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Original Article

Plasma angiopoietin 2 as a novel prognostic biomarker in alcohol-related cirrhosis and hepatitis☆

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ABSTRACT

Background and aim: Severe alcoholic hepatitis (SAH), the most florid form of alcohol-related liver disease (ALD), has a mortality rate of 16% at 28 days. The angiopoietin-Tie 2 system regulates angiogenesis and inflammation, both of which are implicated in the pathogenesis of ALD. This study examined plasma and hepatic gene expression of angiopoietin 1 (ANG1) and angiopoietin 2 (ANG2) in patients with SAH and ALD and investigated their roles as prognostic biomarkers.
Methods: A case-control study was performed measuring plasma levels of ANG1 and ANG2 by enzyme-linked immunosorbent assay (ELISA) from 30 patients with SAH (Maddrey's discriminant function ≥32), 32 patients with ALD cirrhosis and 15 healthy controls (HC). RNA sequencing for *ANG1*, *ANG2*, *TIE1* (codes for Tie1 receptor) and *TEK* (codes for Tie2 receptor) gene expression from a separate cohort study of 79 patients was also performed.
Results: Plasma levels of ANG1 were lower ($P = 0.010$) and ANG2 were higher ($P < 0.0001$) in patients with ALD/SAH compared to HC. The ANG2: ANG1 ratio was higher in those with ALD/SAH compared to HC ($P < 0.0001$). ANG2 levels were the highest in patients who developed sepsis ($P = 0.030$) and those dying within 90 days ($P = 0.020$). ANG2 levels correlated positively with model for end-stage liver disease (MELD) score ($r = 0.30$, $P = 0.020$), Child-Pugh score ($r = 0.38$, $P = 0.003$), international normalized ratio ($r = 0.41$, $P = 0.001$) and white blood cell count ($r = 0.28$, $P = 0.040$) and inversely correlated with albumin ($r = -0.26$, $P = 0.040$).
ANG1 gene expression from liver biopsies was higher in SAH than that in HC ($P < 0.0001$), and greater in severe disease ($P < 0.0001$). *ANG2* gene expression trended towards being lower in SAH than that in HC ($P = 0.070$) though was upregulated in severe disease ($P = 0.0003$).
Conclusions: Plasma ANG2 is raised in SAH and ALD and could be useful as a prognostic biomarker in this patient population.

1. Introduction

There has been an exponential rise in the incidence of alcohol-related liver disease (ALD) globally, and liver disease is now the fifth most common cause of death in the United Kingdom (UK).[1] Acute alcoholic hepatitis, the most florid form of ALD, is a distinct syndrome involving hepatic inflammation in patients with a history of chronic active alcohol use. Severe alcoholic hepatitis (SAH), defined as a modified Maddrey's discriminant function (DF) ≥ 32,[2] carries a high mortality rate of 16% at 28 days, a 3-month mortality rate of 29% and a 1-year mortality of 56%.[3] Patients are particularly susceptible to infection. Sepsis and endotoxemia occur in up to 50%

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of the patients,[4,5] and frequently progress to multiple organ failure and death.[4,6]

Though the pathogenesis of SAH is not completely understood, increased bacterial translocation from the gut,[7,8] systemic inflammation and immune system dysfunction appear to be central to the development of this state.[9] Alcohol consumption triggers liver parenchymal inflammation and promotes fibrosis and angiogenesis.[10] Increased angiogenesis can result in an underdeveloped vascular system with increased vascular permeability, which worsens inflammation.[11]

There is growing evidence that the angiopoietin-Tie2 system, which behaves as a vital regulator in angiogenesis,[12] is also strongly involved in endothelial cell (EC) activation and inflammation.[13] Angiopoietin 1 (ANG1) is a Tie2 receptor agonist and plays a role in vessel formation and maturation. It has anti-inflammatory effects and reduces the adhesion and transendothelial migration of leucocytes.[14,15] The orphan receptor Tie1 is also involved in this pathway, and has been identified as a positive or negative regulator of ANG1/Tie2 signalling depending on cellular context.[15] ANG2 was initially described purely as a competitive antagonist of ANG1/Tie2, but recently has been more shown to act as both a Tie2 agonist and antagonist, depending on the situation. Inflammation (precipitated by infection, tumour necrosis factor alpha (TNF-α) or endotoxin administration) has been shown to provoke a critical change in the effects of ANG2 — encouraging the move from agonist to antagonist.[16] *In vitro* studies have revealed that inflammation induces shedding of the Tie1 ectodomain rendering ANG2 a Tie2 antagonist.[16,17]

When referring to the antagonistic effects of ANG2 on ANG1/Tie2 signaling, ANG2 can be described as a proinflammatory factor.[18] In this context, ANG2 competitively inhibits ANG1 and downregulates Tie2.[19] Furthermore, when activated by stimuli such as inflammatory cytokines, ECs release Wiebel-Palade bodies, which liberate ANG2. ANG2 renders the endothelium responsive to the effects of further stimulation by cytokines and disrupts cell—cell adhesion which increases vascular permeability.[20] This allows the influx of inflammatory cells from the circulation propagating a systemic inflammatory response and accelerating the progression towards multiple organ failure.

Studies in septic patients, and those exhibiting the systemic inflammatory response syndrome (SIRS), have found that ANG2 levels correlate with increased fluid overload, acute kidney injury (AKI) and hepatic and coagulation dysfunction.[21,22] Furthermore, higher ANG2 levels have been found to be associated with a greater 28-day mortality in patients with both sepsis and SIRS.[23,24,25] Conversely, ANG1 levels have been found to be associated with a lower rate of AKI and reduced mortality.[21,25]

The angiopoietin-Tie2 system has also been implicated in the pathogenesis of chronic liver disease. It has been shown to play a role in both inflammation and angiogenesis.[26] Particular interest has been paid to the roles of ANG1 and ANG2 in the pathogenesis of hepatocellular carcinoma (HCC),[27–29] and, more recently, non-alcoholic steatohepatitis (NASH),[30] acute liver failure and decompensated cirrhosis.[31,32] Lefere *et al.*[30] found that serum ANG2 levels were raised in patients with biopsy proven NASH compared to those with steatosis. Furthermore, ANG2 levels correlated with hepatic CD34 immunoreactivity, used as a marker of hepatic angiogenesis.[30]

Patients presenting with SAH have evidence of a systemic pro-inflammatory plasma milieu. Clinical features of SAH share many similarities with patients with sepsis.[4] We therefore postulated that plasma levels of ANG1 and ANG2 would serve as surrogate markers of EC activation in patients with SAH and investigated their role as potential prognostic biomarkers. RNA sequencing for *ANG1*, *ANG2*, *TIE1* (codes for Tie1 receptor) and *TEK* (codes for Tie2 receptor) from a separate cohort study of 79 patients was also performed.

2. Patients and methods

2.1. ANG1 and ANG2 plasma levels

2.1.1. Study design and inclusion criteria

Thirty patients with SAH were recruited from the Liver Unit and Liver Intensive Care Unit at King's College Hospital, London. Patients were recruited if they were between 18 and 75 years of age with SAH defined as total bilirubin >80 μmol/L, a modified Maddrey's DF $\geq$ 32,[2] a history of excess alcohol (>80 g/day for men and >60 g/day for women) and the absence of other causes of liver disease.

Thirty-two patients with ALD cirrhosis (14 actively drinking and 18 abstinent) were also recruited. Abstinence was defined as > 6 months without alcohol consumption. ALD cirrhosis was defined as the presence of clinical, radiological or histological diagnosis of cirrhosis with a history of excess alcohol and the absence of other causes of liver disease.

Fifteen non-smoking healthy volunteers with no history of liver disease were recruited as healthy controls (HC).

2.1.2. Exclusion criteria

Patients with SAH were excluded if there was a history of abstinence from alcohol for >6 weeks. Patients with SAH or ALD were excluded if there was evidence of pregnancy, malignancy, any coexisting history of immunodeficiency, HIV infection, active inflammatory disease, concomitant use of antioxidants or other immunomodulatory therapies within the last 6 months, and aspartate aminotransferase (AST) > 500 IU/L or alanine aminotransferase (ALT) > 300 IU/L.

2.1.3. Consent and data collection

This study was performed in accordance with the Declaration of Helsinki. Ethical approval was granted by the North East London Research Ethics Committee (Ref. No. 08/H0702/52). After obtaining fully informed written consent, or assent from a patient consultee for patients lacking capacity, clinical, biochemical and physiological data were collected. Data collected included alcohol and tobacco use, medication history, antibiotic use, microbiology results, electrolytes and renal function, liver function, differential leucocyte count and clotting parameters. Model for end-stage liver disease (MELD) score and Child-Pugh grade were also calculated. Patients were followed longitudinally for 90 days,[33,34] and further data on subsequent development of sepsis or death were recorded.

2.1.4. Sample collection

Venous blood was obtained by venepuncture from patients/HC into ethylenediaminetetraacetic acid (EDTA) containing vacutainer tubes. Plasma was obtained by centrifuging whole blood, within 30 min of sample collection, at 3940 *g* for 10 min at 4 °C. Plasma samples were then stored at −80 °C until subsequent ANG1 and ANG2 determination by enzyme-linked immunosorbent assay (ELISA).

2.1.5. ANG1 and ANG2 quantification

Plasma levels of ANG1 and ANG2 were measured in duplicate by commercially available ELISA kits (Human Angiopoietin-1 DuoSet ELISA (DY923) and Human Angiopoietin-2 DuoSet ELISA (DY623), R&D systems, Minneapolis, USA). The ratio between ANG2 and ANG1 was then quantified.

2.2. Hepatic gene expression of ANG1, ANG2, TIE1 and TEK

2.2.1. Study design, consent and data collection

Human liver samples were obtained from the Human Biorepository Core from the National Institutes of Health (NIH)-funded international InTeam consortium (7U01AA021908-05; USA). Patients with early alcoholic hepatitis were recruited from Cliniques Universitaires Saint-Luc (Brussels, Belgium). All patients included gave written informed consent. Research protocols were approved by the local Ethics Committees and the central Institutional Review Board of the University of Carolina at Chapel Hill.

Seventy-nine patients were included and selected according to clinically relevant groups: (i) patients with early alcoholic hepatitis, non-obese with high alcohol intake presenting with a mild elevation in transaminases and histologic criteria of steatohepatitis (alcoholic hepatitis, AH, $n = 12$); (ii) patients with severe histologically confirmed alcoholic hepatitis, biopsied before treatment (SAH, $n = 18$); (iii) explants from patients with severe alcoholic hepatitis who underwent early liver transplantation following a well-defined protocol (explants of alcoholic hepatitis, exAH, $n = 11$).[35] These groups were compared with non-diseased human liver fragments (HC, $n = 10$) and disease controls; biopsies from patients with non-alcoholic fatty liver disease (NAFLD) (defined by Keiner's criteria) without alcohol excess (NAFLD, $n = 9$),[36] patients with non-cirrhotic HCV infection (HCV, $n = 10$) and patients with compensated HCV-related cirrhosis (HCV−C, $n = 9$). Patients with malignancies were excluded from the study.

2.2.2. RNA extraction and sequencing

As described previously by Argemi *et al.*,[37] total RNA from flash-frozen liver tissue was extracted by phenol/chloroform separation (TRIzol, Thermox, Massachusetts, USA). Purity and quality of RNA were assessed by automated electrophoresis (Bioanalyser, Agilent, Santa Clara, USA). The TruSeq Stranded Total RNA Ribo-Zero GOLD (Illumina, San Diego, California, USA) was used to build libraries. RNA was sequenced using Illumina HiSeq2000 platform. Sequencing was paired-end (2 × 100 base pairs (bp)) and multiplexed. Ninety-four paired-end sequenced samples obtained an average of 36.9 million total reads with 32.5 million (88%) mapped to GRCh37/hg19 human reference. Short read alignment was performed using Spliced Transcripts Alignment to a Reference (STAR) alignment algorithm with default parameters.[38] To quantify expression from transcriptome mappings RNA-Seq by Expectation Maximization (RSEM) was employed.[18] Principal component analysis (PCA) was performed using made4 library.[18] Differential expression analysis was performed using the Limma package.[18] Cyclic loss normalisation was applied, followed by log transformation of the counts per million and mean-variance adjustment using the voom function.

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 for Mac (GraphPad Software, San Diego, CA, USA). Data are presented as number, percentages or medians with corresponding 25th and 75th percentiles. Comparisons of non-parametric continuous variables between two groups were made with the Mann−Whitney *U* test. The Kruskal−Wallis test was used when three or more groups were compared simultaneously. Dunn's multiple comparison test was used for post hoc analysis. Nominal variables were compared with the Chi-Squared test. Spearman's rank correlation test was used for the assessment of association between non-parametric variables. The log rank (Mantel-Cox) test was used to analyse differences in survival curves. Area Under the Receiver Operator Curves (AUROC) analysis was performed to determine the discriminatory power of ANG2 in distinguishing surviving and non-surviving patients at 90 days. All tests were two-tailed and significance was accepted as $P < 0.05$. Available-case analysis was employed to address missing data, and number of subjects with missing data is indicated for each relevant variable in the results section.

3. Results

3.1. ANG1 and ANG2 plasma levels study

3.1.1. Patient demographics

Thirty patients with SAH and 32 with ALD cirrhosis (14 actively drinking alcohol and 18 abstinent for at least 6 months) were recruited (total patients $n = 62$) and compared with 15 HCs. Patient characteristics are summarised in Table 1. As expected, patients in the SAH group had significantly higher biochemical markers of liver disease severity (bilirubin and international normalized ratio (INR)) and significantly increased indicators of systemic inflammation (white blood cell count and C-reactive protein) compared to the ALD cirrhosis group.

In total, 24.2% patients developed positive bacterial culture sepsis, and 9.7% patients developed culture positive fungal sepsis. Thirty-day mortality rate was 23.3% in the SAH group and 9.4% in the ALD cirrhosis group. Total 30-day mortality rate was 16.1%. Ninety-day mortality rate was 30.0% in the SAH group and total 90-day mortality rate was 19.4%. A Kaplan−Meier graph (Fig. 1) demonstrates a significant difference in survival curves between the SAH group and ALD cirrhosis group at 90 days (log rank Mantel-Cox $P = 0.020$). No patients were transplanted within 90 days of study enrollment.

3.1.2. Plasma levels of angiopoietin

Plasma levels of ANG1 were significantly lower in the patient group (SAH and ALD cirrhosis combined) (5.10 (3.20−7.30) ng/mL) compared to HC group (7.70 (5.10−22.30) ng/mL ($P = 0.01$) (Fig. 2A). Plasma levels of ANG2 were significantly higher in the patient group (1.90 (1.10−3.20) ng/mL) compared to HC group (0.30 (0−0.70) ng/mL) ($P < 0.0001$) (Fig. 2B). On subgroup analysis, ANG2 levels trended towards being higher in the SAH group (2.10 (1.40−3.20) ng/mL), when compared to the ALD cirrhosis group (1.50 (0.70−3.20) ng/mL) ($P = 0.080$) (data not shown). The ANG2: ANG1 ratio was higher in the patient group (0.3 (0.2−0.6)), when compared to HC group (0.05 (0−0.10)) ($P < 0.0001$) (Fig. 2C). There were no differences in ANG1 or ANG2 levels between actively drinking and abstinent patients.

3.1.3. Plasma angiopoietin levels and sepsis

ANG2 levels were higher in septic patients (defined as positive bacterial and/or fungal culture) (2.57 (1.67−4.17) ng/mL) compared to non-septic patients (1.70 (0.89−3.00) ng/mL) ($P = 0.030$) (Fig. 2D).

3.1.4. Plasma angiopoietin levels and outcome

Non-surviving patients at 30 days had higher levels of ANG2 (2.70 (1.89−4.16) ng/mL) than surviving patients (1.80 (0.94−3.01) ng/mL) ($P = 0.040$) (data not shown). Similarly, ANG2 levels were higher in non-surviving patients at 90 days (2.74 (1.96−3.66) ng/mL) than surviving patients (1.70 (0.93−2.91) ng/mL) ($P = 0.020$) (Fig. 2E). No differences were found in ANG1 levels or ANG2: ANG1 ratio between surviving and non-surviving patients.

ANG2 level on recruitment was a predictor of survival at 90 days from baseline, on ROC curve analysis AUC 0.72 (95% CI: 0.59−0.85) ($P = 0.020$) (Fig. 3). An ANG2 level of >1.98 ng/mL predicted 90-day mortality with 75% sensitivity and 62.5% specificity.

Table 1
Clinical characteristics of the patients.

Study data[a]	HC group ($n = 15$)	SAH group ($n = 30$)	ALD cirrhosis group ($n = 32$)	Total patients in study group ($n = 62$)
Female, *n* (%)	9 (60)	15 (50)	8 (25)	23 (37)
Age (years)	34 (27–37)	43 (35–50)	51 (47–58)	48 (42–54)
Current alcohol excess[b], *n* (%)	–	30 (100)[c]	14 (44)	44 (71)
Total bilirubin (μmol/L)	–	298.0 (203.0–422.0)[c]	30.5 (19.3–62.8)	111.0 (29.0–283.5)
Creatinine (μmol/L)	–	64.0 (51.0–149.5)	65.0 (52.5–81.5)	64.0 (52.0–88.5)
Albumin (g/L)	–	27 (25–29)[c]	32 (27–37)	29 (26–35)
INR	–	1.87 (1.71–2.27)[c]	1.42 (1.18–1.63)	1.67 (1.37–2.02)
WBC ($\times 10^9$/L)	–	10.80 (6.76–15.26)[c]	4.73 (3.41–5.95)	5.80 (4.33–9.87)
CRP (mg/L)	–	44.0 (17.6–59.0)[c]	10.0 (3.6–21.7)	21.0 (9.3–52.0)
Ascites, *n* (%)	–	19 (63.3)	23 (71.9)	42 (67.7)
Child-Pugh score	–	11 (9–12)[c]	8 (6–11)	10 (8–11)
MELD	–	27 (22–29)[c]	12 (10–17)	20 (12–27)
Maddrey's discriminant function	–	59.5 (39.5–70.0)	–	–
Antibiotic use, *n* (%)	–	20 (66.7)[c]	11 (34.4)	31 (50.0)
Positive bacterial culture, *n* (%)	–	8 (26.7)	7 (21.9)	15 (24.2)
Positive fungal culture, *n* (%)	–	5 (16.7)	1 (3.1)	6 (9.7)
30-day mortality, *n* (%)	–	7 (23.3)	3 (9.4)	10 (16.1)
90-day mortality, *n* (%)	–	9 (30.0)	3 (9.4)	12 (19.4)

Data are presented as *n* (%) or median (25^{th} and 75^{th} percentiles).
Abbreviations: ALD, alcohol-related liver disease; CRP, C-reactive protein; HC, healthy controls; INR, international normalized ratio; MELD, model for end-stage liver disease; SAH, severe alcoholic hepatitis; WBC, white blood cell.
[a] Missing data for each variable: creatinine ($n = 1$ from SAH group), albumin ($n = 1$ from SAH group), WBC ($n = 5$ from SAH group), CRP ($n = 11$ from SAH group, $n = 17$ from ALD cirrhosis group), ascites ($n = 1$ from SAH group), Child-Pugh score ($n = 1$ from SAH group), MELD ($n = 1$ from SAH group).
[b] > 80 g/day for men and >60 g/day for women.
[c] $P < 0.05$ between SAH and ALD cirrhosis group.

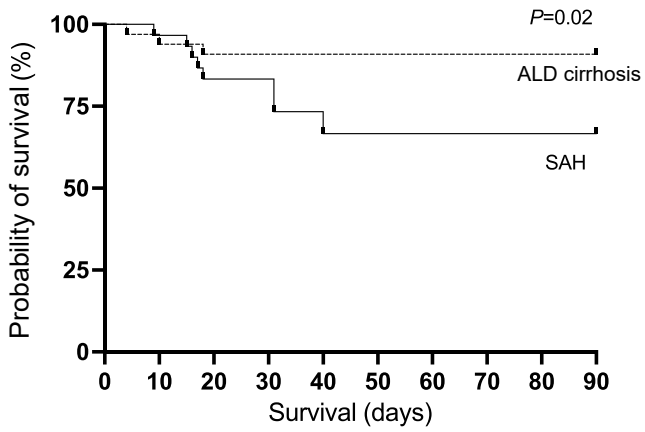

Fig. 1. Kaplan–Meier survival curve of all-cause mortality for all SAH patients and ALD cirrhosis patients at 90 days (log rank Mantel-Cox $P = 0.020$). Abbreviations: ALD, alcohol-related liver disease; SAH, severe alcoholic hepatitis.

3.1.5. Plasma angiopoietin levels and liver disease severity

ANG2 levels correlated positively with MELD score (r = 0.30, $P = 0.02$) (Fig. 4A), Child-Pugh score (r = 0.38, $P = 0.003$) (Fig. 4B), INR (r = 0.41, $P = 0.001$) (Fig. 4C) and white blood cell count (r = 0.28, $P = 0.040$) and were negatively correlated with albumin (r = −0.26, $P = 0.040$) (Fig. 4D). There was no correlation between ANG1 or ANG2: ANG1 ratio and any of the variables. There was no correlation between ANG1, ANG2 or ANG2:1 ratio and DF in the SAH group.

3.2. Hepatic gene expression of ANG1, ANG2, TIE1 and TEK study

3.2.1. Patient demographics

Ribonucleic acid (RNA) sequencing for *ANG1*, *ANG2*, *TIE1* and *TEK* gene expression from total RNA isolated from liver biopsies was performed. Seventy-nine patients were included. Baseline data is shown in Table 2.

Forty-four percent ($n = 8$) of the SAH group were responders to prednisolone treatment. There was a 39% ($n = 7$) mortality rate in the SAH group at 3 months.

3.2.2. ANG1 gene expression

Differences in *ANG1* gene expression between groups are shown in Fig. 5A. There was a significant increase in *ANG1* gene expression in the SAH and exAH groups compared to all other groups. *ANG1* gene expression was higher in SAH compared to HC ($P < 0.05$) and higher in severe disease (AH *vs.* SAH, $P < 0.0001$). No differences were seen in *ANG1* gene expression between SAH treatment responders and non-responders, nor between SAH survivors and non-survivors.

3.2.3. ANG2 gene expression

Differences in *ANG2* gene expression between groups are shown in Fig. 5B. *ANG2* gene expression was significantly higher in the SAH group compared to AH, HCV and HCV-C groups, and in the exAH group compared to HCV and HCV-C. *ANG2* gene expression was also significantly higher in the HC group compared to AH, HCV and HCV-C groups. *ANG2* gene expression trended towards being lower in the SAH group compared to HC group ($P = 0.070$). *ANG2* gene expression was higher in more severe disease (AH *vs.* SAH, $P < 0.05$). No differences were seen in relation to SAH treatment response or mortality.

3.2.4. TIE1 gene expression

Differences in *TIE1* gene expression between groups are shown in Fig. 6A. *TIE1* gene expression was significantly higher in the SAH and exAH groups compared to all other groups. *TIE1* gene expression was higher in SAH than HC ($P < 0.0001$) (Fig. 6A) and higher in severe disease (AH *vs.* SAH, $P < 0.01$). No differences were seen in *TIE1* gene expression between SAH treatment responders and non-responders, nor between SAH survivors and non-survivors.

3.2.5. TEK gene expression

Differences in *TEK* gene expression between groups are shown in Fig. 6B. *TEK* gene expression was significantly higher in HC group

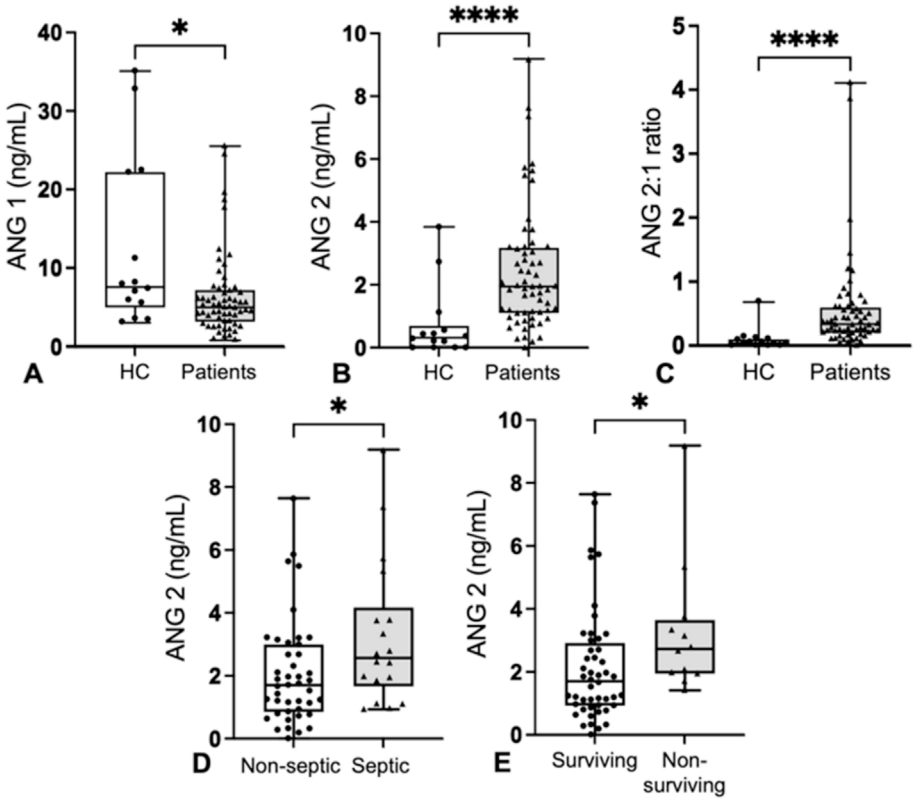

Fig. 2. Plasma ANG levels in HC and patients (SAH and ALD cirrhosis). Plasma **(A)** ANG1 and **(B)** ANG2 levels in HC and patients. **(C)** Plasma ANG2: ANG1 ratio in HC and patients. **(D)** Plasma ANG2 levels in non-septic and septic patients. **(E)** Plasma ANG2 levels in surviving and non-surviving patients at 90 days.
For box-and-whisker plots: midline, median; perimeters, 25th to 75th centile; whiskers, minimum to maximum values. The Mann–Whitney *U* test was used for statistical analysis. *$P < 0.05$, ****$P < 0.0001$.
Abbreviations: ALD, alcohol-related liver disease; ANG, angiopoietin; HC, healthy control; SAH, severe alcoholic hepatitis.

compared to AH, HCV and HCV-C groups. There was no difference in *TEK* gene expression between SAH and HC. *TEK* gene expression was higher in more severe disease (AH *vs.* SAH, $P = 0.002$). No differences were seen in *TEK* gene expression between SAH treatment responders and non-responders, nor between SAH survivors and non-survivors.

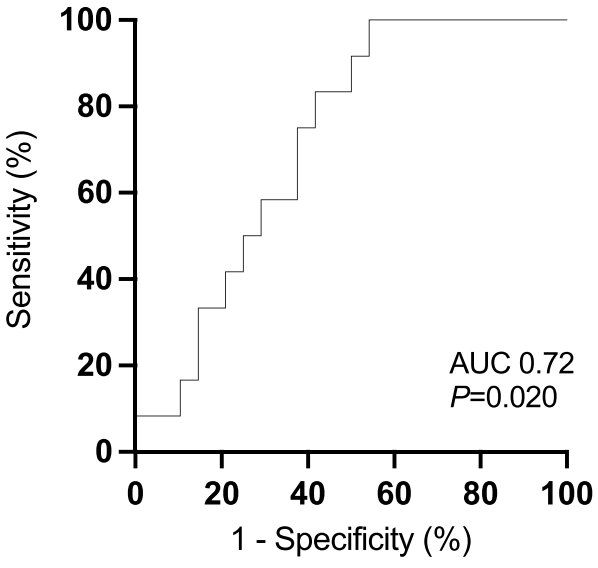

Fig. 3. ROC analysis showing the discriminatory power of ANG2 between surviving and non-surviving patients at 90 days (AUC 0.72; 95% CI: 0.59–0.85; $P = 0.020$).
Abbreviations: ANG, angiopoietin; AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic.

4. Discussion

This study shows that plasma ANG2 is elevated in patients with alcohol-related liver disease (SAH and ALD) and ANG2 levels correlate with several markers of liver dysfunction.

The angiopoietin-Tie2 system is intrinsically involved in angiogenesis and the development of inflammation,[26] both of which have been implicated in the progression of ALD and outcome. ANG1 acts to stabilise the vascular endothelium and inhibit inflammatory gene expression and has an anti-inflammatory role and it is therefore not surprising that plasma levels of ANG1 were significantly lower in the patient group.[19] ANG1 protects against endotoxemia during shock and inhibits vascular leakage and the low levels seen in those with SAH/ALD in this study may in part explain the low grade systemic inflammation observed in these patients.[39] Interestingly, our study is at odds with a prior study which has shown no difference in ANG1 levels between controls and patients with ALD, although patients in this study had a lower median MELD score of 17.5 (12–20),[40] which may account for this disparity.

Plasma ANG2 levels were significantly higher in patients with SAH/ALD. This in keeping with previous studies investigating ANG2 levels in cirrhosis,[28,40] acute liver failure and NASH.[30,31] Whilst not significantly different, ANG2 levels trended towards being higher in

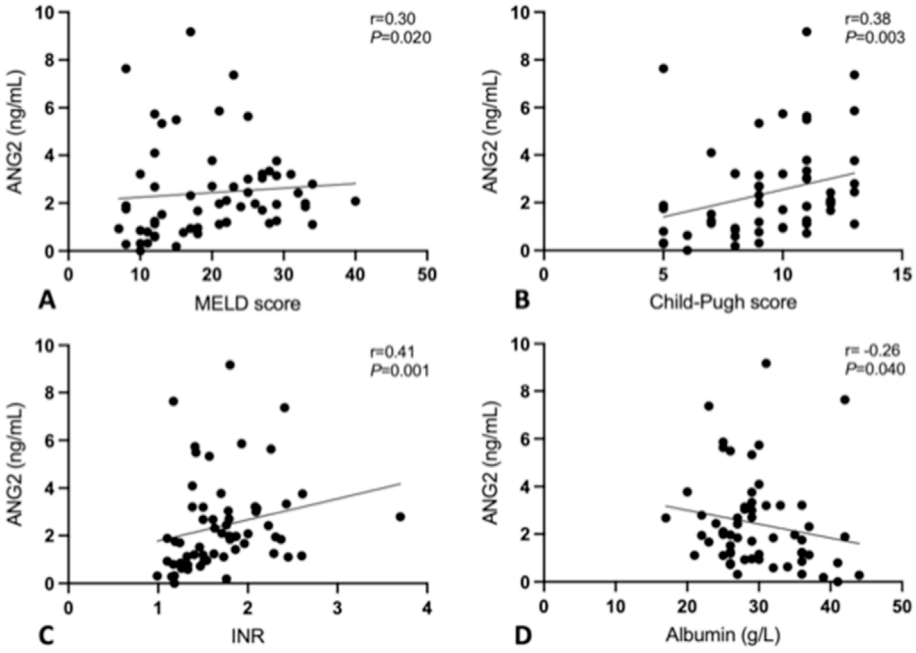

Fig. 4. Correlation between plasma ANG2 levels and liver disease severity scores and biochemical markers of severity. (A) Correlation between MELD score and ANG2 levels in patient group (r = 0.30, $P = 0.020$). **(B)** Correlation between Child-Pugh score and ANG2 levels in patient group (r = 0.38, $P = 0.003$). **(C)** Correlation between INR and ANG2 levels in patient group (r = 0.41, $P = 0.001$). **(D)** Correlation between albumin and ANG2 levels in patient group (r = −0.26, $P = 0.040$).
Spearman's rank correlation test was used for statistical analysis. One patient (SAH) was not included in analysis for MELD score, Child-Pugh score and albumin due to missing data.
Abbreviations: ANG, angiopoietin; INR, international normalized ratio; MELD, model for end-stage liver disease.

Table 2
Characteristics of hepatic gene expression study patients.

Study data	HC ($n = 10$)	AH ($n = 12$)	SAH ($n = 18$)	exAH ($n = 11$)	NAFLD ($n = 9$)	HCV ($n = 10$)	HCV-C ($n = 9$)
Sex (female)	3	5	7	4	7	5	3
Age (years)	32 (29–51)	52 (48–59)	51 (47–58)	49 (48–56)	50 (43–53)	48 (43–63)	61 (51–66)
Total bilirubin (μmol/L)	10.26 (8.55–11.97)	20.52 (11.97–25.66)	324.97 (210.37–456.67)	278.79 (189.85–415.62)	10.26 (6.84–15.39)	12.83 (10.26–17.10)	18.81 (13.34–28.22)
Creatinine (μmol/L)	70.74 (65.43–79.58)	53.05 (52.17–68.08)	69.85 (53.94–88.42)	61.01 (46.86–64.55)	92.84 (74.27–97.26)	88.42 (86.65–97.26)	88.42 (73.39–97.26)
Albumin (g/L)	46 (43–46)	45 (42–47)	29 (23–33)	24 (20–30)	45 (44–46)	44 (42–47)	41 (43–45)
INR	1.03 (0.99–1.06)	1.00 (0.90–1.00)	1.60 (1.20–3.30)	1.80 (1.60–2.60)	1.19 (1.06–1.35)	1.21 (1.01–1.40)	1.24 (1.16–1.38)
Ascites (g/L)	0	0	14	7	0	0	0
Child-Pugh score	N/A	N/A	11.0 (9.0–11.7)	10.7 (9.0–12.0)	N/A	N/A	5.0 (5.0–5.0)
MELD	N/A	N/A	24.0 (22.0–27.7)	24.5 (21.4–27.2)	N/A	N/A	10.0 (8.5–12.0)

Data are presented as n or median (25^{th} and 75^{th} percentiles).
Abbreviations: AH, alcoholic hepatitis group; exAH, explants from patients with SAH who underwent transplantation; HC, healthy control; HCV, non cirrhotic HCV infection group; HCV-C, compensated HCV-related cirrhosis group; INR, international normalized ratio; MELD, model for end-stage liver disease; NAFLD, non-alcoholic fatty liver disease group; SAH, severe alcoholic hepatitis group.

patients with SAH than those with ALD, which would be expected given the more pronounced inflammatory state in SAH. Plasma ANG2 levels were higher in patients with positive (bacterial or fungal) culture sepsis ($P = 0.030$) which is in keeping with the extensive literature base on ANG2 and sepsis.[22,23,25,41,42] Moreover, ANG2 levels positively correlated with several markers of liver disease severity.

Non-surviving patients at 30 days and 90 days had higher plasma ANG2 levels than surviving patients ($P = 0.040$ and $P = 0.020$, respectively). ROC analysis revealed that ANG2 levels had an adequate prognostic accuracy for 90-day mortality (AUC 0.72, $P = 0.020$). ANG2 levels have been shown to be closely related to increased mortality in sepsis.[22,23,25,42] Recent studies have found that ANG2 levels are associated with increased mortality and morbidity in decompensated cirrhosis and acute liver failure and lack of improvement in liver fibrosis stage post viral eradication in HCV.[29,31,32] Our observations add further weight to the possible use of ANG2 as a prognostic biomarker, specifically in patients with alcohol-related cirrhosis and hepatitis.

The results from the hepatic gene expression sub-study are very interesting, with contrasting findings to the plasma study. The gene expression of *ANG1* was greater in patients with alcoholic hepatitis (SAH and exAH), and was still higher in severe disease. The liver in alcoholic hepatitis undergoes a striking ductular reaction with massive expansion of liver progenitor cells (LPCs) and hepatomegaly hence increased levels of *ANG1* may be expected. Perhaps the lower levels of plasma ANG1 could be a result of liver concentration and internalisation of ANG1.[43] The results from *ANG2* RNA-seq were surprising, with higher levels in HCs. It may be that there is stable synthesis of ANG2 in the normal liver, and that when inflammation ensues (independent of aetiology) the first response is to downregulate *ANG2* as a protective mechanism, explaining the reduced expression seen in disease controls and early alcoholic hepatitis (AH). *ANG2* expression was then upregulated in severe

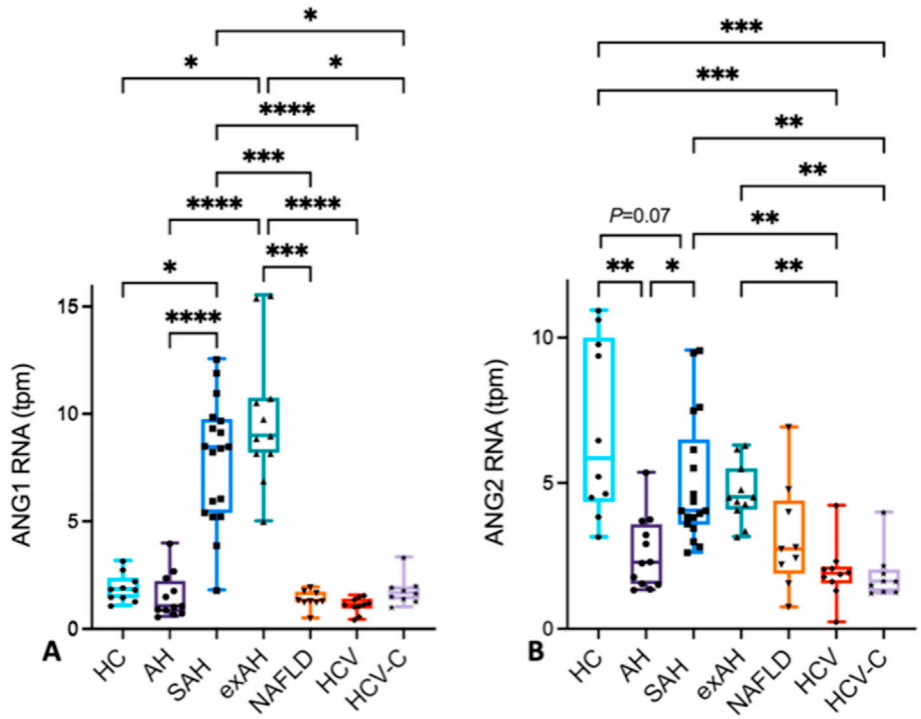

Fig. 5. *ANG1* RNA and *ANG2* RNA results. **(A)** *ANG1* expression in all groups. **(B)** *ANG2* expression in all groups. Gene expression levels are presented as transcripts per million (tpm). For box-and-whisker plots: midline, median; perimeters, 25th to 75th centile; whiskers, minimum to maximum values.
The Kruskal-Wallis test and Dunn's multiple comparison test were used for statistical analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.
Abbreviations: AH, alcoholic hepatitis; ANG, angiopoietin; exAH, explants from patients with SAH who underwent transplantation; HC, healthy control; HCV, non cirrhotic HCV infection group; HCV-C, compensated HCV-related cirrhosis group; NAFLD, non-alcoholic fatty liver disease; RNA, ribonucleic acid; SAH, severe alcoholic hepatitis.

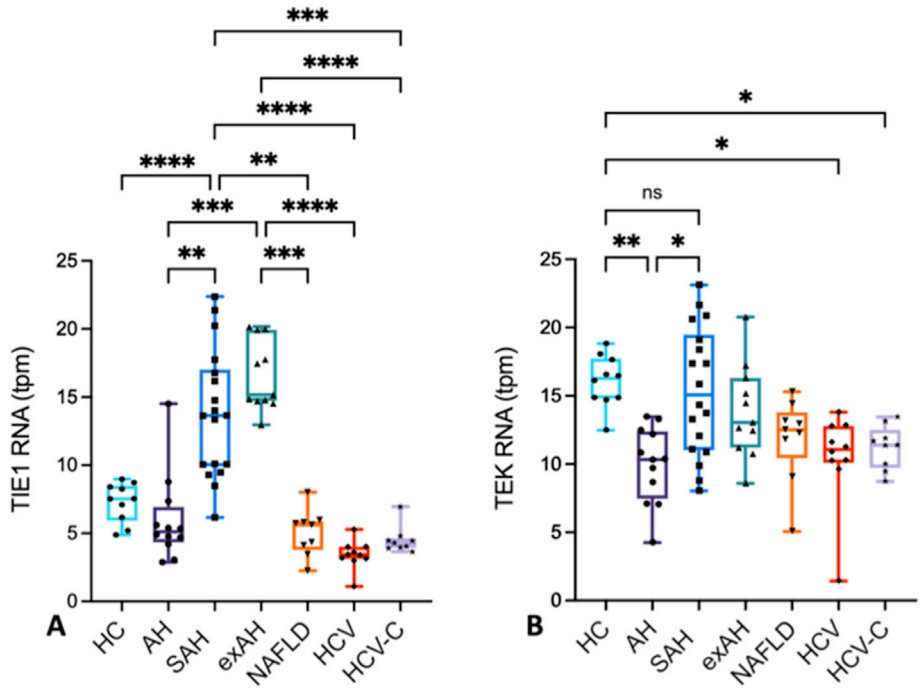

Fig. 6. *TIE1* RNA and *TEK* RNA results. **(A)** *TIE1* expression in all groups. **(B)** *TEK* expression in all groups. Gene expression levels are presented as transcripts per million (tpm). For box-and-whisker plots: midline, median; perimeters, 25th to 75th centile; whiskers, minimum to maximum values.
The Kruskal–Wallis test and Dunn's multiple comparison test were used for statistical analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
Abbreviations: AH, alcoholic hepatitis group; ANG, angiopoietin; exAH, explants from patients with SAH who underwent transplantation; HC, healthy controls; HCV, non cirrhotic HCV infection group; HCV-C, compensated HCV-related cirrhosis group; NAFLD, non-alcoholic fatty liver disease; ns, not significant; RNA, ribonucleic acid; SAH, severe alcoholic hepatitis.

disease (SAH) and perhaps this relative increase is harmful. This is in keeping with the literature; inflammation and high levels of ANG2 provoke a critical switch to ANG2 behaving as a Tie2 antagonist, whereby it exerts detrimental proinflammatory effects.[15–17] The expression of *TIE1* and *TEK*, similar to that of *ANG2*, revealed a relative downregulation during the asymptomatic, compensated and chronic liver disease phenotypes, with a relative upregulation in SAH patients.

Gene expression of *ANG1*, *ANG2*, *TIE1* and *TEK* was not related to mortality or response. This indicates that the mechanism leading to poor prognosis is systemic, and not local. This is supported by existing literature; multiple inflammatory mediators are activated in SAH and contribute to SIRS. SIRS has been shown to be a major predictor of disease severity and organ failure in SAH and, together with impaired hepatic regeneration, leads to multiple organ failure and death.[4,43]

Treatments targeting the angiopoietin-Tie2 system, including Tie2 receptor agonists and recombinant human ANG1, have been shown to be protective in murine models of sepsis.[44–47] A recent study investigated the role of ANG2 as a therapeutic target in NASH. The ANG2/Tie2 receptor inhibiting peptibody L1–10 was evaluated in methionine-choline deficient (MCD) and streptozotocin-western diet NAFLD mouse models. L1–10 treatment reduced hepatocyte ballooning and fibrosis in MCD diet-fed mice, was associated with reduced hepatic angiogenesis and vasculature micro-architecture normalisation. L1–10 treatment reversed NASH and attenuated HCC progression in the streptozotocin-western diet model.[48] Treatments targeting the angiopoietin-Tie 2 system in cirrhosis therefore require further attention.

This study is limited by the relatively small sample size and short follow up interval. Larger studies are required for validation of results before wider application. The recruitment of larger numbers of patients with SAH may actually show that ANG2 levels are discriminatory between those with SAH and ALD.

5. Conclusions

In summary, we have demonstrated that plasma levels of ANG2 are raised in patients with SAH and ALD cirrhosis and are higher in septic and non-surviving patients. Furthermore, ANG2 levels correlated positively with MELD score, Child-Pugh score, INR and white blood cell count and inversely correlated with albumin. Such results raise the possibility of the use of ANG2 as a prognostic biomarker in this patient cohort.

Our hepatic gene expression study generated interesting results, revealing that *ANG1* hepatic gene expression was higher in SAH than controls, whilst *ANG2* hepatic gene expression trended towards being lower in SAH than controls, though was then upregulated in more severe disease. This suggests that in liver disease ANG2 may first be downregulated as a protective mechanism but is then paradoxically upregulated in severe disease with detrimental results. Further work is required to define the role of the angiopoietin-Tie2 system in liver disease.

Authors' contributions

D. L. Shawcross designed the study. V. T. Kronsten led the study, recruited the patients, undertook data collection and performed all the analyses. J. Argemi performed all RNA sequencing. A. S. Kurt and G. M. Vijay assisted with the laboratory analyses. J. M. Ryan assisted with patient recruitment and the laboratory analyses. The manuscript was written by V. T. Kronsten and critically revised by R. Bataller and D. L. Shawcross. All co-authors approved the final submitted manuscript.

Data deposition

RNA data is deposited in the Database of Genotypes and Phenotypes (NIH) with the reference phs001807.v1.p1.

Declaration of competing interest

D. L. Shawcross has participated in advisory boards for Norgine Pharmaceuticals Ltd, Kaleido Biosciences, Mallinckrodt Pharmaceuticals Ltd and Shionogi and has delivered paid lectures for Norgine Pharmaceuticals Ltd and Falk Pharma. The other authors declare that they have no conflict of interest.

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