnification x300.

D, E & F Tissue from a warfarin treated C57BL/6 mouse (**D**) with evidence of fibrous spur formation but no nodule formation. Tissue from a non-warfarin treated C57BL/6 mouse (**E**) with evidence of bridging fibrosis. FvL mutant tissue (**F**) showing nodule formation approaching established cirrhosis. Original magnification x150.

G, H & I High magnification images of CAB Trichrome stained liver tissue from a warfarin treated C57BL/6 mouse (**G**), a non-warfarin treated C57BL/6 animal (**H**) and an FvL mutant mouse (**I**). Original magnification x300.

Figure 2: Differential Gene Expression between Study Groups Relative to C57BL/6

Pro-collagen type 1 was analysed by quantitative rt-PCR (n = 4-7 mice per group). Normalised fold change values relative to C57BL/6 are shown \pm SEM. Pro-collagen type 1 expression was significantly reduced in C57BL/6 mice treated with warfarin and increased in FvL mutant mice.

Figure 3: Western Blot of Alpha-Smooth Muscle Actin Protein Expression

Image of Western blot stained with primary antibodies to α SMA and skeletal Actin (control). The corrected area under the optical density curve of each band was measured using Image J software and is shown as a fold change relative to C57BL/6 mice (FvL 2.1, C57BL/6 1.0, C57BL/6 + Warfarin 0.9, FvL + Warfarin 1.8). After 4 weeks of CCl₄ exposure, FvL mutant mice have greater α SMA expression, and hence hepatic stellate cell activation, than C57BL/6 mice. Anticoagulation was shown to reduce α SMA expression. (n = 4-7 mice per group).

Figure 4: Immunohistochemical Staining of Liver Tissue for Alpha-Smooth Muscle Actin

Tissue samples from some male animals culled at 4 weeks were stained for α SMA. Relative to C57BL/6 control mice (**B**), these demonstrated reduced numbers of α SMA positive cells in warfarin treated C57BL/6 mice (**A**) and increased numbers of α SMA positive cells in FvL mutant animals (**C**).

Supplementary Figure A: Degree of Hepatic Fibrosis by Histological Score and Digital Analysis at 4 Weeks

Four Week Histological score (**A**) and Mean Percentage Area Fibrosis (**B**) for Male Mice.

Data are represented as mean \pm SEM. Carriage of the FvL mutation significantly increases and warfarin treatment significantly reduces histological score and mean percentage area fibrosis in C57BL/6 mice.

The reduction in liver fibrosis seen in warfarin treated FvL mice did not reach statistical significance.

Table 1: Modified Histological Scoring System for Quantifying Liver Fibrosis

Score	Description
0	No fibrosis
1	Fibrous expansion (spurs) around some central veins
2	Fibrous expansion around >50% central veins
3	Fibrous expansion around most central veins plus some CV-to-CV bridging
4	Fibrous expansion around most central veins plus marked (>50%) CV-to-CV bridging
5	Marked bridging fibrosis plus some nodules
6	Cirrhosis (>50% nodularity)

Table 2: Effect of CCl₄ on Histology & Digital Image Analysis Scores for each Study Group

Study Group	Gender	Histology Score (maximum 6)						Mean Percentage Area Fibrosis					
		2 Weeks			4 Weeks			2 Weeks		4 Weeks			
		Mean	(SEM)	n	Mean	(SEM)	n	Mean	(SEM)	n	Mean	(SEM)	n
C57BL/6	M	3.00	(0.45)	6	4.00	(0.26)	6	1.76	(0.2)	6	2.76	(0.09)	6
	F	2.00	(0.32)	5	3.14	(0.14)	7	1.40	(0.29)	5	2.38	(0.37)	7
FvL	M	4.11	(0.2)	9	4.86	(0.26)	7	3.18	(0.28)	9	4.47	(0.4)	7
	F	2.78	(0.15)	9	4.15	(0.15)	13	2.97	(0.32)	9	4.14	(0.21)	13
C57BL/6 + Warfarin	M	2.60	(0.4)	5	1.75	(0.25)	4	1.43	(0.11)	5	1.84	(0.27)	4
	F	2.40	(0.25)	5	2.88	(0.29)	8	1.89	(0.27)	5	2.11	(0.14)	8
FvL + Warfarin	M	3.83	(0.48)	6	4.57	(0.2)	7	2.68	(0.27)	6	3.82	(0.18)	6
	F	3.40	(0.4)	5	4.17	(0.31)	6	2.92	(0.33)	5	3.70	(0.35)	6

Table 3: Differences in Mean Percentage Area Fibrosis and Mean Histology Score Following Two or Four Weeks CCl₄ Exposure

Study Group	Variable	MALE Mean Difference Relative to C57BL/6				FEMALE Mean Difference Relative to C57BL/6			
		2 Weeks Mean (SEM)		4 Weeks Mean (SEM)		2 Weeks Mean (SEM)		4 Weeks Mean (SEM)	
FvL	% Area Fib.*	1.42 (0.38)	0.002	1.71 (0.45)	0.003	1.57 (0.48)	0.007	1.76 (0.39)	0.000
	Hist. Score†	1.11 (0.44)	0.031	0.86 (0.37)	0.044	0.78 (0.30)	0.034	1.01 (0.24)	0.001
C57BL/6 + Warfarin	% Area Fib.*	-0.33 (0.24)	0.207	-0.92 (0.24)	0.005	0.49 (0.39)	0.252	-0.27 (0.37)	0.480
	Hist. Score†	-0.40 (0.61)	0.492	-2.25 (0.38)	0.007	0.40 (0.40)	0.339	-0.27 (0.34)	0.429
		Mean Difference Relative to FvL				Mean Difference Relative to FvL			
FvL + Warfarin	% Area Fib.*	0.50 (0.41)	0.238	0.65 (0.47)	0.196	0.05 (0.5)	0.921	0.44 (0.39)	0.275
	Hist. Score†	0.28 (0.46)	0.746	0.29 (0.33)	0.424	-0.62 (0.43)	0.101	-0.01 (0.31)	0.918

P-values derived from Student's t-test (*) or Mann Whitney U test (†).

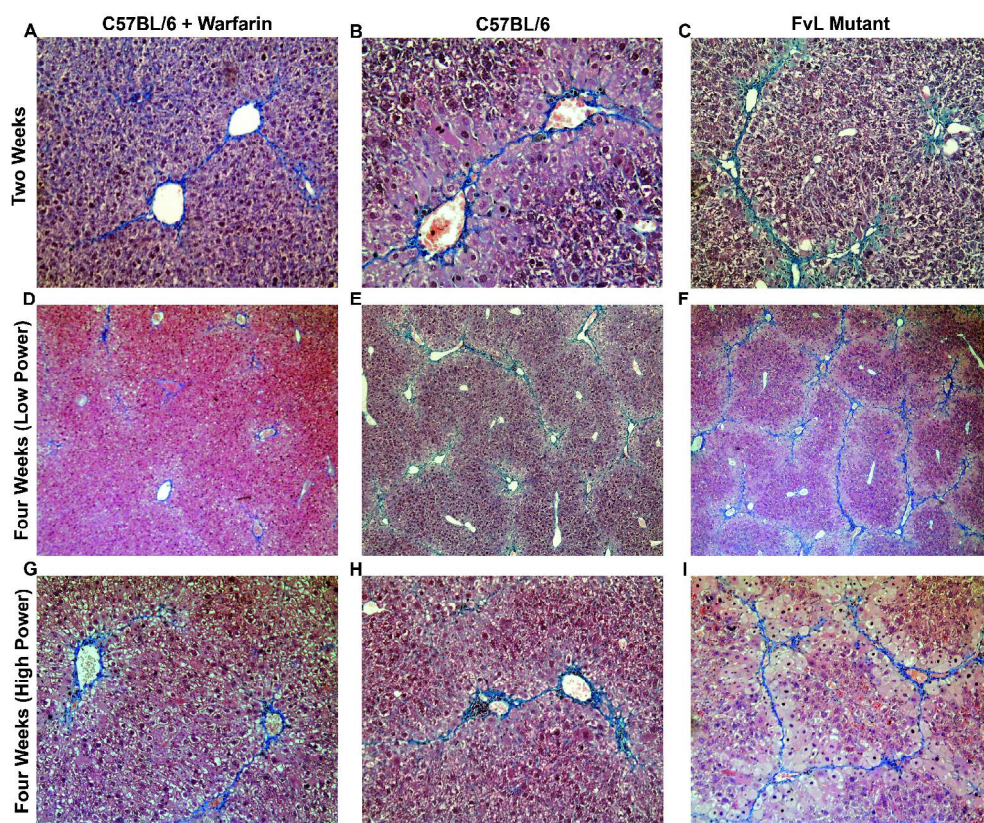
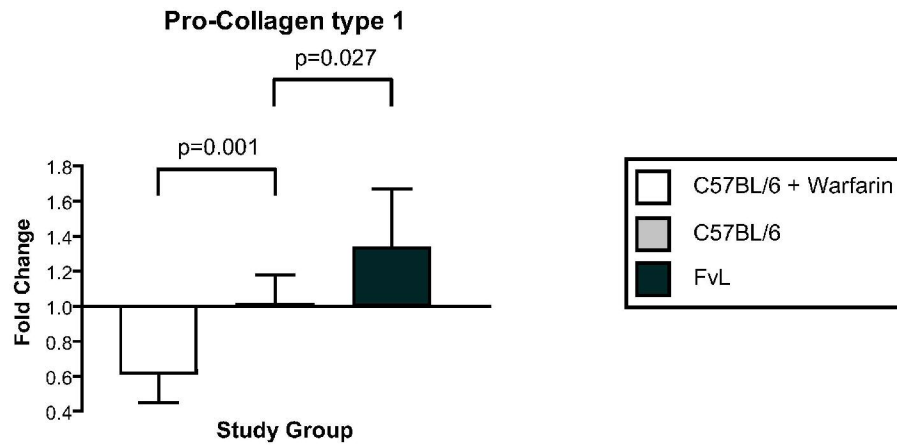


Figure 1: Representative Histological Images Liver tissue sections were stained with CAB trichrome to delineate fibrosis. A, B & C Warfarin treated (A) and non-warfarin treated (B) C57BL/6 mice after 2 weeks CCl₄ exposure. FvL mutant tissue (C) showing evidence of bridging fibrosis and early nodule formation after 2 weeks CCl₄ exposure. Original magnification x300. D, E & F Tissue from a warfarin treated C57BL/6 mouse (D) with evidence of fibrous spur formation but no nodule formation. Tissue from a non-warfarin treated C57BL/6 mouse (E) with evidence of bridging fibrosis. FvL mutant tissue (F) showing nodule formation approaching established cirrhosis Original magnification x150. G, H & I High magnification images of CAB Trichrome stained liver tissue from a warfarin treated C57BL/6 mouse (G), a non-warfarin treated C57BL/6 animal (H) and an FvL mutant mouse (I). Original magnification x300.

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22 **Figure 2: Differential Gene Expression between Study Groups Relative to C57BL/6 Pro-**
23 **collagen type 1 was analysed by quantitative rt-PCR (n = 4-7 mice per group).**
24 **Normalised fold change values relative to C57BL/6 are shown \pm SEM. Pro-collagen type 1**
25 **expression was significantly reduced in C57BL/6 mice treated with warfarin and**
26 **increased in FvL mutant mice.**

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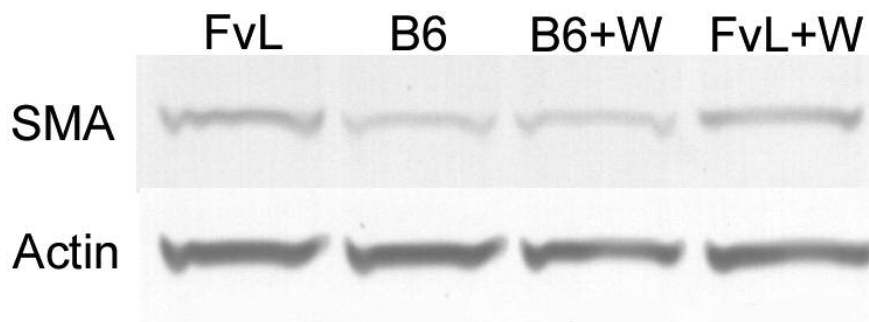


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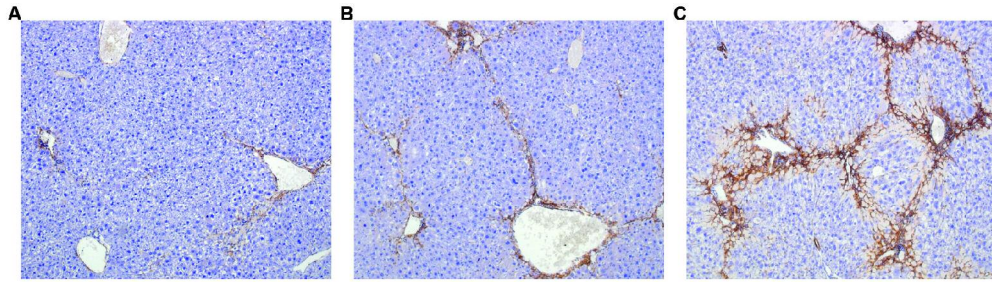
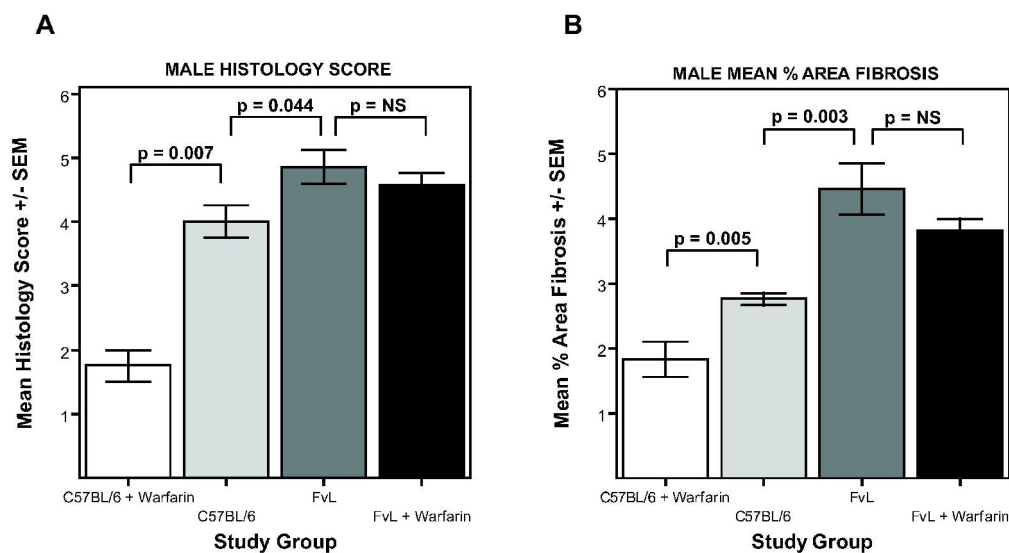


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Supplementary Figure A: Degree of Hepatic Fibrosis by Histological Score and Digital Analysis at 4 Weeks Four Week Histological score (A) and Mean Percentage Area Fibrosis (B) for Male Mice. Data are represented as mean±SEM. Carriage of the FvL mutation significantly increases and warfarin treatment significantly reduces histological score and mean percentage area fibrosis in C57BL/6 mice. The reduction in liver fibrosis seen in warfarin treated FvL mice did not reach statistical significance.

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