Global quality assessment of liver allograft C4d staining during acute antibody mediated rejection in formalin-fixed paraffin-embedded tissue

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ABSTRACT

Discussion of liver antibody mediated rejection during the 2011, 2013 and 2015 Banff liver sessions raised concerns over reliability of complement fragment 4d (C4d) staining, precipitating a global survey followed by a tissue microarray staining quality assessment study among centers on formalin-fixed, paraffin-embedded tissue. Tissue microarray sections containing tissue plugs of resected native and allograft (with acute antibody mediated rejection) liver, heart and kidney (n=33 total cores) were sent to 31 centers for C4d staining using local method (s) and pathologist scoring. Digital whole slide images (n=40) were then semi-quantitatively scored by 7 experts for background, distribution and intensity of portal vein and capillary, hepatic artery, sinusoidal, and central vein endothelia and portal and central stromal staining. Results showed that strong and diffuse portal vein and capillary C4d staining, as determined by both local and central pathologists, clearly distinguished allografts showing acute antibody mediated rejection from native livers and from those with evidence of weaker donor specific antibody. Downstream vascular endothelial cell C4d staining and assessment were more variable and difficult to identify. C4d staining in the majority of laboratories reliably detects acute liver allograft antibody mediated rejection in formalin-fixed, paraffin-embedded tissues. Assessment should focus on portal veins and capillaries, sinusoids and central veins present in peripheral core needle biopsies. C4d staining in one organ does not always translate to staining in another.

KEYWORDS: Complement fragment 4d, liver allograft, antibody mediated rejection, method, tissue microarray

1. INTRODUCTION

Despite Western World abandonment of ABO-incompatible liver transplantation because of acute antibody mediated rejection (AMR) in untreated recipients; AMR occurrence in ABO-compatible orthotopic liver transplant (OLTx) is still debated because of its low incidence in OLTx [1-7].

However, accumulating case reports [8-13], cohort studies [14-17] and reviews [7,18], show that AMR can cause liver allograft dysfunction and rarely, failure. Based on published studies and intense discussion over a 6-year period AMR has also been incorporated into the Banff scheme for liver allograft pathology [19].

C4d staining is an important, although limited [20], tool used to assist in establishing an AMR diagnosis in all solid organ allografts. Optimal methods of tissue preservation, staining, and compartment scoring of C4d deposition have yet to be established for liver allografts, but in general, immunofluorescence (IF) on frozen tissue is considered the gold standard. Unfortunately, most liver centers obtain only formalin-fixed paraffin embedded (FFPE) tissues for C4d immunohistochemistry (IHC). Kozlowski et al [16] suggested that IHC antigen retrieval pH is an important determinant of C4d staining sensitivity and that staining patterns qualitatively differ between IF and IHC, and only the former might be reliable.

AMR discussions during the last 3 Banff meetings [Paris, France (2011), Comandatuba, Brazil (2013) and Vancouver, Canada (2015)] prompted a survey to gauge practices and attitudes as to the relevance of AMR in OLTx; C4d usefulness in liver allograft AMR diagnosis; and frozen versus FFPE sections. Based on survey results this TMA reliability was carried out using native and AMR-positive kidney, heart plugs, and liver plugs.

The aims of the study were to: 1. Elicit current views on AMR in OLTx; 2. Determine if C4d staining on FFPE sections is able to identify acute AMR in “gold standard” cases; 3. Identify C4d staining methods and structures that might be used to standardize an acute liver allograft AMR diagnosis.

2. MATERIALS AND METHODS

2.1. Surveys

Two internet surveys were initially conducted (see supplementary material for details), both in 2013: The first focused on current understanding and attitudes of hepatologists, surgeons, immunologists and pathologists toward liver allograft AMR; the second queried centers regarding C4d staining methods, reliability and interpretation for liver allografts on FFPE sections. Banff-participating and other larger international OLTx centers were targeted as key opinion leaders, but participants were encouraged to disseminate the survey.

2.2. Tissue microarray production

Failed allografts are required to obtain enough tissue to create a TMA for multicentre analysis; diagnostic biopsies, after clinical assessment do not provide enough tissue. Unfortunately, liver allografts that failed from AMR are sparse, often historical, lacking complete solid phase HLA DSA testing and having no matched fresh frozen tissue for comparison to “gold standard” IF. A 33-plug tissue microarray (TMA) using FFPE tissues was developed and included peri-hilar and peripheral plugs of 5 liver allografts from sensitized recipients which failed within 1-month post-transplant, with a strong suspicion of acute AMR as the cause of graft failure. Details of the OLTx histological, immunology and clinical features are shown in Table 1. As controls, the TMAs included 5 non-transplant (native) hearts, 5 native kidneys, 5 native livers (with a peri-hilar and peripheral plug from each), 2 cardiac AMR cases and 6 kidney AMR cases. Native organs with an immunological disease process and allografts without AMR were avoided because: a) simultaneous donor specific antibody (DSA) was felt to be necessary; and b) a complement deposition role in other diseases has not been thoroughly investigated, especially for livers. Two kidney AMR cases were excluded because of extensive necrosis of the plug, which made comparisons unreliable. Chronic AMR was not considered.

2.3. Tissue microarray staining and scoring

Sixty-eight TMA unstained sections were mailed to 31 centers: 2 sections were sent to 25 centers and 3 sections to 6 centres that used 2 methods for C4d staining. TMAs were then stained using

local C4d method(-s), scored locally using a centrally-devised scoring template with instructions, between 33 and 38 local scores were received for each plug. Slides were returned to the University of Pittsburgh Medical Center for creation of digital whole slide images (WSI). Forty stained slides from 31 centers (13 North America, 13 Europe, 2 Japan, 2 Australia and 1 South America) were assigned an anonymous TMA number and converted to WSI.

All WSIs were semi-quantitatively scored by 7 central pathologists blinded to any meta-data. Each TMA plug was assessed for background staining (0 no background, 1 some background that does not; or 2 high background that does, interfere with interpretation). Heart interstitial capillaries and kidney peritubular and glomerular capillaries were graded from 0-3 based on a combination of distribution and intensity: 0 no staining, 1 equivocal staining, 2 weak focal staining and 3 moderate/strong diffuse staining.

Eight separate liver compartments were scored separately for distribution and intensity: portal vein (PV), portal capillary (PC), hepatic artery (HA), sinusoids (SIN) and central vein (CV) endothelium, and portal and central/perivenular stroma. Distribution was scored as 0: no staining; 1: <10%; 2: 10-50%; and 3: >50% structures stain positively. Intensity was scored as: 0 no staining; 1: weak; 2: moderate; and 3: strong staining.

Local pathologists (25 at 1 center, 5 at 2 and 2 at 3), also blinded to meta-data, scored 32 TMAs using glass slides. Median values were used for >1 local pathologist. Hepatocyte cytoplasmic staining was ignored.

2.4. Collection of methodology used

Each center provided detailed C4d staining methodology including fixative, manual or automated (company) staining method; method of antigen retrieval (heat or enzyme); antigen retrieval pH; endogenous peroxidase blocking; wash buffer; and primary and secondary sources (suppliers and catalogue numbers) and concentrations.

2.5. Determining compartmental specificity of C4d in acute liver allograft AMR?

Staining distribution and intensity were compared between peripheral and peri-hilar plugs to determine if staining intensity decreased peripherally via absorption.

2.6. Selection of the “best” TMA staining method

The peripheral plugs of OLTx 1 and 2, which had the strongest and most diffuse staining by the majority of centers (figure 1) were used in the first step. For each compartment (PV, PC, HA, SIN and CV) the methods that showed strong and diffuse staining with no background were identified. Of these, only the subset that adequately stained all compartments were determined. Methods that also showed staining of structures in OLTx 3, 4 or 5 with weak background without interference with interpretation were added. These “best” methods showed the most sensitive and specific staining.

2.7. Do the methods that stain liver also work on kidney and heart?

Median scores for each positive AMR plug was then assessed for each TMAs identified as “best” by assessment of the liver plugs.

2.8. Statistical analysis

Statistical analysis was performed using SPSS version 22 (IBM analytics). A Kruskal-Wallis test was used to assess distribution or intensity differences between any OLTx plugs or native liver plugs for each TMA. A Mann-Whitney tests was used to assess differences between native and Tx plugs. Wilcoxon Rank sum test assessed difference between the peri-hilar and peripheral plugs. Within peripheral plugs a Friedman test assayed for differences in both distribution and intensity of staining throughout the vascular tree. Statistical analysis of kappa values was performed using STATA v10, StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP, on the best stained TMA (30) and best liver Tx plug (OLTx1), between central reviewers.

3. RESULTS:

3.1. General internet AMR survey (Survey 1)

Fifty-six participants (35 pathologists, 12 hepatologists, 7 surgeons and 2 immunologists) from 41 centers (performing a median range of 76-100 OLTx/year from a minimum <25 to a maximum >175)

in at least 13 countries (17 North America, 17 Europe, 2 Australia, 1 Japan, 1 South America and 3 unstated country) responded to survey #1.

Ninety-one percent of responders (51/56) considered evidence supporting occurrence of liver allograft AMR at least moderately convincing, but only 46% thought AMR was definitely a cause of graft injury. Forty-nine percent (20/41) routinely performed some testing for anti-donor antibodies pre-transplant: 4/41 (10%) directly tested for HLA DSA while 10/41 (24%) used crossmatch testing to monitor anti-donor reactivity and 6/41 (15%) used both methods. Fifteen (15/41; 36%) centers did no testing. No center prophylactically treats recipients based on preformed DSA results regardless of testing methods. However, in up to 50%, DSA results influence management when there is no other cause of graft dysfunction and other evidence of AMR. Post-Tx protocol DSA testing is uncommon (7% by protocol and 10% commonly if unexplained graft dysfunction) with about half the centers only rarely testing for DSA in the event of unexplained graft dysfunction. Despite this most centers (76%) have made an acute AMR diagnosis and 75% believe current acute liver allograft AMR criteria are adequate: suspicious histology; positive C4d; appropriate clinical setting; proven DSA; and exclusion of other possible causes.

The majority 38/41 (93%) of centers conduct liver allograft biopsy C4d staining, at least occasionally, mostly 20/38 (53%) to further investigate otherwise unexplained graft dysfunction; 11/38 (29%) perform on all cases where the histology is suspicious and 6/38 (16%) perform routinely. Most centers 30/41 (73%) perform C4d on FFPE sections only.

3.2. Pathologist survey (Survey 2)

15 pathologists representing 12 centers (median number transplants: 76-100) undertook survey 2 and 6 other pathologists from different centers provided some extra relevant information in the first survey included here (North America (n:8); Europe (n:7); Japan (n:1); Australia (n:1). One pathologist/center used only C4d IF and was excluded.

Participating laboratories had experience with C4d immunostaining in other, non-liver, allografts: 11/13 (85%) also stained kidney, 9/13 (69%) heart, 5/13 (38%) lung, 5/13 (38%) pancreas and 1/13

(8%) intestine Tx biopsies. Most 9 (69%) labs used the same C4d staining method in livers as other organs, but 2 used IF on kidney and heart. Most centers (35/37, 95%) used FFPE sections for liver C4d staining, but 5 also used frozen section, and 2 use only frozen sections. Five centers did not answer or did not know. Nine of 15 (60%) pathologists assess at least 1 other organ.

Pathologists were uncertain about expected C4d staining patterns during acute AMR: 9/14 (64%) considered PC, 8/14 (57%) PV, 2/14 (14%) PS, 3/14 (21%) HA, 7/14 (50%) SIN, 3/14 (21%) CV positive in acute AMR. Nobody considered hepatocyte cytoplasmic staining to be an acute AMR feature, as this occurs in any necrotic cell as a result of opsonisation of dead/dying cells by CRP with activation of complement [21,22, 23] (Figure 2). Despite a majority of centers employing C4d immunostaining only one of fourteen (7%) pathologists was satisfied with the results; 7 (50%) were dissatisfied with sensitivity compared to other organs and/or compared to histological suspicion. The majority 10 (71%) felt uneasy evaluating the C4d stain because of a lack of correlative DSA data and/or infrequency of use.

3.3. TMA results

3.3.1. Background staining

The vast majority (39/40) TMAs, had weak or no background that did not interfere with interpretation.

3.3.2. Native control liver plugs

No staining of any structures in any TMA method was seen in peripheral native plugs yielding a median score of "0". Occasional "false positives" were detected in peri-hilar native plugs: 1) weak focal PV staining in one TMA with high background; 2) weak focal PC staining in multiple TMAs, as assessed by two central scorers; 3) HA staining in three native livers in many TMAs; 4) weak portal stromal staining in four native plugs in many TMAs. Sinusoids, CVs and central stroma were occasionally missing from peri-hilar plugs, but when present were negative.

3.3.3. Peri-hilar versus peripheral staining and native versus transplant staining

There was significantly more diffuse and intense staining of OLTx than native liver plugs for all structures in peripheral and peri-hilar plugs, except for portal and central stroma in the latter.

Peri-hilar and peripheral Tx plug staining for each structure was similar: there was significant, but minimally (same median values), more diffuse and intense staining of peri-hilar plugs than peripheral plugs for PV (median score 1), PC (median score 1), HA (median score 0) and portal stromal (median score 0) staining for both distribution and intensity ($p < 0.001$ for all).

3.3.4. Peripheral plugs

Within peripheral plugs, staining detected diminishes along the flow of blood ($p < 0.001$) with the greatest staining in portal vascular structures (PV, PC and HA) compared to more peripheral vascular structures (sinusoids and CVs). OLTx plug staining varied in intensities and distributions (Figure 1A), as expected. OLTx1 and OLTx2 showed the strongest and most diffuse staining, particularly of PVs, PC and HA endothelium (not elastic lamina; Figure 3). Staining variability existed among different methods (Figure 1B): some methods working well and others not working, at all, even on plugs that stained strongest at other centers.

All 40 TMAs showed diffuse (100%) and 38/40 (95%) strong (grade 3) PV staining in OLTx1 (Figure 4a); the remaining 2 showing moderate intensity staining. Most (84%) showed diffuse and at least moderate 31/40 (77.5%) C4d staining of OLTx2, with 15/40 (37.5%) strong. Two TMAs failed to detect C4d staining in OLTx2, 1 showed weak/suboptimal staining for both intensity and distribution and 5 showed weak, but at least moderately diffuse, staining and one plug had fallen off the slide. Two TMAs showed staining of PVs in OLTx 4, with no staining detected by any TMA for OLTx 3 or 5, however, half of the OLTx3 plugs had fallen off the slide. Median central pathologist PV scores were similar with a kappa value of 0.78 (good) for distribution and 1 (very good) for intensity.

PC showed diffuse and strong staining (Figure 4b) in 38 of 40 (95%) TMAs with the remaining 2 showing diffuse moderate staining on OLTx1. For OLTx2 1 plug had fallen off, 37 of 39 (94.9%) TMAs showed diffuse (>50%) staining of OLTx2, 2/39 moderately diffuse (10-50%) and 1 focal (<10%) staining, with 35/39 (89.7%) showing at least moderate staining. One TMA showed suboptimal

staining and 2 weak staining of OLTx2. Two (5%) TMAs show weak and focal staining of OLTx5, but also had weak background staining. There was no staining of OLTx3 or 4.

Individual central pathologist PC scores showed a kappa value of 0.62 (good) for distribution and 0.81 (very good) for intensity.

HA endothelium staining was at least moderately diffuse (10-50%) and moderately intense by all methods in OLTx1 compared with 32/39 (82%) at least moderately diffuse and intense in OLTx2. One method did not stain OLTx2, 6 were suboptimal and 1 plug had fallen off. Two methods stained HAs in OLTx3 and 1 in OLTx5, none showed any staining of OLTx4. Two central pathologists “underscored” the HA compartment compared to the other 5 with kappa values of 0.39 (fair) for both distribution and intensity.

No portal stromal staining was detected by any method of OLTx1 or 4, however, 7 methods showed generally weak and focal portal stromal staining in OLTx2, 2 of OLTx3 and 1 of OLTx5.

Sinusoidal staining was detected in OLTx1 and OLTx2 (Figure 5 and 6), but was weaker and less diffuse compared to portal structures (Figure 1). Seven TMAs showed sinusoidal staining in both OLTx1 and 2 and 13 more showed sinusoidal staining in either OLTx1 or 2. Central pathologists tended to cluster into 2 groups (Figure 6): 3 detected sinusoidal staining and 4 did not. Therefore, kappa agreement values were 0.29 (fair) and 0.3 (fair) for distribution and intensity respectively.

Reassessing the data using only the 3 central pathologists who identified sinusoidal staining showed that all TMAs displayed sinusoidal staining in OLTx1 and 2 with a median of 1 (<10%) for distribution and median of 2 (moderate) for intensity of staining. Although 56% of OLTx3 sections could not be assessed, sinusoidal staining was detected in 24.5% of the remaining plugs. Two TMAs (8 & 14) were the strongest: median of 2 for distribution and 1 and 2 for intensity, respectively. Of note PV and PC staining was not detected in OLTx3 (Figure 4).

CV endothelial staining (Figure 3) was seen in 7 TMAs in OLTx1: median distribution (focal <10%) and intensity (weak). No CV staining was detected in the other 4 Tx plugs. Central pathologist CV scoring

variability showed a kappa value of 0.44 (moderate agreement) for distribution and 0.63 (good agreement) for intensity. No central stromal staining was detected by any method or any scorer.

Hepatocyte cytoplasmic staining was not graded due to a unanimous agreement that cytoplasmic staining was a non-specific feature of necrosis, but hepatocyte surface membranous C4d was seen in some AMR livers (Figure 7).

3.3.5. Comparison of local and central scores

Central and local pathologist (median scores) generally agreed (Table 2). When variances occurred, local pathologist scores drifted higher, especially for sinusoidal scoring: 25.3% higher, 2.5% lower compared to central pathologist medians. Of the total of 1920 different scores for the 5 peripheral liver plugs for PV, PC, HA, SIN and CV for the 32 TMAs: local scores were higher in 271 (14.2%) and lower in 79 (4.1%). HA showed similar rates of increased and decreased scores (11.9% v 10.9%).

3.3.6. Best methods and performance in kidney and heart

Six methods (TMAs 10, 16, 20, 30, 37 and 39) showed the strongest and most diffuse staining combined with the cleanest background of OLTx1 and 2 and with staining of structures within other liver Tx plugs (Figure 8 and Table 3). Results showed equal numbers of high and low pH of antigen retrieval; Cell Marque is the most common primary antibody. Of the two deemed “best” based on the strongest staining of more structures (TMA10 and TMA 30) TMA 10 is fully automated whilst TMA 30 included a manual antigen retrieval step, which is not ideal for a clinical lab.

Conceding difficulties with sinusoidal staining and interpretation, median scores were reassessed using only 3 pathologists detecting sinusoidal staining. Three, TMAs 8, 14 and 39 (also included in the above list), were best for sinusoidal staining and TMA 37 shows broader staining of PV and PC.

C4d staining correctly identified the AMR-positive heart controls (median of 3 for both intensity and distribution) with minimal background staining. The four “best” liver methods (TMA 14, 16, 20 and 37) did not perform optimally on the heart with false negative staining of positive controls, the

remainder (TMA 8, 10, 30, 39) worked well with strong diffuse staining of positive controls. TMA 30 was the cleanest with no equivocal staining of the native plugs.

Only 6 methods showed weak focal or moderate diffuse staining of PTCs (TMA 8, 10, 14, 17, 24, 25) and 3 (TMA 10, 14, 16) weak focal or moderate diffuse of glomerular capillaries, with 2 showing moderate/strong staining of both (TMA 10, 14). Both of these worked well on liver. The remaining 6 “best” liver methods are suboptimal for kidney with false negatives.

Overall, TMA 10 (Table 3) produced the “best” results for detecting AMR in kidney, liver, and heart allografts.

4. DISCUSSION

This study accomplished its intended goals, it: 1) elicited views on acute liver allograft AMR with the majority of centers agreeing that acute AMR has the potential to damage liver allografts; 2) showed FFPE C4d IHC is able to detect specific staining for acute liver allograft AMR in gold standard cases; 3) identified C4d staining methods and structures that can be used to standardize the diagnosis. In addition, for many pathologists involved this was the first unequivocal liver allograft C4d staining they had seen. It also identified several centers in need of C4d staining method improvements.

Uncertainty about a diagnosis of acute AMR [24] is likely attributable to a combination of factors including rarity of occurrence, inattention to portal microvascular changes, and lack of C4d staining and DSAs testing on a regular basis. However, indication C4d staining is substantially more widespread than post-Tx DSA testing (93% versus 61%) to investigate otherwise unexplained graft dysfunction. Protocol C4d staining of all biopsies (16%) is also more widespread than protocol DSA testing (7%), both well-short of kidney and heart Tx practices [20].

IF on frozen tissue is the gold standard for staining because antigens are not altered by fixation rendering the approach more sensitive than IP on FFPE liver sections [14,15]. We identified that most (79%) liver centers do not routinely collect a second biopsy fragment for freezing, and FFPE sections are also used for C4d staining in other organs at the majority of centers. Whilst 3 – 4 mm of

tissue could be diverted to IF studies from needle cores > 2.0 cm length without compromising H&E diagnosis, this necessitates the tissue arriving unfixed which may not always happen, thus a method that works on FFPE is desirable.

This study showed that IP on FFPE is able to detect gold standard acute AMR cases, however, less florid cases might be missed. The staining pattern can be different between IF and IP with the latter showing predominantly portal microvascular endothelial staining and less sensitive sinusoidal staining [15]. In addition, intensely positive C4d staining might be more difficult to detect in “old” cases stored in paraffin blocks for > 10 years, particularly with the current use of automated platforms as OLTx cases 3-5 were historically C4d positive. Portal stromal C4d staining, a feature of severe AMR in ABO-incompatible OLTx [25], is often present in AMR in ABO compatible OLTx, as previously suggested [26,27], perhaps related to usually more severe damage seen in ABO-I grafts. Most acute liver AMR cases with strong and diffuse PV and PC staining also showed weak patchy sinusoidal C4d staining. One case, however, showed a sinusoidal-predominant staining pattern, similar to that described by Bellamy [27]. Perhaps a different antigenic target (non-HLA DSA) is responsible for the sinusoidal staining. Sinusoidal staining also seems more difficult for pathologists to recognize as evidenced by the lower kappa values, compared to the PV and PC staining. However, it should still be assessed, routinely, along with all other endothelial and stromal cells compartments. Hepatocyte membranous staining detected in some liver AMR plugs might represent anti-class I DSA or non-HLA DSA.

Strong and diffuse smaller portal tract PV and PC endothelial C4d staining in peripheral core needle biopsies, recognized equally-well by local and central pathologists, clearly distinguished acute liver allograft AMR plugs from native livers. Staining in larger portal tracts should be interpreted with caution as occasional “false” positives were seen in most structures in peri-hilar native plugs. Decreased staining intensity from the proximal to distal liver circulation suggests that the large liver mass endothelial surface absorbs circulating antibody and staining becomes weaker distally,

consistent with increased risk for acute AMR in reduced-sized grafts [28]. The abrupt transition between PV and PC C4d positivity and weak sinusoidal may relate to absorption of immune complexes and antibody via Fc receptors by both Kupffer and liver sinusoidal endothelial cells [29] which may contribute to liver allograft AMR resistance and protection of simultaneous kidneys and heart allografts from the same donor [30,31]. HA C4d staining might be weaker than the PV and PC because of higher pressures and faster arterial flows [32] that interfere with DSA-antigen binding.

Only one C4d staining method, TMA 10, worked well on all organs tested, suggesting that most multi-organ centers should modify their technique. Antigen retrieval pH was found to be dependent on the local approach with both high and low pH acceptable in different protocols and no single primary anti-C4d antibody source stood out. Only a few centers were alerted to the need to improve their local C4d staining method.

A study weakness is that primarily only “gold standard” acute OLTx AMR cases, all obtained from liver allografts that failed within 1 month, were included. However, this purposeful design ensured a majority of major centers would be able to detect such cases. C4d staining of the AMR cases lacked apparent sensitivity suggesting that false positive staining requiring a native control with an immunological disease process and liver allografts that have failed from non-AMR rejection (something difficult to prove with the historic lack of DSA data) was not an issue. Following optimisation of staining techniques further studies of other immunological processes occurring within liver allografts eg recurrent or de novo viral hepatitis and recurrent autoimmune diseases are warranted to rule out any potential source of positive staining which would result in false positive staining for AMR. Chronic AMR with waxing and waning DSA and C4d staining needs further study including histological criteria refinement [33].

5. CONCLUSIONS

The concept of acute liver allografts AMR is widely accepted and C4d IHC staining of FFPE liver biopsies appears to be a suitable substitute for detecting gold standard cases. Small portal tract PV

and PC endothelial cell C4d staining are the most sensitive and reliable target structures, and sinusoidal staining can occur in isolation. Further multicentre TMA studies based on failed allografts with contemporaneously proven DSAs, using current solid phase assays, with ideally both fresh frozen and FFPE tissue to be able to compare IF and IP would be useful to confirm and refine these findings, however such samples are sparse.

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FIGURE AND TABLE LEGENDS

Figure 1: Comparison of Native and Transplant C4d staining and the influence of method used

A. Median central pathologist Complement fragment 4d (C4d) intensity (int) and distribution (dist) staining scores for all liver plugs for all methods. OLTx 1 and 2 plugs had stronger and more diffuse staining portal microvascular endothelial cell staining. No staining was detectable in peripheral native livers. **B.** Staining differences among methods (centers) on different liver structures: some methods (centers) were suboptimal or failed to work in comparison to others. Colour codes for each of the 40 tissue micro arrays (TMAs) (sites/centers) shown below the graph.

Figure 2: Ischemic coagulative necrosis.

C4d immunostained section showing geographic areas of ischemic coagulative necrosis staining with C4d. The staining in necrotic hepatocytes is cytoplasmic.

Figure 3: Portal and Central C4d staining.

Liver transplant plug showing strong and diffuse Complement fragment 4d (C4d) staining of portal vein and capillary staining within a portal tract (PT) and weaker staining of central vein (CV) endothelial cells.

Figure 4: Median scores of central scorers for portal veins and capillaries.

Central scorer median for **A.** Portal vein (PV); and **B.** Portal capillaries (PC) staining in each liver transplant plug by each method. Transplant (Tx) 1 and Tx2 show diffuse and strong PV staining by all, but two, methods whilst staining is not detected in Tx3 and 4 and only 2 methods detect staining in Tx5. Tx1 and 2 show PC staining by most methods whilst staining is not detected in Tx3 and 4 and only 2 methods detect staining in Tx5. Colour codes for each of the 40 tissue microarrays (TMAs) shown below the graft. Distribution (dist) and intensity (int).

Figure 5. Sinusoidal C4d staining

There is linear staining of sinusoidal endothelium of a liver transplant plug with patchy intrasinusoidal serum staining.

Figure 6: Median scores of central scorers for sinusoids

A. Central scorer medians for sinusoidal staining for each liver transplant plug by each method. Many methods fail to detect sinusoidal staining in Transplant (Tx)1 and 2. Colour codes for each of the 40 tissue microarrays (TMAs) shown below the graft. Distribution (dist). Intensity (int) **B.** Median scores for distribution and intensity of sinusoidal staining for each central scorer for each liver plug. Three central scorers detected sinusoidal Complement fragment 4d (C4d).

Figure 7. Hepatocyte C4d staining

Membranous hepatocyte staining (red arrows) was seen in some liver transplant plugs with negative hepatocytes also seen (green arrows). Focal sinusoidal (blue arrow) is also seen. Strong staining of portal capillaries is seen within a portal tract (PT).

Figure 8: Best methods

Graphs showing the central scorer medians for the 6 best methods as judged by central scorers. These methods showed the strongest and cleanest staining of the majority of compartments, predominantly of Transplant (Tx)1 and Tx2. Distribution (dist). Intensity (int).

Table 1: Immunological details of the failed liver allograft plugs, further clinical details and histological features supportive of AMR (antibody mediated rejection). Microvasculitis (portal or sinusoidal) refers to the accumulation of inflammatory cells within the vessel with or without swelling of the endothelial cells. C4d Complement fragment 4d; DSA donor specific antibody; MHC major histocompatibility complex; HAT hepatic artery thrombosis; AHG anti-human globulin; DTT diethiothreitol. * Modified AMOS method involves the addition of washing steps after the initial incubation of donor cells with the patient serum prior to the addition of complement described in Transplantation 1969; 7(3): 220–223.

Table 2: The number and percent of local reads for both intensity or distribution for each structure that are either increased (↑) or decreased (↓) compared to the median score of the central pathologists for the 32 TMAs where at least one local pathologists scored the TMA. There are a total

of 320 reads for each structure (32 TMAs with 5 peripheral plugs and 2 reads / plug (distribution and intensity)). The local pathologists tended towards the higher end of the central pathologists range of scores. PV portal vein; PC portal capillary; HA hepatic artery; SIN sinusoid; CV central vein.

Table 3: 8 methods were identified as the best out of the 40 TMAs for staining of acute AMR in liver allografts, of these only TMA 10 works well on liver, kidney and heart.

Manufacturer details: Ventana Medical Systems Inc. A member of the Roche Group, Basel, Switzerland; Leica Biosystems, Buffalo Grove, Illinois; Dako, Santa Clara, California; Cell Marque, Rocklin, California; DB Biotech, Kosice, Slovakia; AbD Serotech part of Bio Rad Laboratories Inc, Hercules, California; ALPCO, Salem, New Hampshire; Biomedica Medizinprodukte, Vienna, Austria

SUPPORTING INFORMATION

1. **Survey 1:** The questions asked in the first survey to gauge current understanding and attitudes of hepatologists, surgeons, immunologists and pathologists toward liver allograft AMR.
2. **Survey 2:** The questions asked in the 2nd survey to pathologists utilising formalin fixed paraffin embedded sections for C4d staining about method, assessment and reliability of staining.

TABLE 1. IMMUNOLOGICAL AND CLINICAL DETAILS OF LIVER TRANSPLANT PLUGS

OLTx number	Day post-Tx failed	Antibody detection method	Further comment	Histologic supportiv
1	17	Pre-solid phase era. 99% panel reactive antibody, complement dependent cytotoxicity +ve, B cell flow crossmatch +ve	Combined liver/kidney transplant. Kidney is KTx1. Both organs failed from AMR	Portal mi ductular r microvasc
2	18	DSA +ve for MHC class I and II. Sum mean fluorescence intensity 18000	HAT present	Portal mi venulitis
3	23	Pre-solid phase era. Strong +ve B cell crossmatch using modified AMOS method* before transplantation	AMR felt to be at least partially responsible at the time of allograft failure with	Portal mi ductular r positive s (2000)
4	24	Pre-solid phase era. Negative T and B cell crossmatch using modified AMOS method* at the time of transplantation, but B cell crossmatch became strongly positive several days before allograft failure	HAT present, AMR felt to be at least partially responsible; (2003)	lymphocy microvasc ductular r microvasc
5	30	Pre-solid phase era. Strong positive T cell crossmatch using AHG and DTT before transplantation	AMR felt to be at least partially responsible at the time of allograft failure	Arteritis, microvasc prominen portal oe sinusoida C4d posit time (200

TABLE 2.

LOCAL VERSUS CENTRAL MEDIAN SCORES FOR PERIPHERAL LIVER TRANSPLANT PLUGS

	PV		PC		HA		SIN		CV	
	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓
NUMBER	63	11	42	14	38	35	81	8	47	17
PERCENT	19.7%	3.4%	13.1%	4.4%	11.9%	10.9%	25.3%	2.5%	14.7%	4%

TABLE 3.

DETAILS OF THE “BEST” METHODS FOR C4D STAINING IN LIVER ANTIBODY MEDIATED REJECTION

TMA	Manual step	pH antigen retrieval	Platform	1° Antibody	Company	Concentration	Detection
8	No	high	Ventana Benchmark Ultra	Polyclonal (rabbit)	Cell Marque	Prediluted	Ultraview
10	No	high	Leica Bond III	Cocktail monoclonal (SP91) + polyclonal (rabbit)	Cell Marque	Both 1:50	Bond polymer refine
12	No	High	Dako PT link	Clonal (rabbit)	DB Biotech	1:4000	Envision
14	No	Low	Ventana XT	Clonal (rabbit)	DB biotech	1:100	Ultraview
16	No	Low	Leica BondMax ER1	Clonal (rabbit)	DB Biotech	1:100	Bond polymer refine
30	Yes (waterbath antigen retrieval)	Low	Dako PT link	Monoclonal (SP91)	Cell Marque	Neat	Envision
37	No	Low	Ventana XT Benchmark	Polyclonal (rabbit)	AbD serotec	1:15	Ultraview
39	No	High	Ventana	Polyclonal (rabbit)	ALPCO/ Biomedica	1:50	Ultraview

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Figure 6

A

B

Pathologists scores for sinusoidal staining

Figure 6

Figure 7

Figure 7

Figure 8

Figure 8

HIGHLIGHTS

- C4d detects liver antibody mediated rejection in formalin fixed sections
- C4d predominantly stains portal veins and capillaries in formalin fixed tissues
- C4d methods do not work equally across all transplanted organs
- The most sensitive C4d staining methods which work across all organs are provided

A

C4d staining in peripheral transplant and native plugs

B

Influence of method on staining of peripheral transplant plugs

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 28 29 30 31 32 33 34 35 36 37 38 39 40

Figure 1

Figure 2

Figure 3

Portal Vein C4d Staining

A

Portal Capillary C4d staining

B

Figure 4

Figure 5

A

Sinusoidal C4d Staining

B

Pathologists scores for sinusoidal staining

Distribution

Intensity

Figure 6

Figure 7

TMA 10

TMA 16

TMA 30

TMA 37

TMA 39

Figure 8