In vivo imaging of hepatic neutrophil migration in severe alcoholic hepatitis with 111In-radiolabelled leucocyteS

Article (Accepted Version)


This version is available from Sussex Research Online: http://sro.sussex.ac.uk/id/eprint/75704/

This document is made available in accordance with publisher policies and may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the URL above for details on accessing the published version.

Copyright and reuse:
Sussex Research Online is a digital repository of the research output of the University.

Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable, the material made available in SRO has been checked for eligibility before being made available.

Copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

http://sro.sussex.ac.uk
In vivo imaging of hepatic neutrophil migration in severe alcoholic hepatitis with $^{111}$In-radiolabelled leucocytes

Jonathan R Potts$^{1,2}$, Neda Farahi$^3$, Mark R Howard$^4$, Mark R Taylor$^4$, Sarah Heard$^5$, Arun N Shankar$^6$, Graeme J Alexander$^6$, Edwin R Chilvers$^4$, Sumita Verma$^{1,2,*}$, A Michael Peters$^7*$

1. Department of Gastroenterology and Hepatology, Brighton and Sussex University Hospitals NHS Trust, Brighton, UK
2. Department of Medicine, Brighton and Sussex Medical School, Brighton, UK
3. Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, UK
4. Department of Histopathology, Brighton and Sussex University Hospitals NHS Trust, Brighton, UK
5. Department of Nuclear Medicine, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
6. Department of Hepatology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
7. Division of Clinical and Laboratory Investigation, Brighton and Sussex Medical School, Brighton, UK

* joint senior authors

Correspondence:
Professor A M Peters
Brighton and Sussex Medical School
Brighton BN2 5BE, UK
01273 523360
a.m.peters@bsms.ac.uk

Running title: Imaging severe alcoholic hepatitis

Word count of main text: 3566

Author contributions:
JRP patient identification, recruitment, study procedures, data collection and analysis and writing of the initial draft
MRH: histopathology analysis
MT: histopathology analysis
NF: microautoradiography development
SH: nuclear medicine support
ANS: patient identification and recruitment
GJA: study design
ERC: study design
SV: study concept, design and critical revisions
AMP: study concept, design and critical revisions.

All authors contributed to and approved the final draft of the manuscript.

Funding: The study was funded by a project grant from Brighton and Sussex Medical School
ABSTRACT The study aim was to image severe alcoholic hepatitis (SAH) using $^{111}$In-labelled leucocytes with two objectives in mind: firstly for non-invasive diagnosis and secondly to provide a platform for experimental therapies aiming to inhibit intrahepatic neutrophil migration. $^{111}$In-leucocyte scintigraphy was performed 30 min and 24 h post-injection in 19 patients with SAH, 14 abstinent patients with alcohol-related cirrhosis and 11 normal controls. Eleven with SAH and 7 with cirrhosis also had $^{99m}$Tc-nanocolloid scintigraphy. Change in hepatic $^{111}$In radioactivity was expressed as decay-corrected 24 h:30 min count ratio and, in SAH, compared with histological grading of steatohepatitis and expression of granulocyte marker, CD15. Hepatic microautoradiography on biopsy specimens obtained 24 h post-injection of $^{111}$In-leucocytes was performed in one patient. Median 24 h:30 min hepatic $^{111}$In activity ratio was higher in SAH (2.5 [IQR 1.7-4.0]) compared with cirrhotics and normal controls (1.0 [0.8-1.1] and 0.8 [0.7-0.9] respectively, $P<0.0001$). In SAH, it correlated with CD15 expression ($r=0.62, P=0.023$) and was higher in marked versus mild/moderate steatohepatitis (4.0 [3.0-4.6] versus 1.8 [1.5-2.6], $P=0.006$). Hepatic-to-splenic $^{99m}$Tc count rate ratio was reduced in SAH (0.5 [0.4-1.4]) compared with cirrhotics (2.3 [0.6-3.0]) and 3 historic normal controls (4.2 [3.8-5.0]; $P=0.003$), consistent with impaired hepatic reticuloendothelial function. Scintigraphic findings in SAH included prominent lung radioactivity at 30 min, likely the result of neutrophil priming. Microautoradiography demonstrated cell-associated $^{111}$In in areas of parenchymal neutrophil infiltration. In conclusion, $^{111}$In-leucocyte scintigraphy can non-invasively diagnose SAH and could provide a platform for evaluation of novel treatments aiming to inhibit intrahepatic neutrophil migration.
Key words: alcohol-related liver disease; cirrhosis; $^{111}$In-labelled leucocytes; liver biopsy; inflammation

List of abbreviations: ALP alkaline phosphatase, ALT alanine aminotransferase, ARLD alcohol-related liver disease, AST aspartate aminotransferase, CS corticosteroids, DF discriminant function, ECBL early change in bilirubin level, GAHS Glasgow alcoholic hepatitis score, GI gastrointestinal, $\gamma$GT Gamma glutamyl transpeptidase, HE hepatic encephalopathy, HRS hepatorenal syndrome, INR international normalised ratio, IQR interquartile range, MELD model for end-stage liver disease, PI post intravenous injection, PTX pentoxifylline, SBP spontaneous bacterial peritonitis, severe AH severe alcoholic hepatitis, SIRS systemic inflammatory response syndrome
INTRODUCTION

Alcohol-related liver disease (ARLD) is a growing clinical problem. In the UK, rates of hospitalisation and death have risen by almost 50% over the past decade, a trend which is projected to continue (1). Acute alcoholic hepatitis (AH) is the most florid form of alcohol-related liver disease (ARLD). It presents with jaundice and systemic upset in heavy drinkers. Clinically mild AH may resolve with abstinence but in-hospital mortality in severe AH (SAH), defined by a discriminant function (DF) ≥32, approaches 40% (2). Prompt diagnosis is key to improving survival, enabling timely supportive care, nutritional support and potentially specific medical therapies such as corticosteroids (3). Although histology is the gold standard for diagnosing SAH, liver biopsy is controversial because its accuracy varies from 50-90% (4–6). In most instances, coagulopathy and ascites mandate transjugular biopsy (TJB), an invasive procedure with recognised morbidity and mortality (7). Histological features include a neutrophil-rich parenchymal inflammatory infiltrate surrounding ballooned hepatocytes, often with eosinophilic Mallory-Denk inclusion bodies (8). Biopsy may yield prognostic information; for example, neutrophil infiltration correlates positively with corticosteroid response (9) and short-term survival (10). Doppler ultrasonography and scintigraphy with $^{99m}$Tc-colloids (11,12) may support the diagnosis but lack specificity. There is therefore a recognised need for non-invasive diagnostic tests (3).

The predominating inflammatory cell infiltrating the liver in SAH is the neutrophil (8). Almost all inflammatory diseases involving neutrophils have been imaged at some time with radiolabelled leucocytes, most notably inflammatory bowel disease in a gastroenterological context (13). The labelled neutrophil is the effective ingredient
in labelled leucocytes, which therefore depict not only neutrophil-predominant inflammation but also physiological neutrophil kinetics, especially neutrophil transit through the lungs (14), pooling (i.e. slow intravascular transit) in tissues (specifically, liver, spleen and bone marrow) (15), and sites of physiological neutrophil destruction (again, liver, spleen and bone marrow) (16). Despite their widespread use in other scenarios, labelled leucocytes have never previously been used to diagnose hepatic inflammation, probably because the liver is a major site of physiological neutrophil pooling and destruction, and consequently regarded as beyond such inflammation-targeted imaging.

In SAH, however, we speculated that physiological neutrophil pooling and destruction would be sufficiently impaired to expose pathological neutrophil migration in the liver and thereby allow imaging of inflammation with labelled leucocytes or purified neutrophils. This would open up two potentially important applications: firstly, diagnosing SAH non-invasively in the appropriate clinical setting, and secondly providing a platform for testing new anti-inflammatory therapies, analogous to the use of $^{111}$In-labelled neutrophils and eosinophils in inflammatory lung disease (17,18). The aim of the current study, therefore, was to investigate the potential of $^{111}$In-labelled leucocytes to non-invasively image SAH.

**METHODS**

This was a prospective study involving 3 groups of patients.

1. **Subjects with SAH (n=19)** who were recruited from consecutive hospital admissions. SAH was suspected on clinical grounds from an admission DF ≥32 in active or recently abstinent heavy alcohol consumers with jaundice for ≤3 months,
and confirmed wherever possible by TJB. All patients were screened to exclude other causes of liver disease, co-existent biliary obstruction and focal hepatic lesions. Medical therapy for SAH with corticosteroids (prednisolone 40 mg daily) and/or pentoxifylline (1200 mg daily) was prescribed at the discretion of the managing hepatologist. The response to treatment was determined by a fall in bilirubin level following a week of therapy (19). Two patients received no specific treatment for SAH and 3 were given blinded therapy as part of another placebo-controlled study (20), preventing assessment of treatment response. Therapy was started after imaging in 5 patients, while in the remaining 9 the median duration of treatment at the time of scintigraphy was 2 [1.5-6.5] days.

2. Patients with alcohol-related cirrhosis but without SAH (n=14) who were recruited following abstinence from alcohol for ≥6 months to ensure resolution of subclinical steatohepatitis. Cirrhosis was diagnosed from the combination of cutaneous stigmata of chronic liver disease, radiological findings (irregular liver margin with or without splenomegaly) and corroborative histology, where available.

3. Controls without liver disease (n=11) who were referred for $^{111}$In-leucocyte scintigraphy for suspected prosthetic joint infection. They had normal liver biochemistry, no risk factors for liver disease and no evidence of inflammatory pathology on $^{111}$In-leucocyte scintigraphy, so could be regarded as normal controls. Those reporting alcohol use exceeding Royal College of Physicians guidelines on safe consumption were excluded. The study received ethical approval via the NHS National Research Ethics Service. (Reference Number 08/H1107/36).
**Leucocyte labelling**

Autologous leucocytes from peripheral venous blood were labelled *in vitro* under sterile conditions with the lipophilic chelating agents, $^{111}$In-oxine ($n = 41$) or $^{111}$In-tropolonate ($n = 3$, all with SAH) according to published guidelines (21). Briefly, erythrocytes were allowed to sediment from 35 ml anticoagulated blood, aided by the addition of 1% methylcellulose. A leucocyte-rich, platelet-deplete cell pellet was obtained by centrifugation of the supernatant. The cells were re-suspended in saline ($^{111}$In-oxine) or plasma ($^{111}$In-tropolonate) and incubated with approximately 25 MBq of $^{111}$In-chelate for 15 min, before addition of autologous platelet-poor plasma. The labelled leucocytes were pelleted, supernatant aspirated, and cell-associated and unbound radioactivity measured to calculate labelling efficiency. $^{111}$In-labelled leucocytes were re-suspended in a further 3 ml of platelet-poor plasma and injected intravenously. The administered radioactivity was ~20 MBq, giving a radiation exposure of ~7 mSv.

**$^{111}$In-leucocyte imaging protocol**

Static planar images of the chest and abdomen were obtained 30 min and 24 h after administration of labelled leucocytes using a dual-headed gamma camera (SMV DSTXL or GE Discovery NM630) with medium energy collimators and a 10-min acquisition time. To assess labelled cell viability, the recovery of cell-bound label was determined from peripheral venous blood samples obtained 45 min post-injection and expressed as a percentage of administered activity.

**$^{111}$In-leucocyte image analysis**
Tissue-associated radioactivity (expressed as mean counts per pixel) was determined from manually defined regions of interest (ROI) over homogeneous areas of liver, spleen and lungs. The geometric mean of anterior and posterior counts was corrected for physical radionuclide decay and background activity. The change in liver-associated radioactivity between the two imaging times was expressed as the 24 h:30 min ratio of activities.

**99mTc-nanocolloid scintigraphy**

99mTc-nanocolloid scintigraphy was performed after completion of 111In-leucocyte scintigraphy in 11 patients with SAH and 7 abstinent patients with cirrhosis. Abdominal gamma camera images (SMV DSTXL) were acquired 20 min post-injection of 80 MBq 99mTc-nanocolloid (Nanocoll, GE Healthcare, Amersham, UK; radiation exposure ~1 mSv) using low-energy high-resolution collimation. Downscatter from the 111In photopeak accounted for <15% of 99mTc tissue activity. Geometric means of counts per pixel were calculated for liver and spleen in the same ROI as above and expressed as liver:spleen ratio.

**Liver biopsy**

TJB (radiation exposure ~10 mSv) was performed in patients with SAH within 1 week of 111In-leucocyte scintigraphy. Independent biopsy interpretation was undertaken by two histopathologists blinded to the other’s findings and to scintigraphy. SAH was defined as the coexistence of steatosis, hepatocyte ballooning and lobular neutrophil infiltration (3). Histological features of steatohepatitis, including hepatocyte ballooning, lobular inflammation, steatosis, canalicular cholestasis, ductular cholestasis and cholangiolitis, were assessed according to
recently described criteria (4). The severity of steatohepatitis was semi-quantitatively classified as mild, moderate or marked according to whether foci of infiltrating parenchymal neutrophils were sparse, moderate or abundant. Cells positive for granulocyte marker CD15 were counted in 5-10 non-consecutive high-power fields in 13 patients in whom sufficient biopsy material was available for immunohistochemical staining, and the median count averaged between the two histopathologists.

**Microautoradiography**

To determine the intrahepatic fate of $^{111}$In-leucocytes in SAH, microautoradiography was performed on biopsy specimens obtained 24 h post-injection of labelled leucocytes in a single patient. Formalin-fixed liver sections (3 µm thickness) were placed on positively charged adhesive slides, de-waxed and a radiosensitive emulsion (Ilford K2, Harman Technology, UK) applied in dark room conditions. Micro-autoradiographs were exposed in dry, dark conditions for two weeks, following which they were fixed, developed, dried, post-stained with H&E and inspected for foci of radioactivity.

**Statistical analysis**

Data are presented as mean ± standard deviation or median [interquartile range]. Parametric data were compared using Student’s t-test or analysis of variance (ANOVA) and non-parametric data using Mann-Whitney U or Kruskal-Wallis tests. Correlation was quantified using Pearson’s correlation coefficient ($r$) or Spearman’s rho ($\rho$) for parametric and non-parametric data, respectively. Inter-observer agreement
of categorical histopathology data was assessed using the kappa statistic ($\kappa$). Reported $P$ values are two-tailed.

**RESULTS**

**Patients**

Baseline characteristics and demographic data are shown in Table 1. Median alcohol consumption prior to admission was 144 g/day [80-200] for 10 [5-15] years. Four abstinent cirrhotics had histologically confirmed disease. The diagnosis in the remaining 7 rested on clinical and radiological grounds.

In patients with SAH, the median admission DF was 52 [44-75]; the Glasgow Alcoholic Hepatitis Score (GAHS) (22) was ≥9 in 12 and Modified End-stage Liver Disease (MELD) (23) score ≥21 in 16. Four (21%) had evidence of the systemic inflammatory response syndrome and two had active infection (limb cellulitis and spontaneous bacterial peritonitis). Peripheral blood leucocyte counts were significantly higher in SAH than in either of the other groups (Table 1; $P<0.001$). Normal controls were significantly older than either cohort with liver disease ($P = 0.009$), consistent with the population referred for investigation of prosthetic joint pain.

**$^{111}$In-labelled-leucocyte scintigraphy**

Labelling efficiency was broadly similar between the 3 patient groups: 89 (85-92)%,$\quad$ 84 (81-85)% and 86 (81-88)%.$\\$ Labelled cell recovery, however, was significantly higher in SAH (57 [48-63]%) compared with abstinent cirrhotics and normal controls (38 [26-46]% and 38 [34-44]%, respectively, $P = 0.002$),
Normal $^{111}$In-leucocyte scintigraphy 30 min post-injection shows prominent activity in liver and spleen, and faint diffuse activity in the lungs. At 24 h, the diffuse lung activity disappears and bone marrow activity becomes evident (Fig 1). Liver activity falls slightly while spleen remains largely unchanged, suggesting that leucocyte pooling is broadly matched by neutrophil destruction (13).

The 24 h:30 min activity ratio in the liver was significantly higher in SAH (2.5 [1.7-4.0] compared with abstinent cirrhotics (1.0 [0.8-1.1]) and controls (0.8 [0.7-0.9], $P < 0.0001$; Figs 2 and 3). Scintigraphic appearances in SAH were similar between $^{111}$In-oxine and $^{111}$In-tropolone radiolabelling (Fig 2). Two abstinent cirrhotics had decompensated liver disease with diuretic-refractory ascites and liver synthetic impairment (both Child-Pugh class B). The 24 h:30 min ratio in these two individuals (1.08 and 1.37) was not significantly higher than in compensated cirrhosis (median 0.96 [0.78-0.99], $P = 0.067$) and remained substantially lower than the cohort with SAH ($P = 0.025$).

Prominent diffuse pulmonary activity at 30 min, which cleared by 24 h, was a frequent finding in SAH (Fig 2) and occurred to a lesser degree in abstinent cirrhotics. Median lung-associated radioactivity at 30 min (expressed relative to splenic activity) was significantly higher in SAH (20 [15-26]%) than normal controls (14.5 [13-16]%; $P = 0.02$), but did not differ significantly from abstinent cirrhotics (18 [12.5-25]%, $P = 0.38$). By 24 h, lung radioactivity had decreased similarly in all 3 groups but could not be quantified because of overlying physiological activity in chest wall bone marrow.
Abnormal gut-associated radioactivity 24 h post-injection was observed in 4 patients with SAH (Fig 2). Delayed imaging at 48-72 h demonstrated distal transit of $^{111}$In within bowel, in keeping with access of labelled leucocytes into bowel.

**Liver histology in SAH**

Clinical deterioration after $^{111}$In-leucocyte scintigraphy precluded TJB in 2 patients with SAH, leaving 17 with histology. The majority had underlying advanced hepatic fibrosis or cirrhosis and all exhibited some degree of steatohepatitis, judged to be marked in 7 (Table 2). Inter-observer agreement of fibrosis stage, steatosis grade, steatohepatitis severity and presence of Mallory’s hyaline was high ($\kappa \geq 0.5$), but there was substantial variability in assessment of ballooning grade, degree of canalicular cholestasis and the presence of cholangiolitis and Councilman bodies ($\kappa \leq 0.2$ in all).

There was agreement between histopathologists regarding severity of parenchymal neutrophil infiltration in 16 of 17 patients. Steatohepatitis was deemed marked in 7 patients, moderate in 7 and mild in 2. The 24 h:30 min hepatic activity ratio was significantly higher in marked steatohepatitis (4.0 [3.0-4.6]) compared with moderate (1.9 [1.6-3.0]) and mild (1.45 [1.2-1.7]) steatohepatitis ($P = 0.022$; Fig 4). The 24 h:30 min ratio in patients with histologically mild steatohepatitis was similar to that in abstinent cirrhotics with hepatic decompensation ($P = 0.25$).

There was good inter-observer agreement in parenchymal granulocyte quantification determined from CD15-positive cell counts ($r = 0.85$, $P < 0.001$). CD15-positivity count correlated modestly with the 24 h:30 min ratio (Fig 4; $r = 0.62$; $P = 0.023$).
Examples of $^{111}$In-leucocyte scintigraphy and corresponding liver biopsy sections in mild versus marked steatohepatitis are shown in Fig 5. No association was observed between the 24 h:30 min activity ratio and fibrosis stage.

**Clinical parameters, treatment response and survival**

There was no correlation between the 24 h:30 min activity ratio and serum bilirubin ($\rho = 0.02, P = 0.9$), peripheral blood leukocyte counts ($\rho = 0.28, P = 0.25$) or clinical measures of disease severity, including Child-Pugh score ($\rho = -0.17, P = 0.5$), DF ($\rho = 0.16, P = 0.5$), GAHS ($\rho = 0.29, P = 0.23$) or MELD ($\rho = 0.26, P = 0.29$). Assessment of treatment response in SAH was precluded in 3 patients who were treated as part of another placebo-controlled trial. Of the remaining 16, 14 received corticosteroids and/or pentoxifylline, of whom 10 exhibited a clinical response following a week of therapy. No significant difference was observed in the 24 h:30 min activity ratio between patients who responded to therapy compared with non-responders (2.4 [1.6-3.8] versus 2.3 [1.7-2.6]). Inpatient mortality (21%) was not predicted by imaging findings. Furthermore, there was no association between histological findings and either treatment response or in-hospital survival.

**Microautoradiography**

H&E stained autoradiography undertaken in one patient with SAH demonstrated foci of cell-associated radioactivity in areas of parenchymal neutrophil infiltration with low level background hepatic radioactivity (Fig 6). The 24 h:30 min hepatic activity ratio in this patient was 4.5.
**99mTc-nanocolloid scintigraphy**

Hepatic-to-splenic uptake ratio of $^{99m}$Tc-nanocolloid was significantly lower in SAH (median 0.5 [0.4-1.4]) compared with abstinent cirrhotics (2.3 [0.6-3.0]) and 3 historic controls without liver disease (4.2 [3.8-5.0]; $P = 0.003$; Fig 7). In SAH patients, however, there was no correlation between $^{99m}$Tc-nanocolloid liver/spleen ratios and either the 24 h:30 min activity ratio ($r = -0.04$, $P = 0.9$) or histological severity of steatohepatitis ($r = -0.4$, $P = 0.3$).

**DISCUSSION**

This proof-of-concept study shows for the first time that hepatic neutrophil migration can be successfully imaged and semi-quantified using $^{111}$In-leucocyte scintigraphy. This provides not only a potential means of diagnosis and outcome prediction in SAH, but also, importantly, a platform for the assessment of new anti-inflammatory therapies for a range of acute inflammatory liver disorders. An analogous scenario is inflammatory pulmonary disease in which there is interest in using labelled neutrophils and eosinophils for assessing new therapies. Whilst labelled ‘mixed’ leucocytes are shown here to be appropriate for routine clinical use, labelled purified neutrophils would probably be preferable for research applications.

Current European guidelines recognise the need for non-invasive diagnostic tools for SAH (3). Abnormal leucocyte scintigraphy in SAH was characterised by an increase in liver-associated radioactivity between 30 min and 24 h, not seen in abstinent cirrhotics or normal controls. The 24 h:30 min hepatic activity ratio portrays intrahepatic neutrophil migration as shown by correlation with measures of neutrophil infiltration on liver biopsy.
We hypothesised that, for whatever mechanistic reason but almost certainly including reduced hepatic blood flow, physiological hepatic accumulation of $^{111}$In, both at 30 min as a result of impaired pooling, and 24 h as a result of impaired destruction, would be sufficiently decreased in SAH to expose hepatic neutrophil migration as an increase in liver-associated radioactivity between 30 min and 24 h. Consistent with impaired neutrophil destruction, hepatic accumulation of $^{99m}$Tc-nanocolloid was suppressed in SAH (Fig 7), corroborating the findings of earlier studies indicating impaired Kupffer cell phagocytic function (12,24). The increase in liver-associated $^{111}$In activity is almost certainly, therefore, the result of neutrophil migration. This interpretation is supported by microautoradiography of biopsy material obtained 24 h post-injection of $^{111}$In-leucocytes, which demonstrated, albeit in only one individual, discrete foci of cell-associated radioactivity in areas of neutrophil satellitosis within the lobular parenchyma. Moreover, although it provides no insight into the mechanisms of cellular extravasation, CD15 expression correlated significantly with the 24 h:30 min activity ratio.

Several extrahepatic features were noted on $^{111}$In-leucocyte scintigraphy. Prominent diffuse lung radioactivity at 30 min, clearing by 24 h, was a consistent finding in SAH, suggesting pulmonary vascular entrapment of primed neutrophils without extravascular migration. These imaging features have been reported in several other disorders associated with systemic neutrophil priming, including vasculitis (25) and severe inflammatory bowel disease (26). Leucocyte kinetics associated with priming are different from those seen following labelling-induced leucocyte injury, which gives immediate lung activity, markedly reduced 45 min recovery and decreased
physiological uptake in the spleen and bone marrow. Our scintigraphic findings of priming add to the existing ex vivo evidence for systemic neutrophil priming in SAH (27) and suggest that priming also occurs, albeit to a lesser degree, in patients with compensated chronic liver disease.

Labelled leucocyte recovery is probably the best benchmark of cell ‘health’ following labelling. The marginated neutrophil pool (which in health resides in the liver, spleen and bone marrow) (16) is in equilibrium with the circulating pool, and represents approximately 50% of the total blood neutrophil pool. Maximum recovery is therefore ~50% of administered activity. The increased recovery in SAH, despite increased pulmonary margination, suggests a pathophysiological shift in granulocyte margination away from the liver, in keeping with clearly reduced hepatic activity at 30 min post-injection in SAH (Fig 2). On the other hand, delayed pulmonary transit would be expected to reduce recovery.

Abnormal gut radioactivity was observed in a fifth of patients with SAH, in the absence of gastrointestinal symptoms or apparent disease. Because ‘free’ $^{111}$In does not enter the gut, distal transit of $^{111}$In on delayed imaging suggests luminal transit of labelled cells within faeces. The cause remains unclear and warrants further study, although potential explanations include disposal of intrahepatic neutrophils cells via bile, or more likely a sequel of portal hypertension.

We did not identify patients with clinically suspected SAH where histology refuted or altered the diagnosis, as others have shown (4,6). Our data, however, demonstrated significant heterogeneity in the severity of steatohepatitis that was independent of
clinical and laboratory disease markers, but predicted by $^{111}$In-leucocyte scintigraphy. No correlation was observed between clinical prognostic scores commonly used to judge disease severity and either the 24:30 min activity ratio or histological severity of steatohepatitis. However, most patients had clinically advanced disease making it difficult to further stratify according to disease severity.

Although recent clinical trials have questioned the benefit from corticosteroids (20), they remain widely used in medical management of SAH. Previous authors described a positive association between parenchymal neutrophil infiltration and clinical corticosteroid response in SAH (9). Predicting response to therapy would be useful, particularly given the high rates of infection and poor survival in corticosteroid non-responders (28). We did not demonstrate a relationship between scintigraphic findings and either treatment response or in-hospital mortality. Furthermore, we saw no association between steatohepatitis severity and either treatment response or survival, probably because of heterogeneity in prescribed therapies and small patient numbers. A larger study, powered to assess these specific end points, would be required to assess the prognostic value of $^{111}$In-leucocyte scintigraphy.

Further study limitations are as follows. For the convenience that would be important in routine clinical studies, we used labelled ‘mixed’ leucocytes. However, although mixed leucocytes clearly demonstrate the feasibility of imaging hepatic inflammation, $^{111}$In-labelled purified neutrophils would be preferable for research applications. $^{111}$In is preferable to $^{99m}$Tc because of its greater cell labelling stability. Moreover, $^{111}$In preferentially labels neutrophils, unlike $^{99m}$Tc-hexamethylpropylamine oxime (HMPAO), which is strongly selective for eosinophils (29).
Differentiation of SAH from other acute deteriorations of chronic liver disease, particularly end-stage cirrhosis or decompensation due to infection, is often challenging and any non-invasive diagnostic test for SAH would need to distinguish it from decompensation. Although they will form the cohort for future studies, we did not include a control group with decompensated cirrhosis at this stage of our work as our primary aim was to establish the feasibility of imaging hepatic inflammation with radiolabelled leucocytes in homogeneous patient cohorts. A decompensated group would be highly heterogenous and likely to include patients with ongoing or recent alcohol use, in whom subclinical alcoholic hepatitis would be a significant unmeasured confounder. Confident inclusion of such patients, moreover, would have required TJB for the sole purpose of excluding those with histologically active steatohepatitis, and would therefore have raised significant ethical issues. TJB in SAH, in contrast, is clinically justifiable and indeed standard of care in some centres. Liver biopsy is not common practice in other forms of ARLD, which are invariably associated with ongoing alcohol use. Given their high prevalence of asymptomatic subclinical steatohepatitis, active heavy drinkers [30], other than SAH patients, were not included. Patients with cirrhosis were therefore recruited following at least 6 month’s abstinence, to allow sufficient time for resolution of underlying steatohepatitis.

In conclusion, we have demonstrated how $^{111}$In-labelled leucocyte scintigraphy can be exploited to detect hepatic neutrophil migration in SAH. The technique has potential clinical application as a non-invasive means of diagnosing this condition and assessing the severity of liver inflammation without the need for liver biopsy.
Importantly, it also has the potential to provide a platform for experimental therapies aiming to inhibit intrahepatic neutrophil migration. Evaluation of leucocyte kinetics in other forms of acute and acute-on-chronic liver disease would be of interest.

REFERENCES


15. Peters AM, Saverymuttu SH, Bell RN, Lavender JP. Quantification of the


Biomarkers of eosinophilic inflammation in asthma. Expert Rev Respir Med.

al. Quantification of neutrophil migration into the lungs of patients with chronic

al. Early change in bilirubin levels is an important prognostic factor in severe
9.

Prednisolone or pentoxifylline for alcoholic hepatitis. N Engl J Med. 2015 Apr

21. Roca M, de Vries EFJ, Jamar F, Israel O, Signore A. Guidelines for the labelling
of leucocytes with (111)In-oxine. Inflammation/Infection Taskgroup of the


<table>
<thead>
<tr>
<th></th>
<th>Severe AH (n=19)</th>
<th>Abstinent cirrhosis (n=14)</th>
<th>Normal controls (n=11)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>50.3 ±9.8</td>
<td>55.7 ±8.9</td>
<td>64.0 ±14.9*</td>
<td>0.009</td>
</tr>
<tr>
<td>Male gender</td>
<td>14 (73.7%)</td>
<td>11 (78.6%)</td>
<td>7 (63.6%)</td>
<td>0.702</td>
</tr>
<tr>
<td>Ascites</td>
<td>11 (57.9%)*</td>
<td>2 (14.3%)</td>
<td>-</td>
<td>0.015</td>
</tr>
<tr>
<td>SIRS</td>
<td>4 (21.1%)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Child-Pugh Score</td>
<td>10 (8-11)*</td>
<td>5 (5-6)</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MELD score</td>
<td>23 (22-27)*</td>
<td>8.8 (7.4-9.3)</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DF</td>
<td>52 (44-75)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GAHS</td>
<td>9 (8-10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GAHS ≥9</td>
<td>12 (63.2%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leukocyte count (x10⁹/l)</td>
<td>12.6 (9.3-15.9)*</td>
<td>6.6 (5.6-7.6)</td>
<td>7.0 (6.3-8.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophil count (x10⁹/l)</td>
<td>9.8 (7.1-13.3)*</td>
<td>3.9 (2.6-4.9)</td>
<td>4.3 (3.7-5.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet count (x10⁹/l)</td>
<td>158 (106-264)</td>
<td>136 (91-194)*</td>
<td>239 (174-287)</td>
<td>0.047</td>
</tr>
<tr>
<td>INR</td>
<td>1.8 (1.6-2.1)*</td>
<td>1.1 (1.1-1.2)</td>
<td>1.1 (1.0-1.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>288 (202-451)*</td>
<td>10 (8-17)</td>
<td>7 (4-11)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>29 (27-34)*</td>
<td>43.5 (37-46)</td>
<td>43 (42-44)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>56 (47-102)</td>
<td>80.5 (62-107)</td>
<td>78 (58-81)</td>
<td>0.085</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>63 (49-80)*</td>
<td>17.5 (15-32)</td>
<td>20 (15-22)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>171 (117-220)*</td>
<td>36.5 (25-46)</td>
<td>18 (14-20)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are presented as mean ±standard deviation, median (IQR) or number (%), *P<0.05
SIRS: ≥2 features of the systemic inflammatory response syndrome at recruitment, MELD: Modified end-stage liver disease score, DF: Discriminant function, GAHS: Glasgow Alcoholic Hepatitis Score, INR: international normalised ratio, ALT: alanine aminotransferase, AST: aspartate aminotransferase
Normal values: bilirubin 0-21 µmol/l, INR 0.8-1.2, albumin 35-52g/l, ALP 40-129 iu/l, ALT 0-41 iu/l, AST 0-40 iu/l, γGT 10-71 iu/l, urea 1.7-8.3 mmol/l, creatinine 62-106 µmol/l, leukocyte count 4-11 x10⁹/l, neutrophil count 2-7.5 x10⁹/l, platelet count 150-450 10⁹/l
<table>
<thead>
<tr>
<th>Histological parameter</th>
<th>Pathologist #1 n (%)</th>
<th>Pathologist #2 n (%)</th>
<th>Agreement κ (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ishak fibrosis stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>9 (52.9%)</td>
<td>10 (58.8%)</td>
<td>0.538</td>
</tr>
<tr>
<td>Stage 5</td>
<td>7 (41.2%)</td>
<td>2 (11.8%)</td>
<td>(P &lt;0.0001)</td>
</tr>
<tr>
<td>Stage 4</td>
<td>-</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td>Stage 3</td>
<td>-</td>
<td>3 (17.6%)</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>1 (5.9%)</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Steatosis (parenchymal involvement)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5%</td>
<td>1 (5.9%)</td>
<td>2 (11.8%)</td>
<td>0.750</td>
</tr>
<tr>
<td>5-33%</td>
<td>6 (35.3%)</td>
<td>6 (35.3%)</td>
<td>(P &lt;0.0001)</td>
</tr>
<tr>
<td>&gt;33-66%</td>
<td>7 (41.2%)</td>
<td>5 (29.4%)</td>
<td></td>
</tr>
<tr>
<td>&gt;66%</td>
<td>3 (17.6%)</td>
<td>4 (23.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Severity of lobular neutrophil infiltrate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>2 (11.8%)</td>
<td>3 (17.6%)</td>
<td>0.904</td>
</tr>
<tr>
<td>Moderate</td>
<td>8 (47.1%)</td>
<td>7 (41.2%)</td>
<td>(P &lt;0.0001)</td>
</tr>
<tr>
<td>Marked</td>
<td>7 (41.2%)</td>
<td>7 (41.2%)</td>
<td></td>
</tr>
<tr>
<td><strong>Parenchymal involvement by ballooning</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No ballooning</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>&lt;5%</td>
<td>1 (5.9%)</td>
<td>2 (11.8%)</td>
<td>0.183</td>
</tr>
<tr>
<td>&gt;5-10%</td>
<td>1 (5.9%)</td>
<td>4 (23.5%)</td>
<td>(P =0.160)</td>
</tr>
<tr>
<td>&gt;10-20%</td>
<td>6 (35.3%)</td>
<td>3 (17.6%)</td>
<td></td>
</tr>
<tr>
<td>&gt;20-50%</td>
<td>8 (47.1%)</td>
<td>7 (41.2%)</td>
<td></td>
</tr>
<tr>
<td>&gt;50%</td>
<td>1 (5.9%)</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Canicular cholestasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5 (29.4%)</td>
<td>5 (29.4%)</td>
<td>0.174</td>
</tr>
<tr>
<td>&lt;5</td>
<td>-</td>
<td>9 (52.9%)</td>
<td>(P =0.033)</td>
</tr>
<tr>
<td>&gt;5-10</td>
<td>1 (5.9%)</td>
<td>1 (5.9%)</td>
<td></td>
</tr>
<tr>
<td>&gt;10-20</td>
<td>8 (47.1%)</td>
<td>2 (11.8%)</td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>3 (17.6%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Ductular cholestasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>14 (82.4%)</td>
<td>14 (82.4%)</td>
<td>0.218</td>
</tr>
<tr>
<td>1</td>
<td>2 (11.8%)</td>
<td>3 (17.6%)</td>
<td>(P = 0.285)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>1 (5.9%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Mallory’s hyaline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>14 (82.4%)</td>
<td>15 (88.2%)</td>
<td>0.767</td>
</tr>
<tr>
<td>Cholangiolitis</td>
<td></td>
<td></td>
<td>(P = 0.001)</td>
</tr>
<tr>
<td>Polymorphs around &amp; in ductule lumen</td>
<td>13 (76.5%)</td>
<td>12 (70.6%)</td>
<td>-0.053</td>
</tr>
<tr>
<td>Councilman bodies</td>
<td>9 (47.1%)</td>
<td>4 (23.5%)</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>(P = 0.893)</td>
</tr>
</tbody>
</table>
Figure Legends

Fig 1. Gamma camera images (anterior and posterior projections) obtained 30 min and 24 h after injection of autologous $^{111}$In-labelled leucocytes in a control patient. Liver: open arrows; spleen: closed arrows. Hepatic and splenic activities reflect physiological leucocyte pooling at 30 min and cell destruction at 24 h. Hepatic activity remains unchanged or decreases between the two time points. Lung activity can be seen faintly at 30 min.

Fig 2. Anterior gamma camera images following $^{111}$In-leucocyte administration with corresponding 24 h:30 min hepatic activity ratios in a control patient (a), abstinent alcohol-related cirrhotic patient (b), and patients with SAH using $^{111}$In-oxine labelling (c) and $^{111}$In-tropolonate labelling (d). Extrahepatic findings in SAH included prominent lung activity at 30 min post-injection (arrows) and gut activity at 24 h (circled) (e).

Fig 3. The 24 h:30 min hepatic activity ratio in patients with SAH, abstinent cirrhotics and normal controls (bars depict median value, whiskers show IQR). The 24 h:30 min ratios were significantly higher in SAH.

Fig 4. Comparison between $^{111}$In-leucocyte scintigraphy and histology in SAH. The 24 h:30 min hepatic activity ratios were significantly higher in marked versus mild and moderate steatohepatitis [pathologist #1 (a) and pathologist #2 (b)]. CD15 quantification of parenchymal neutrophil infiltration correlated with 24 h:30 min activity ratio (c).
**Fig 5.** $^{111}$In-leucocyte scintigraphy in patients with mild (a) and marked (b) steatohepatitis with corresponding H&E stained liver biopsy sections (c and d respectively). Note increased diffuse lung activity at 30 min post-injection and hepatomegaly, plus a hint of gut activity in the left mid-abdomen, in (b). The 24 h:30 min hepatic activity ratios were 1.2 and 3.5, respectively. Note that in (b) lung activity clears by 24.

**Fig 6.** Liver biopsy microautoradiographs in SAH at 40x (a) and 100x (b) objective magnification demonstrating foci of parenchymal granulocyte-associated radioactivity, indicated by overlying clusters of black silver halide crystals, against a low level of background radioactivity. Corresponding gamma camera images 30 min (c) and 24 h (d) demonstrate a prominent increase in liver-associated radioactivity (24 h:30 min activity ratio of 4.5). Note that in (d) lung activity clears by 24.

**Fig 7.** Hepatic:splenic $^{99m}$Tc-nanocolloid activity ratios (geometric mean of counts/pixel) in patients with SAH (a), abstinent cirrhotics (b) and historic normal controls (c) (bars depict median value, whiskers show IQR). The ratios were significantly lower in SAH compared with both abstinent cirrhotics and normal controls, consistent with severely impaired hepatic reticuloendothelial function (d).
Fig 1

Anterior

Posterior

30 min PI

24 h PI
Fig 2

a) Normal control  b) Abstinent cirrhosis  c) Severe AH (oxine)  d) Severe AH (tropolone)  e) Severe AH (oxine)

30 min PI

24 h PI

24h:30min liver ratio  0.7  1.0  3.5  5.4  3.5
Fig 3

24 h : 30 min liver-associated radioactivity ratio

- Severe AH (n=19)
- Abstinent Cirrhosis (n=14)
- Normal controls (n=11)

*P < 0.0001*

*P = 0.09*
Fig 4

a) 24 h: 30 min liver-associated radioactivity ratio

Minimal (n=2)  Moderate (n=8)  Marked (n=7)
Severity of parenchymal inflammatory infiltrate

b) 24 h: 30 min liver-associated radioactivity ratio

Minimal (n=3)  Moderate (n=7)  Marked (n=7)
Severity of parenchymal inflammatory infiltrate

c) CD15 positive cells per high power field

$r = 0.62, P = 0.033$