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Early-onset versus late-onset nonanastomotic biliary strictures post liver transplantation: risk factors reflect different pathogenesis

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Summary

Nonanastomotic biliary strictures (NAS) cause significant morbidity post liver transplantation. Timing of stricture development varies considerably, but the relationship between timing of stricture onset and aetiology has not been fully elucidated. Database analysis was performed on all adult patients undergoing liver transplantation between 1st January 1990 and 31st May 2008. Diagnosis of NAS required demonstration on at least two radiological studies. Early NAS were defined as developing <1 year post transplant (minimum 1-year follow-up) and late NAS developing >1 year post transplant (minimum 10-year follow-up). Ninety-six of 397 patients developed NAS (24%); 54 were early-onset NAS (56%) and 42 late-onset NAS (44%). Primary sclerosing cholangitis (PSC) was the only risk factor for NAS overall ($P = 0.001$). However, when patients with PSC were excluded, older donor age was a significant risk for NAS ($P = 0.003$). Early-onset NAS were associated with advanced donor age ($P = 0.02$), high MELD score ($P = 0.001$) and ABO-identical grafts ($P = 0.02$), whereas late-onset NAS were associated with PSC ($P = 0.0008$), bilio-enteric anastomosis ($P = 0.006$) and tacrolimus ($P = 0.0001$). Advanced donor age is a significant risk for NAS in patients without PSC. Importantly, aetiology of NAS varies depending on time to stricture development, suggesting early-onset and late-onset NAS may have different pathogenesis.

Nonanastomotic biliary strictures (NAS) following liver transplantation are common, with an incidence quoted between 4% and 30% [1]. These strictures cause significant morbidity and mortality, with the attendant costs of increased hospital admissions and procedural interventions [2,3]. NAS are notoriously resistant to therapy, culminating in liver retransplantation in up to 25% and death in 2–5% [4]. Despite risk factors being identified in previous studies [5–15], NAS remain a frequent complication of liver transplantation, and improved understanding of aetiology and preventive strategies are urgently needed.

To date, most studies of NAS have been small retrospective series and many contradictions exist within the published literature. In part, this is owing to variations in diagnostic criteria, periods of follow-up and risk factors studied. Primary sclerosing cholangitis (PSC) recurrence post liver transplantation has been well described [15,16] and is an established risk factor for post-transplant NAS [17]. However, many patients who develop NAS do not have PSC. Risk factors for NAS in the absence of a diagnosis of PSC have been rarely examined in previous studies.

It is also intriguing that the time to NAS onset is highly variable, from within weeks to many years post

transplantation. This has lead our group to consider that perhaps not all NAS are the same; risk factors for NAS development may differ depending on the time of presentation, reflecting potentially different pathogenesis for early-onset and late-onset NAS.

This single centre, retrospective cohort study investigates risk factors for NAS development following liver transplantation. The aims of this study are firstly to identify risk factors for NAS, secondly to identify risks for NAS in patients without PSC and thirdly to establish whether risk factors for early-onset NAS and late-onset NAS differ.

Methods

Study design

This retrospective cohort study was conducted on adult patients undergoing liver transplantation between 1st January 1990 and 31st May 2008 at a single centre. To be included in the study, patients had to be aged 18 years or above with a minimum follow-up time of 30 days. Follow-up of patients was until death or 31st June, 2008. Ethical approval for the study was provided by the institutional ethics committee.

We firstly compared patients with NAS to those without NAS. Next, we compared those patients with and without NAS in the cohort, excluding all patients with PSC. We then compared those with early-onset NAS to those without early-onset NAS. Finally, we separately compared patients with late-onset NAS to those without. To avoid lead-time bias, we restricted our analysis for early-onset strictures to all patients who either developed NAS within 1 year, or who had a minimum follow-up time of 1 year and had not developed NAS within the 1 year period. Similarly, for the late-onset NAS analysis, we only included patients who either developed NAS beyond 1 year post transplant during the study period, or who had a minimum follow-up time of 10 years, but had not developed NAS.

NAS definition

NAS were defined as any stricture, dilatation or irregularity of the intra or extra hepatic ducts, excluding those involving the biliary anastomosis, in the presence of a patent hepatic artery, visualized on at least two consecutive radiological studies. Radiological modalities used for diagnosis were MRCP, ERCP, PTC and biliary catheter cholangiogram. The majority of patients (85%) received protocol biliary catheter cholangiogram within 3 months post transplant, whereas those without biliary catheters received ultrasound imaging. All imaging was reviewed by two radiologists specializing in hepatobiliary imaging, who were blinded to clinical information relevant to NAS. Hepatic artery thrombosis was excluded using either Doppler ultrasound (which was performed routinely daily for the first 3 days post transplant, then again at the time of NAS diagnosis to exclude hepatic artery thrombosis) or angiography (which was performed as a confirmatory test, if Doppler ultrasound did not identify hepatic artery thrombosis). Hepatic artery thrombosis was excluded on duplex Doppler in the presence of completely unobstructed vascular flow waveform. Early-onset strictures were arbitrarily defined as those developing within 12 months of transplantation, as done by others [18]. Late-onset strictures were defined as those occurring beyond 12 months post transplantation.

Clinical definitions

For the purposes of this study, certain clinical definitions were used in data analysis. Acute cellular rejection (ACR) was defined as ACR confirmed on liver biopsy with a BANFF score of 5 or greater, requiring steroid therapy.

ABO match referred to donor-recipient ABO identical grafts, whereas ABO mismatch referred to donor-recipient compatible, but nonidentical grafts.

HLA match was defined as having at least one (of a possible two) allelic match between donor and recipient. Total HLA mismatch referred to the absence of any donor-recipient HLA matches (of a possible total of six for HLA-A, HLA-B and HLA-DR collectively).

Cytomegalovirus (CMV) infection was defined as positive CMV viraemia as detected by PCR post transplant. CMV PCR testing became available and routinely used in our unit from 2000; prior to this, CMV p65 antigen detection was used.

PSC recurrence was diagnosed with a combination of radiological and histopathological findings using standardized criteria [15].

Surgical procedure

All donor organs were procured from deceased donors who were ABO identical or compatible with the recipient. Donor livers that were suspected on macroscopic appearance of being steatotic were biopsied and assessed with frozen section using oil red-O staining. We routinely avoid transplanting liver grafts that show greater than 60% macrovesicular steatosis on liver biopsy and selectively transplant liver grafts that have 30–60% macrovesicular steatosis. Organ procurement was performed using standardized techniques. The common duct was flushed with normal saline antegrade via the cystic duct and retrograde through the transected common bile duct. In all cases, a preflush of low viscosity solution via the aorta

was performed. In 338 cases, the preflush was 4 litres hypertonic citrate solution (Ross solution; Orion Laboratories, Balcatta, WA, Australia), followed by Belzer University of Wisconsin (UW) solution (Viaspan; DuPond Merck Pharmaceutical Co., the Netherlands). In 38 cases, histadine-tryptophan-ketoglutarate (HTK) solution (Custodial; Dr Franz Kohler Chemie Gmbh, Alsbach-Hähnlein, Germany) was used both for preflush and final *in situ* perfusion. *Ex situ* perfusion at the donor back table was performed using the final perfusion solution (UW or HTK), 500 ml to the portal vein, 200 ml to the hepatic artery and 200 ml to the bile duct. All perfusion was performed under gravity feed with a pressure of approximately 1 m water.

Implantation was via a standard piggy-back approach, as described elsewhere [19,20]. Most patients received duct-to-duct biliary anastomosis, however, many patients with PSC required bilio-enteric anastomosis because of the presence of large-duct strictures. Our unit routinely places biliary stents, where feasible. Usually these were size 6 Fr infant feeding catheters placed transcystically across the biliary anastomosis to allow access to the biliary tree post transplant. The transplant surgical team remained constant for the duration of this study and there were no major changes in operative technique within the study period.

Post-operative management

The standard immunosuppression regimen within our unit is triple therapy for the first 6 months (prednisolone, calcineurin inhibitor and azathioprine). Tacrolimus and cyclosporine were both in widespread use throughout the duration of the study period. In patients with renal impairment, calcineurin inhibitors were withheld until creatinine clearance had improved and induction therapy with basiliximab was used (available in our unit since 2005). Since 2005, mycophenolate mofetil has been used in those with renal impairment, to allow reduction in dose of calcineurin inhibitor. To be recorded as having received a given medication, patients had to be on the drug for a minimum of 3 months. CMV prophylaxis with oral valganciclovir was routinely initiated at Day 10 and continued for 90 days if the donor was CMV positive and the recipient CMV negative. Clinically significant ACR was treated with pulsed intravenous methylprednisolone for 3 days. OKT3 was used for patients resistant to methylprednisolone.

If a biliary catheter was inserted, then routine cholangiography was performed between days 10–14 and as clinically indicated. Biliary catheters were clamped after biliary imaging was obtained, and removed 3 months post transplant following catheter cholangiography, if no significant abnormalities were found. MRCP has been used routinely in the diagnosis of biliary strictures since 2005 in our unit.

Data collection and analysis

Patient data were prospectively recorded in the Liver Transplant Unit Victoria database. Data recorded included donor and recipient demographic data, clinical and operative data and post-transplant events including medication. Important clinical events, such as diagnosis of NAS, time of NAS onset, death and retransplantation were also prospectively recorded.

Risk factors analysed were as follow: operative (MELD score, split graft, perfusion solution, cold ischaemic time (CIT), warm ischaemic time (WIT), total operative time, bilio-enteric anastomosis, biliary stent placement), demographic (donor and recipient demographic data including age, gender, race, whether deceased cardiac death donor, reason for transplantation), immunological (HLA match, ABO and rhesus match, B and T cell cross match, CMV IgG status) and post-transplant event data [ACR, cytomegalovirus (CMV) infection, immunosuppressive drug regimen]. Statistical analysis was performed using PASW Statistics 18.0 (IBM Corporation, Somers, NY, USA) and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Categorical data were compared using chi-square for equal proportion and presented as numbers (%). Continuous, normally distributed data were compared using student t-tests and reported as means (SDs), whereas non-normally distributed variables were compared using Wilcoxon rank sum tests and presented as medians (Interquartile Range). Survival and time to development of strictures were compared using log-rank tests. Multivariate analysis was performed using logistic regression for binomial outcomes with results reported as Odds Ratios (95% CI), whereas to time event analysis was performed using Cox proportional hazards regression with results reported as Hazard Ratios (95% CI). Multivariate models were constructed using both stepwise selection and backwards elimination procedures with all variables with a P-value <0.10 considered for model inclusion. A two-sided P-value of 0.05 was considered to be statistically significant.

Results

Prevalence and clinical characteristics of NAS

A total of 397 patients were included in the study with a median follow-up time of 7.5 years (IQR: 3.8–12.0 years). Forty-three of 397 patients (11%) had follow-up for less than 1 year. All donor grafts were from deceased donors, with three (0.7%) being split grafts and two (0.5%) being grafts donated after cardiac death (DCD).

Ninety-six of 397 patients developed NAS, an incidence of 24%. Figure 1 demonstrates time to development of strictures, which demonstrates an early peak in the first year post transplant. The median time to onset of NAS was 175 days (IQR: 73–1875.5). New diagnoses of NAS continued up to 17 years post transplant. The majority of patients in this study had clinically significant NAS. Eighty-seven of the 96 patients with NAS (91%) had at least one hospital admission for complications of NAS (excluding admissions solely for investigations or procedures) and 69 of 96 (72%) required radiological interventions within the study period.

Of the 96 patients with strictures, 56% developed early-onset NAS and 44% late-onset NAS. Median time to onset was 78 days for early-onset NAS (IQR: 34–127), whereas median time to onset for late-onset NAS was 2024 days (IQR: 871.5–3545.5).

Risk factors for NAS overall

All patients were included in this analysis. Results of univariate analysis are outlined in Table 1. On univariate analysis, we identified PSC, bilio-enteric anastomosis and tacrolimus use as risks for NAS, whereas CMV IgG positivity of the recipient and hepatitis C infection appeared protective. However, PSC was the only risk for NAS identified on multivariate analysis ($P = 0.001$, OR: 2.8, 95% CI: 1.54–5.14). Fifty-one patients had PSC, of whom 22 developed NAS (43%). All 22 patients who developed NAS were diagnosed with recurrent PSC.

Risk factors for NAS, excluding PSC

Risk factors for NAS excluding patients with PSC are outlined in Table 2. Of the 346 patients who did not have PSC, 74 (21%) developed NAS. Univariate analysis demonstrated tacrolimus use, advanced donor age and a high MELD score were risks for NAS, whereas CMV IgG positive recipient status was protective. On multivariate analysis, advanced donor age ($P = 0.003$, OR: 1.03, 95% CI: 1.01–1.04) was the only factor significantly associated with NAS, whereas CMV IgG positive recipient status ($P = 0.02$, OR: 0.5, 95% CI: 0.29–0.88) appeared protective.

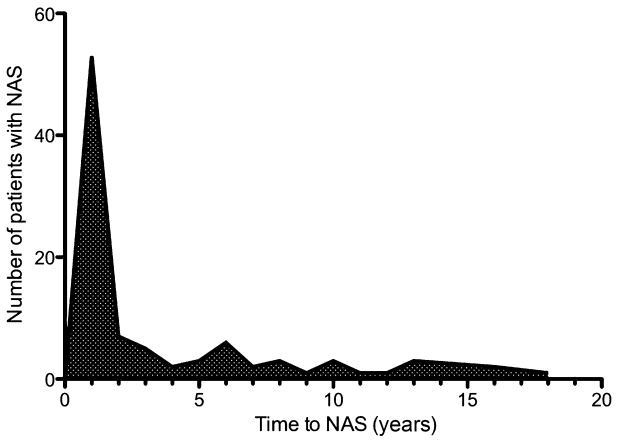

Figure 1. Time to development of non-anastomotic biliary strictures (years).

Risk factors for early-onset NAS

Of the 96 patients with NAS, 54 had early-onset NAS (56%). When compared with those 311 patients without early-onset NAS and with at least 1 year of follow-up, we identified several risk factors for early-onset NAS (Table 3). High MELD score ($P = 0.001$, OR: 1.1, 95% CI: 1.04–1.16), advanced donor age ($P = 0.02$, OR: 1.03, 95% CI: 1.01–1.05) and identical ABO status of donor and recipient ($P = 0.02$, OR: 4.1, 95% CI: 2.78–13.19) were all significant risk factors for early-onset NAS on multivariate analysis. The incidence of early-onset NAS was lowest in those with donor age less than or equal to 50 years and MELD score less than or equal to 20 (5%), and highest in those with donor age greater than 50 years (22%). In those with donor age less than 50 years, MELD score greater than 20 considerably increased the risk of early-onset NAS (20% vs. 5%), whereas in those with donor age greater than or equal to 50 years, MELD score did not affect the incidence of early-onset NAS (22%).

Risk factors for late-onset NAS

Forty-two patients of the overall cohort of 397 patients had late-onset NAS (11%). One hundred and twenty-four patients had the appropriate follow-up time to be included in this analysis and 29 (23%) of this group had late-onset NAS. Univariate analysis (Table 4) identified PSC ($P = 0.0008$, OR: 5.8, 95% CI: 2.11–16.14), tacrolimus use ($P = 0.0001$, OR: 5.59, 95% CI: 2.29–13.67) and bilio-enteric anastomosis ($P = 0.006$, OR: 3.6, 95% CI: 1.48–8.94) as significant risk factors for late-onset NAS, whereas cyclosporine use was protective ($P = 0.0006$, OR: 0.21, 95% CI: 0.08–0.51). There were inadequate patient numbers to perform multivariate analysis in this patient group.

Discussion

NAS remain the Achilles' heel of liver transplantation, resulting in significant morbidity and cost [12]. Since the paradigm of ischaemic-type biliary strictures was first described in the setting of hepatic artery thrombosis [21],

Table 1. Comparison of variables (univariate and multivariate) of liver transplantation with and without nonanastomotic biliary strictures.

Characteristic	NAS (*n* = 96)	No NAS (*n* = 301)	*P*-value	OR	95% CI
Donor variables					
Age (years, mean ± SD)	39.8 ± 16.3	36.8 ± 15.9	0.12		
Gender (M/F)	41/55	118/183	0.41		
DCD donor	1 (1%)	1 (0.3%)	1.00		
Recipient variables					
Age at transplant (years, mean ± sd)	47.2 ± 10.1	48.6 ± 10.3	0.26		
Gender (M/F)	34/62	100/201	0.69		
Race (%)					
Asian	5 (5)	27 (9)	0.29		
Caucasian	87 (91)	268 (89)	0.55		
African	0	2 (<1)	1.00		
Polynesian	1 (1)	0	0.24		
Hispanic	1 (1)	0	0.24		
Disease (%)					
HBV	14 (15)	50 (17)	0.64		
HCV	17 (18)	91 (30)	0.02	0.5	0.28-0.89
Alcohol	16 (17)	68 (23)	0.22		
HCC	14 (15)	49 (16)	0.69		
AHN	10 (10)	19 (6)	0.18		
PSC	22 (23)	29 (10)	0.001	2.8	1.51–5.14
AIH	4 (4)	19 (6)	0.44		
PBC	10 (10)	19 (6)	0.18		
NASH	1 (1)	5 (2)	0.67		
Cryptogenic	9 (9)	24 (8)	0.67		
Metabolic	4 (4)	12 (4)	0.94		
Storage	1 (1)	3 (1)	0.97		
Biliary atresia	2 (2)	3 (1)	0.42		
Haemachromatosis	1 (1)	1 (0.3)	0.42		
Other	3 (3)	10 (3)	0.93		
MELD score (mean ± SD)	20.9 ± 7.86	21.0 ± 6.76	0.26		
Immune variables (%)					
CMV IgG positive donor	61 (67)	187 (62)	0.42		
CMV IgG positive recipient	50 (56)	192 (68)	0.03	0.6	0.36–0.95
CMV IgG mismatch (donor–recipient)	59 (69)	192 (68)	0.96		
ABO match‡ (donor-recipient)	84 (88)	238 (79)	0.07		
Rhesus match (donor–recipient)	76 (81)	223 (79)	0.66		
Positive B cell cross match	14 (20)	75 (27)	0.21		
Positive T cell cross match	11 (15)	43 (15)	0.96		
HLA-match§	61 (71)	159 (65)	0.40		
HLA-A match§	58 (68)	155 (63)	0.61		
HLA-B match§	21 (24)	81 (33)	0.22		
HLA-DR match§	29 (37)	80 (37)	0.95		
Surgical variables (%)					
Split graft (Y/N)	1 (1)	2 (0.6)	0.71		
Perfusion solution (%)					
HTK	13 (14)	25 (9)	0.12		
UW	7 (86)	261 (91)	0.12		
CIT (min, mean ± sd)	501 ± 204	477 ± 157.8	0.50		
WIT (min, mean ± SD)	51 ± 25	49 ± 14.5	0.21		
Total op. time (min, mean ± sd)	529 ± 162	518 ± 139.4	0.55		
Bilio-enteric anastomosis	29 (31)	45 (15)	0.001	2.4	1.43–4.19
Biliary stent	73 (88)	259 (87)	0.89		
Post operative variables (%)					
ACR	25 (26)	68 (23)	0.53		
CMV disease†	11 (11)	33 (11)	0.92		
Cyclosporine	45 (50)	170 (58)	0.16		

Table 1. continued

Characteristic	NAS (*n* = 96)	No NAS (*n* = 301)	*P*-value	OR	95% CI
Tacrolimus	57 (63)	142 (49)	0.02	1.8	1.11–2.95
Azathioprine	90 (98)	290 (97)	0.58		
Mycophenolate	38 (42)	128 (43)	0.77		
Multivariate analysis					
PSC			0.001	2.8	1.51–5.14

DCD, donation after cardiac death; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; AHN, acute hepatic necrosis; PSC, primary sclerosing cholangitis; AIH, auto-immune hepatitis; PBC, primary biliary cirrhosis; NASH, nonalcoholic steatohepatitis; Haemachom, haemachromatosis; CMV, cytomegalovirus; HTK, histidine-tryptophan-ketoglutarate; UW, University of Wisconsin solution; CIT, cold ischaemia time; WIT, warm ischaemia time; Total Op. Time, total time of operation; ACR, acute cellular rejection requiring treatment with steroids.
*CMV IgG mismatch means donor and recipient IgG status were not the same.
†CMV disease means positive CMV viraemia on PCR.
‡ABO match means donor and recipient ABO identical.
§HLA-match means at least one allele identical between donor and recipient.

Table 2. Analysis: risk factors for NAS excluding PSC patients.

Characteristic	NAS (*n* = 74)	No NAS (*n* = 272)	*P*-value	OR	95% CI
Univariate analysis					
Tacrolimus	46 (66%)	125 (47%)	0.007	2.10	1.23–3.69
Donor age	42.3 ± 16.3	36.9 ± 15.9	0.011	1.02	1.01–1.04
MELD Score	21.9 ± 8.1	19.9 ± 6.8	0.034	1.04	1.01–1.08
CMV IgG positive recipient	39 (56%)	203 (68%)	0.047	0.58	0.34–0.99
Multivariate analysis					
Donor age			0.003	1.03	1.01–1.04
CMV IgG positive recipient			0.016	0.50	0.29–0.88

NAS, nonanastomotic stricture; CMV, cytomegalovirus.

Table 3. Analysis: risk factors for early-onset NAS (within 1 year post transplant).

Characteristic	Early NAS (*n* = 54)	Late NAS (*n* = 311)	*P*-value	OR	95% CI
Univariate analysis					
HTK	12 (24%)	21 (7%)	<0.0001	3.94	1.80–8.64
MELD	22.7 ± 8.1	19.8 ± 6.8	0.006	1.06	1.01–1.10
Tacrolimus	34 (65%)	147 (50%)	0.038	1.90	1.02–3.52
HLA-B match†	7 (15%)	78 (31%)	0.036	0.42	0.18–0.97
Donor age	41.3 ± 17.0	36.9 ± 15.8	0.061	1.82	0.20–9.0
ABO match	49 (91%)	273 (79%)	0.061	2.54	0.98–6.64
Multivariate analysis					
MELD			0.001	1.10	1.04–1.16
Donor age			0.024	1.03	1.01–1.05
ABO match*			0.020	4.10	2.78–13.19

NAS, nonanastomotic stricture; HTK, histidine-tryptophan-ketoglutarate.
*ABO Match means donor and recipient ABO identical.
†HLA-B Match means at least one allele identical between donor and recipient.

many studies have focussed on operative and demographic risks for NAS [22]. The potential role of immunological risk factors has only been considered in recent years [23] and immunological factors, such as donor-recipient HLA matching and immunosuppressive regimens have been less well studied. We therefore included these factors in our study design, along with other clinical factors that have been variably demonstrated to be associated with NAS in the current published literature.

One of the key areas of discrepancy in the NAS literature is the variability in reported incidence rates, ranging from 4% to 30%, depending upon the criteria used [1].

Table 4. Analysis: risk factors for late-onset NAS (beyond 1 year post transplant, minimum follow-up 10 years).

Characteristic	Late NAS (n = 29; %)	No late NAS (n = 95; %)	P-value	OR	95% CI
Univariate analysis					
Tacrolimus	17 (59)	19 (20)	0.0001	5.59	2.29–13.67
PSC	11 (38)	9 (9)	0.0008	5.81	2.11–16.14
Bilio-enteric anastomosis	13 (45)	17 (18)	0.006	3.60	1.48–8.94
Cyclosporine	14 (48)	77 (82)	0.0006	0.21	0.08–0.51

NAS, nonanastomotic stricture; PSC, primary sclerosing cholangitis.

The incidence of NAS in our unit was 24%, which is higher than in some previous studies [23,24]. Many studies have included only NAS cases diagnosed with interventional procedures, such as ERCP [25], or at cholangiogram in the early postoperative period when clinical evidence of biliary obstruction developed [4]. More recent studies, such as ours have used MRCP in addition to ERCP and PTC, which may improve early detection of NAS in patients with mild cholestatic LFTs but not severe enough to warrant endoscopic or percutaneous interventions. In addition, biliary catheter cholangiography is used routinely in our unit, resulting in improved early detection of strictures. Previous reports with lower prevalence have in general had shorter duration of follow-up [26,27]. In this study the median follow-up period was 7.5 years, which no doubt contributes to the higher stricture prevalence we have reported. There has also been an observed increase in incidence of NAS internationally over the past 10 years, which has been attributed in part to improvements in diagnostic radiological imaging technology and the increased use of marginal donors worldwide [28].

PSC has long been identified as a risk for NAS and PSC recurrence is now well-recognized [15,17]. There is, however, some controversy in defining PSC recurrence from *de novo* NAS development post transplant, despite the development of standardized diagnostic criteria [15]. This is because of lack of specificity of both the radiological and histopathological hallmarks of recurrent PSC and the relative paucity of pathognomonic histological features in most patients [25]. Furthermore, MRCP has been less well validated for diagnosis of PSC recurrence [29]. For this reason, in our study we did not distinguish between PSC patients with de-novo NAS compared with PSC recurrence, preferring to include the patients together. It is for this reason that we performed the analysis excluding PSC patients, a method first employed by Heidenhhain *et al* [25].

In patients without PSC, advanced donor age was the variable most strongly associated with NAS development (Table 2). The mechanism linking donor age with subsequent stricture development remains unclear. Age has been shown to affect the function of donor hepatocytes and other donor parenchymal cells [30,31]. It has also been demonstrated that advancing age is associated with pseudocapillarization of sinusoids resulting in a reduction in hepatocyte oxygen delivery [32–34]. It is quite possible therefore that older donor livers are more susceptible to ischaemic injury, leading to ischaemic biliary stricture formation.

Interestingly, our analysis also demonstrated that recipient CMV IgG positive status was significantly protective against NAS (P = 0.02, Table 3). This may reflect a reduced susceptibility to *de novo* CMV infection therefore averting an important cause of liver inflammation and damage. However, we did not identify CMV IgG mismatch between donor and recipient as an independent risk factor for NAS (Table 1, P = 0.96). Patients with CMV IgG status donor-recipient mismatch were given CMV prophylaxis, which may account for the absence of CMV IgG mismatch as a risk factor. CMV immunoglobulin testing was available in our unit prior to highly sensitive CMV PCR testing to detect viraemia, which may be why CMV infection per se was not detected as a significant risk for strictures in our study. This theoretical explanation requires further investigation.

A further aim of our study was to explore whether risk factors for NAS may differ depending on the time of onset, reflecting differing aetiologies for early-onset and late-onset NAS [34]. Our data demonstrated that risk factors for early-onset and late-onset NAS differ markedly from each other. A high MELD score (P = 0.001), advanced donor age (P = 0.02) and donor-recipient ABO match (P = 0.02) were all significant risk factors for early-onset NAS on multivariate analysis (Table 3). High MELD score recipients and advanced donor age grafts have been shown by other groups to be risks for NAS [28] being associated with a more complicated operative course and higher risk of ischaemia-preservation injury. These complications occur early in the postoperative period and would therefore seem feasible contributors to the incidence of early-onset NAS.

Donor–recipient ABO identical grafts being a risk for early-onset NAS was a surprise finding and difficult to

explain in the context of the current literature. Some groups have identified ABO incompatibility as a risk for NAS [14,35]. ABO incompatibility has been associated with hepatic artery thrombosis [36], a mechanism thought to be important in the onset of ischaemic NAS. However, these studies describe ABO incompatible grafts, not ABO compatible, but nonidentical grafts. There were no ABO incompatible transplants performed in our study. As such, our findings are novel. A potential explanation may be that within our unit, ABO compatible, but nonidentical grafts would not receive donor blood transfusion during the perioperative period, whereas we routinely use donor blood transfusion (after cross matching) in our ABO-identical patients during the transplantation procedure. It may be that ABO identical grafts are a marker of this practise. It is possible that donor blood transfusion leads to immunoreactivity and subsequent thrombosis of the microvasculature through mismatch of other blood antigens not explored in our study. Within our unit, we have previously analysed blood volume donation for both donor blood transfusion and exogenous blood transfusion, and did not find a significant difference between these two groups (data not shown).

Whilst difficult to explain, it is well established that ABO antigen presentation by donor hepatocytes to recipient peripheral immune cells infiltrating the donor liver is a very early event, supporting its role in early-onset rather than late-onset NAS. Donor AB antigens are expressed on vascular endothelium and bile duct epithelial cells up to 150 days post transplant [14] and almost exclusively on large duct biliary epithelial cells and not those of small ducts or hepatocytes [37,38]. This complements the findings of Buis *et al.* [18] showing that early-onset NAS were more likely to involve the central large bile ducts.

In contrast, late-onset NAS were associated on univariate analysis with PSC, bilio-enteric anastomosis and tacrolimus use (Table 4). Owing to small numbers with an adequate duration of follow-up for this arm of our study, multivariate analysis was not performed. PSC and bilio-enteric anastomosis are of course co-dependent variables and well-established risk factors for NAS. The finding of tacrolimus being a risk factor for late-onset NAS and cyclosporine protective (as its corollary) is fascinating, particularly in view of the fact that the role of immunosuppressive regimens in NAS formation has not been extensively studied previously. The increasing use of tacrolimus in recent years may be one explanation for the apparent rising incidence of NAS over time. In our unit, the use of tacrolimus has remained relatively static throughout the study period and inclusion of year post transplant in our multivariate model did not change significance of tacrolimus as a risk. This finding needs to be validated in further studies.

We were surprised to find that cold and warm ischaemia times were not significant risks for NAS in our study, as these have been identified by several other studies to date [9,23,39]. Based on previous data, we have conscientiously minimized cold and warm ischaemia times in our unit and this may be why these were not revealed to be risks in our study. Certainly, several studies have not identified cold and warm ischaemia times as significant risk factors for NAS [13,26,40–43]. We have also altered practise based on the work of Moench *et al.* [7] to improve back-table arterial pressure perfusion. Similarly, there were other risk factors identified in previous studies that we have not found in our work, such as acute rejection [14,44] and auto-immune hepatitis [23], however, these factors have been identified in only one or two studies. Other operative risk factors, such as use of split grafts [8] and DCD grafts [11] were of very small number in our study therefore our analysis was underpowered to identify these as risks.

The work published by Buis *et al.* [18] was the first to consider that early-onset strictures and late-onset strictures may in fact have differing pathogenesis. They identified that early-onset strictures were associated with preservation-related risk factors and most commonly involved the central large bile ducts. In contrast, late-onset strictures were associated with immunological factors, such as PSC and were more likely to be located in peripheral, small ducts. However, in Buis' study, early- and late-onset strictures were directly compared with each other rather than to those without strictures, and analysis was therefore limited to univariate methodology. Whilst this provided important information about how early- and late-onset strictures may differ, the mutually exclusive nature of the comparative analysis could not establish whether early-onset and late-onset strictures are different diagnoses. The current study is therefore the first to analyse these factors as separate clinical entities and utilize multivariate analysis to exclude confounding variables, at least for early-onset strictures.

Our data demonstrate that risk factors for early-onset and late-onset NAS differ markedly. Similar to the work by Buis *et al.* [18], we found that early-onset NAS were associated with factors more likely to affect the perioperative course, such as MELD score and donor age, whereas late-onset NAS were associated with immunological factors, such as PSC, as well as bilio-enteric anastomosis and choice of immunosuppressive agent.

It is important to note the limitations of our study. Our study was retrospective, and incomplete data entry may have inadvertently affected our results. The timing of NAS development and diagnosis is also problematic in a retrospective study. Longer time duration in a study allows increasing diagnosis of NAS as strictures may

develop many years post transplant. We analysed our data using transplant date prior to 2000 versus year 2000 and beyond and not surprisingly found that transplantation prior to 2000 was a significant risk on univariate analysis for NAS overall ($P < 0.0001$, OR: 2.61), NAS in the absence of PSC ($P = 0.0006$, OR: 2.52) and late-onset NAS ($P < 0.0001$, OR: 7.07; data not shown). This is likely because of greater time for NAS development and diagnosis with increased time of follow-up. Importantly, this factor was not significant on any of the multivariate analyses performed and time to diagnosis was incorporated into our statistical model to avoid lead-time bias.

Technological developments have markedly improved our ability to detect NAS over time, including the now-widespread use of MRCP in our centre. However, the routine use of biliary catheter cholangiography throughout our series should reduce the tendency to a higher rate of diagnosis in more recent patients. ERCP was more widely used as a diagnostic tool prior to use of MRCP, which also minimizes the trend to increased diagnosis of NAS in recent years with increasing availability of MRCP technology.

Similarly, methods for identifying potentially important risk factors have changed over the study period, for example the increased sensitivity of multiplex PCR to detect CMV viraemia. CMV PCR was only available for routine use in our unit from 2000, which may have affected our results.

Another important limitation of our study is that early- and late-onset NAS are arbitrarily defined. We have used a definition first described by Buis *et al.* [18] and one that is clinically useful. Support for this definition comes from Fig. 1 which demonstrates the bimodal distribution of NAS (with peaks at 1 and 6 years post transplantation). Finally, the type of analysis used in our study can only suggest risks and highlight potential differences between groups to encourage further prospective research in this area. In particular, for the late-onset NAS cohort we could not perform a multivariate analysis to tease out independent risk factors because of the small number with adequate duration of follow-up.

However, despite these shortcomings, our data identify several important principles which improve our understanding of NAS and potentially provide methods to reduce their incidence. Firstly, we identify that in the absence of PSC, NAS still commonly occur and the key risk is advanced donor age. Unfortunately, in most transplant units worldwide donor age cannot be manipulated. However, we also found recipient CMV IgG positive status is protective, suggesting that greater exploration of CMV immunity and infection prevention may help to reduce the onset of NAS in the absence of PSC.

This study also importantly demonstrates that early-onset NAS and late-onset NAS have different clinical risks and therefore are likely to be discrete clinical entities. Our study suggests that high MELD score predisposes to early-onset NAS and is a stronger risk in those with young donor grafts compared with older donors. By contrast, late-onset NAS were associated with PSC, bilio-enteric anastomosis and tacrolimus immunosuppression. The finding of increased late-onset NAS with tacrolimus use is novel and requires further validation, however, it suggests a new preventive strategy that warrants further investigation.

Conclusion

In the absence of PSC, we have identified that advanced donor age is a significant risk for NAS, whereas recipient CMV IgG positivity is protective. Our data also show significant differences in risks for early-onset compared with late-onset NAS. Early-onset NAS are associated with perioperative variables, whereas late-onset NAS are associated with immunological factors. In addition to providing important clues to intriguing pathophysiological differences between early-onset and late-onset NAS, our data suggest potential preventive strategies for NAS. Whilst avoidance of older donor graft use is currently not an option for most transplant units because of waiting list mortality, manipulation of immunosuppression may be a potential strategy to reduce late-onset NAS and warrants further investigation.

Authorship

JH: data collection, statistics, study design, manuscript writing. PG: study design, manuscript editing. PA: study design, manuscript editing. RJ: study design, data collection. BZW: data collection. MB: statistics. MF: study design, data collection, statistics, manuscript editing.

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