

Supporting Information.

Supporting Material and Methods.

Liver tissue RNA extraction and processing

Total RNA was extracted according to AllPrep® DNA/RNA/Protein Mini protocol (QIAGEN). The samples of liver tissue homogenized in 2 steps, first in bead-milling disruption using Tissue Lyser and second using QIAshredder spin column. For isolation intact DNA, RNA and proteins the homogenization was preparing in buffer RLT, which inactivates DNases and RNases as well as proteases. The quality and quantity were assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies) and Nanodrop ND-1000, respectively. The RNA was then reverse transcribed into cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Gene lists were taken from previous publications and the published results of the Molecular Microscope System from the Alberta Transplant Applied Genomic Centre. (<http://atagc.med.ualberta.ca/Research/GeneLists/Pages/default.aspx>)¹⁻⁵ and supplemented with further genes associated with immune regulation (Table S1).

A preamplification of cDNA over 14 cycles was performed using pooled TaqMan Assays and the TaqMan PreAmp Master Mix following manufacturer's protocol. RT-PCR was performed using the 48.48 and 96.96 Dynamic Array following manufacturer's protocol using a BioMark (Fluidigm Corporation, CA, USA). To quantify transcript levels, target gene Ct values were normalized using Ct values of GAPDH, GUSB, POLR2A as reference genes to generate $-\Delta Ct$ values.

Liver biopsy specimens

Liver biopsies were performed percutaneously under local anesthesia. An approximately 5 mm portion of the needle biopsy was immediately preserved in Allprotect Tissue Reagent (QIAGEN) and then stored at -80° C. The remaining cylinder was fixed in 4% neutral buffered formalin and embedded in paraffin wax.

Supplemental Tables:

Table S1: Histological characteristics of liver biopsies with available intrahepatic gene expression analysis.

		NHR		cTCMR		subTCMR		DSA+ subTCMR		DSA - subTCMR	
		Median (range)	N	Median (range)	N	Median (range)	N	Median (range)	N	Median (range)	N
RAI	total	1 (0-1)	25	4 (3-6)	16	3 (3-6)	36	3.5 (3-6)	8	3.0 (3-6)	20
	portal	1 (0-1)	25	2 (1-2)	16	1 (1-3)	36	1.5 (1-2)	8	1.0 (1-3)	20
	venous endothelial	0 (-)	25	1 (1-2)	16	1 (1-2)	36	1.0 (1-2)	8	1.0 (1-2)	20
	bile duct	0 (0-1)	25	1 (1-2)	16	1 (1-2)	36	1.0 (1-2)	8	1.0 (1-2)	20
Ishak hepatitis activity index	total	1 (0-2)	25	4 (2-9)	15	2.5 (0-7)	36	3.0 (1-6)	8	2.0 (0-7)	20
	A (interface hepatitis)	0 (-)	25	1 (0-3)	15	0 (0-2)	36	0.5 (0-2)	8	0.0 (0-2)	20
	B (confluent necrosis)	0 (-)	25	0 (0-2)	15	0 (-)	36	0.0 (-)	8	0.0 (-)	20
	C (lobular inflammation)	0 (0-1)	25	1 (1-2)	15	1 (0-2)	36	1.0 (0-2)	8	0.5 (0-2)	20
	D (portal inflammation)	0 (0-1)	25	2 (1-3)	15	1 (0-3)	36	2.0 (1-3)	8	1.0 (0-3)	20
Central perivenulitis		0 (0-1)	25	0 (0-2)	14	0 (0-2)	33	1.0 (0-2)	8	0.0 (0-1)	20
Portal microvasculitis		0 (0-1)	25	0 (0-1)	14	0 (0-2)	33	1.0 (0-1)	6	0.0 (0-2)	19
Ductular reaction		0 (0-2)	25	1 (0-2)	14	1 (0-2)	33	1.0 (0-2)	6	1.0 (0-2)	19
Nodular regenerative hyperplasia		0 (0-1)	25	0 (0-2)	14	0 (0-1)	33	0.0 (0-1)	6	0.0 (0-1)	19
NAS	total	0 (0-2)	25	0 (0-1)	16	0 (0-5)	35	0.0 (-)	7	0.0 (-)	20
	steatosis	0 (0-1)	25	0 (0-1)	16	0 (0-2)	35	0.0 (-)	7	0.0 (-)	20
	ballooning	0 (-)	25	0 (-)	16	0 (0-2)	35	0.0 (-)	7	0.0 (-)	20
	lobular inflammation	0 (0-1)	25	0 (-)	16	0 (0-1)	35	0.0 (-)	7	0.0 (-)	20

Ishak fibrosis stage	F (periportal fibrosis)	0 (0-1)	25	1 (0-2)	16	1 (0-4)	35	1.0 (0-4)	8	1.0 (0-3)	20
Liver allograft fibrosis score	total	0 (0-3)	25	1 (0-2)	15	1 (0-8)	36	2.0 (0-8)	8	1.0 (0-4)	20
	portal tract fibrosis	0 (0-1)	25	1 (0-2)	15	1 (0-3)	36	1.0 (0-3)	8	1.0 (0-3)	20
	sinusoidal fibrosis	0 (0-1)	25	0 (0-1)	15	0 (0-2)	36	0.0 (0-2)	8	0.0 (0-1)	20
	perivenular fibrosis	0 (0-1)	25	0 (0-1)	15	0 (0-3)	36	0.0 (0-3)	8	0.0 (0-1)	20

Table S2: Gene sets for the intrahepatic gene expression analysis.

Rejection markers (RM) ¹		Endothelial cells markers (ECM) ¹		Immunoregulation and iron homeostasis markers (IM)		T cell exhaustion markers (TCM) according to McKinney et al. ³		Spontaneous operational tolerance markers (SOTM) according to Bohne et al. ²	
Gene symbol	Official name	Gene symbol	Official name	Gene symbol	Official name	Gene symbol	Official name	Gene symbol	Official name
ABCB1	ATP binding cassette subfamily B member 1	ADCY4	adenylate cyclase 4	BATF	basic leucine zipper ATF-like transcription factor	BCL2	BCL2, apoptosis regulator	CDHR2	cadherin related family member 2
ANXA2	annexin A2	CDH5	cadherin 5, type 2	CD3E	CD3e molecule, epsilon	BTLA	B and T lymphocyte associated	MIF	macrophage migration inhibitory factor
CCL19	C-C motif chemokine ligand 19	COL4A1	collagen, type IV, alpha 1	CD40LG	CD40 ligand	CD86	CD86 molecule	PEBP1	phosphatidylethanolamine binding protein 1
CD2	CD2 molecule	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	CD274	CD274 molecule	CD160	CD160 molecule	SOCS1	suppressor of cytokine signaling 1
CD8A	CD8a molecule	HSPG2	heparan sulfate proteoglycan 2	FOXP3	forkhead box P3	CD244	CD244 molecule, natural killer cell receptor 2B4	TFRC	transferrin receptor
CD19	CD19 molecule	IGFBP7	insulin-like growth factor binding protein 7	FTH1	ferritin heavy chain 1	CTLA4	cytotoxic T-lymphocyte-associated protein 4		
CD34	CD34 molecule	KMT2C	lysine methyltransferase 2C; also known as MLL3	GATA3	GATA binding protein 3	IL7R	interleukin 7 receptor		
CD52	CD52 molecule	LAMB1	laminin, beta 1	HAMP	hepcidin antimicrobial peptide	KAT2B	lysine acetyltransferase 2B		
CD68	CD68 molecule	MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	HAVCR2	hepatitis A virus cellular receptor 2; also known as TIM3	KLRC1	killer cell lectin like receptor C1		
CD83	CD83 molecule	MSL3	male-specific lethal 3	HGF	hepatocyte growth factor	KLRG1	killer cell lectin like receptor G1		
CXCL8	C-X-C motif chemokine ligand 8; also known as IL8	OPN3	opsin 3	IFNG	interferon gamma	LAG3	lymphocyte-activation gene 3		
CXCL9	chemokine (C-X-C motif) ligand 9	PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	IL2	interleukin 2	LILRB4	leukocyte immunoglobulin like receptor B4		
CXCL10	chemokine (C-X-C motif) ligand 10	RGS5	regulator of G-protein signaling 5	IL6	interleukin 6	PDCD1	programmed cell death 1		
DHRS9	dehydrogenase/reductase (SDR family) member 9	SELP	selectin P	IL10	interleukin 10	PTGER2	prostaglandin E receptor 2		
FABP5	fatty acid binding protein 5	S1PR1	sphingosine-1-phosphate receptor 1	IL17A	interleukin 17A	TNFRSF9	TNF receptor superfamily member 9		

GBP2	guanylate binding protein 2, interferon-inducible	TRPV2	transient receptor potential cation channel, subfamily V, member 2	LRRC32	leucine rich repeat containing 32; also known as GARP
GPNMB	glycoprotein (transmembrane) nmb			RORC	RAR related orphan receptor C
GZMB	granzyme B			TBX21	T-box 21
HLA-DMA	major histocompatibility complex, class II, DM alpha				
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1				
HLA-DRA	major histocompatibility complex, class II, DR alpha				
HLA-F	major histocompatibility complex, class I, F				
IL18BP	interleukin 18 binding protein				
IL32	interleukin 32				
IRF1	interferon regulatory factor 1				
ITM2A	integral membrane protein 2A				
LYZ	lysozyme (renal amyloidosis)				
MMP9	matrix metalloproteinase 9				
PARVG	parvin gamma				
PLA2G7	phospholipase A2 group VII				
RFX5	regulatory factor X5				
SLC1A3	solute carrier family 1 member 3				
STAT1	signal transducer and activator of transcription 1				
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)				
TGFB1	transforming growth factor beta 1				
TK1	thymidine kinase 1				
TOP2A	topoisomerase (DNA) II alpha				
TYMS	thymidylate synthetase				
UBD	ubiquitin D				

Table S3: Transcripts with differential expression in grafts with NHR, subTCMR and cTCMR (Figure 2B).

Gene sets (Table S2)	Gene symbol	p value	FDR
ECM	MSL3	0.00998	0.02109
	S1PR1	0.00154	0.00495
	TRPV2	0.00305	0.00782
IM	BATF	0.03434	0.06206
	CD274	0.02883	0.05432
	CD3E	0.01416	0.02862
	FOXP3	0.00236	0.00666
	HAVCR2	0.00075	0.00248
	IFNG	0.00311	0.00782
	IL10	0.00022	0.00099
	IL17A	0.00987	0.02109
	LRRC32	0.01041	0.02151
	RORC	<0.00001	<0.00001
RM	ANXA2	0.00054	0.00192
	CCL19	<0.00001	0.00027
	CD19	0.02532	0.04907
	CD2	0.00020	0.00099
	CD52	0.04522	0.07787
	CD68	0.00230	0.00666
	CD8A	0.00040	0.00157
	CXCL10	<0.00001	<0.00001
	CXCL9	<0.00001	<0.00001
	DHRS9	<0.00001	<0.00001
	GBP2	0.00024	0.00102
	GPNMB	0.00254	0.00674
	GZMB	<0.00001	0.00046
	HLA-DMA	<0.00001	0.00037
	HLA-DRA	<0.00001	<0.00001
	HLA-F	0.03470	0.06206
	IL18BP	0.00022	0.00099
	IL32	0.02056	0.04069
	IL8	<0.00001	<0.00001
	ITM2A	0.03600	0.06318
	LYZ	0.00745	0.01689
	MMP9	0.00518	0.01236
	PARVG	0.00044	0.00162
	PLA2G7	<0.00001	<0.00001
	SLC1A3	0.02920	0.05432
	STAT1	<0.00001	0.00043
	TAP1	0.00208	0.00624

	TK1	<0.00001	<0.00001
	TOP2A	<0.00001	<0.00001
	TYMS	0.00012	0.00073
	UBD	<0.00001	<0.00001
SOTM	SOCS1	0.00954	0.02109
	TFRC	0.00037	0.00150
TCEM	BTLA	0.00056	0.00192
	CD244	0.00502	0.01227
	CTLA4	0.00013	0.00073
	IL7R	0.00542	0.01261
	LAG3	0.00168	0.00522
	PDCD1	0.00022	0.00099
	PTGER2	0.00016	0.00089
	TNFRSF9	0.00251	0.00674

Transcripts from the green cluster are highlighted in green. All other transcripts belong to the red cluster (Fig. 2B)

Table S4: Transcripts with differential expression in grafts DSA+ and DSA-subTCMR (Figure 4B).

Gene sets (Table S2)	Gene symbol	p value	FDR
ECM	ENPP2	0.00734	0.03844
	MFNG	0.00708	0.03844
	TRPV2	0.00734	0.03844
IM	BATF	0.01299	0.04611
	CD3E	0.00282	0.02382
	CD40LG	0.03202	0.08757
	IFNG	0.00335	0.02397
	IL6	0.02117	0.06562
	TBX21	0.00047	0.02167
RM	CCL19	0.01278	0.04611
	CD19	<0.00001	0.00155
	CD52	0.00096	0.02382
	CD68	0.01339	0.04611
	CD83	0.02570	0.07710
	CD8A	0.00141	0.02382
	CXCL10	0.00260	0.02382
	CXCL9	0.00257	0.02382
	DHRS9	0.00756	0.03844
	GBP2	0.00216	0.02382
	GPNMB	0.01619	0.05378
	GZMB	0.02090	0.06562

	HLA-DRA	0.01069	0.04518
	IL18BP	0.00746	0.03844
	IRF1	0.01011	0.04479
	ITM2A	0.01322	0.04611
	MMP9	0.00204	0.02382
	PARVG	0.00320	0.02397
	UBD	0.00870	0.04045
TCM	BTLA	0.00174	0.02382
	CD160	0.00785	0.03844
	CD244	0.00128	0.02382
	CD86	0.01229	0.04611
	LAG3	0.03009	0.08479
	PTGER2	0.02892	0.08404

Table S5: Transcripts with differential expression in grafts with NHR, DSA-subTMCR, DSA+ subTCMR and cTCMR (Figure 4C).

Gene sets (Table S2)	Gene symbol	p value	FDR
ECM	MSL3	0.00057	0.00188
	S1PR1	0.00104	0.00294
	TRPV2	0.00541	0.01259
IM	BATF	0.01194	0.02415
	CD274	0.00951	0.02087
	CD3E	0.01327	0.02571
	FOXP3	0.00428	0.01020
	HAVCR2	0.00029	0.00105
	IFNG	0.00421	0.01020
	IL10	<0.00001	0.00026
	IL17A	0.01146	0.02369
	LRR32	0.01796	0.03274
	RORC	<0.00001	<0.00001
RM	ANXA2	0.00025	0.00096
	CCL19	<0.00001	0.00029
	CD19	0.02646	0.04557
	CD2	0.00016	0.00076
	CD52	0.02949	0.04898
	CD68	0.00056	0.00188
	CD8A	0.00029	0.00105
	CXCL10	<0.00001	<0.00001
	CXCL9	<0.00001	<0.00001
	DHRS9	<0.00001	<0.00001
	GBP2	<0.00001	0.00013
	GPNMB	0.00374	0.00967
	GZMB	<0.00001	0.00029
	HLA-DMA	<0.00001	0.00029

	HLA-DRA	<0.00001	<0.00001
	HLA-F	0.01859	0.03324
	IL18BP	<0.00001	0.00013
	IL32	0.01932	0.03390
	IL8	<0.00001	<0.00001
	ITM2A	0.00984	0.02087
	LYZ	0.00081	0.00236
	MMP9	0.00559	0.01268
	PARVG	0.00015	0.00071
	PLA2G7	<0.00001	<0.00001
	SLC1A3	0.01231	0.02436
	STAT1	<0.00001	<0.00001
	TAP1	0.00187	0.00511
	TK1	<0.00001	<0.00001
	TOP2A	<0.00001	<0.00001
	TYMS	0.00024	0.00096
	UBD	<0.00001	<0.00001
SOTM	CDHR2	0.02793	0.04724
	PEBP1	0.01447	0.02691
	SOCS1	0.01381	0.02622
	TFRC	0.00067	0.00211
TCEM	BTLA	0.00069	0.00211
	CD244	0.00387	0.00972
	CTLA4	0.00023	0.00096
	IL7R	0.00988	0.02087
	LAG3	0.00279	0.00740
	PDCD1	0.00023	0.00096
	PTGER2	<0.00001	0.00033
	TNFRSF9	0.00070	0.00211

Supplemental Figure Legends

Figure S1: Histological phenotype of patient cohorts.

The histological phenotype of representative examples for each patient cohort used in this study (NHR= no histological rejection; subTCMR= subclinical T cell-mediated rejection; cTCMR= clinical T cell-mediated rejection) are outlined as 40x magnification of portal tracts as well as liver lobules (upper row) and 100x magnification (lower row) of portal tracts for the grading with the rejection activity index (RAI).

Figure S2: Longitudinal histological assessment of NHR, subTCMR and cTCMR.

Longitudinal assessment of histological markers of liver inflammation and fibrosis in various compartments in the latest available follow-up biopsy (last Bx) after the first biopsy with NHR (n =19; time from first NHR biopsy to last follow-up biopsy: median (range): 30 (5-79) months; Wilcoxon test for paired samples), subTCMR (n=33; time from first subTCMR biopsy to last follow-up biopsy: median (range): 39 (7-72) months) and cTCMR (n=24; time from first cTCMR biopsy to last follow-up biopsy: median (range): 20 (2-96) months). *p <0.05, **p <0.01, and n.s.: not significant p ≥0.05.

Figure S3: Causes for liver transplantation of liver biopsies for intrahepatic gene expression analysis.

Causes for liver transplantation in the same cohort. (AILD: autoimmune liver disease; ALF: acute liver failure; HBV: Hepatitis B virus infection; HCC: hepatocellular carcinoma; PLD: polycystic liver disease)

Figure S4: Causes for liver transplantation in subTCMR.

Causes for liver transplantation in subTCMR with available testing for DSA are depicted. Further clinical data are listed in Table 3. (AILD: autoimmune liver disease; ALF: acute liver failure; HBV: Hepatitis B virus infection; HCC: hepatocellular carcinoma; PLD: polycystic liver disease)

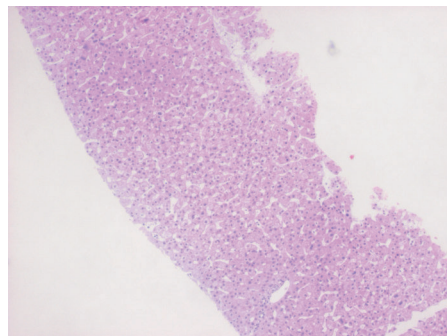
References

1. Taubert R, Danger R, Londono MC, et al. Hepatic Infiltrates in Operational Tolerant Patients After Liver Transplantation Show Enrichment of Regulatory T Cells Before Proinflammatory Genes Are Downregulated. *Am J Transplant* 2016;16(4):1285-1293.
2. Bohne F, Martinez-Llordella M, Lozano JJ, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. *J Clin Invest*. 2012;122(1):368-382.

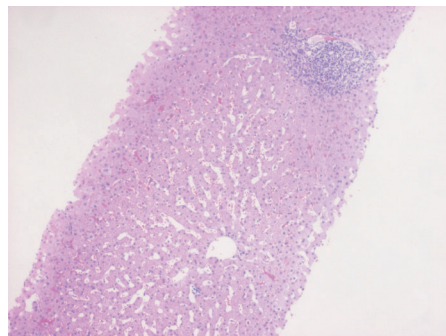
3. McKinney EF, Lee JC, Jayne DR, et al. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature*. 2015;523(7562):612-616.
4. Bonaccorsi-Riani E, Pennycuik A, Londono MC, et al. Molecular Characterization of Acute Cellular Rejection Occurring During Intentional Immunosuppression Withdrawal in Liver Transplantation. *Am J Transplant*. 2016;16(2):484-496.
5. Sis B, Jhangri GS, Bunnag S, et al. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. *Am J Transplant*. 2009;9(10):2312-2323.

Figure S1

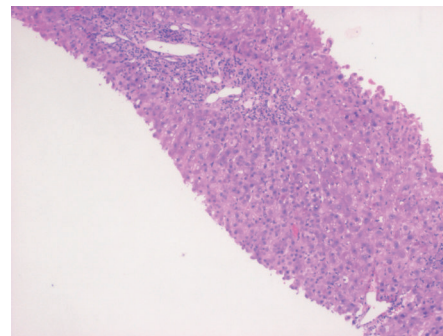
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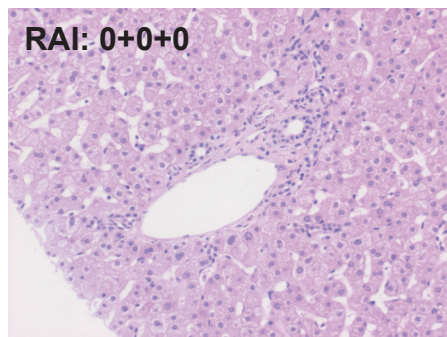
subTCMR



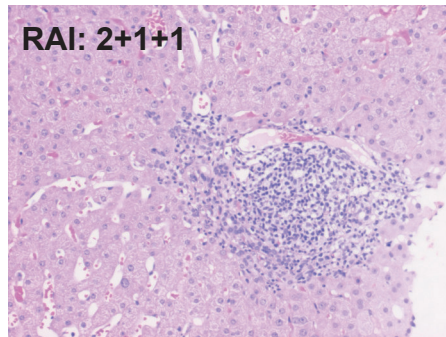
cTCMR



RAI: 0+0+0



RAI: 2+1+1



RAI: 2+2+1

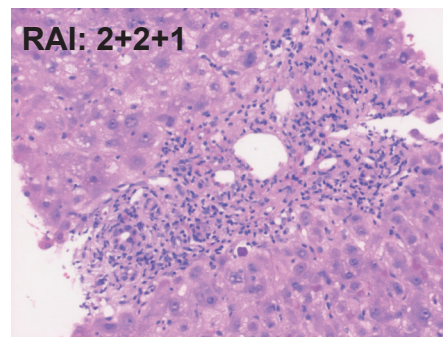


Figure S2

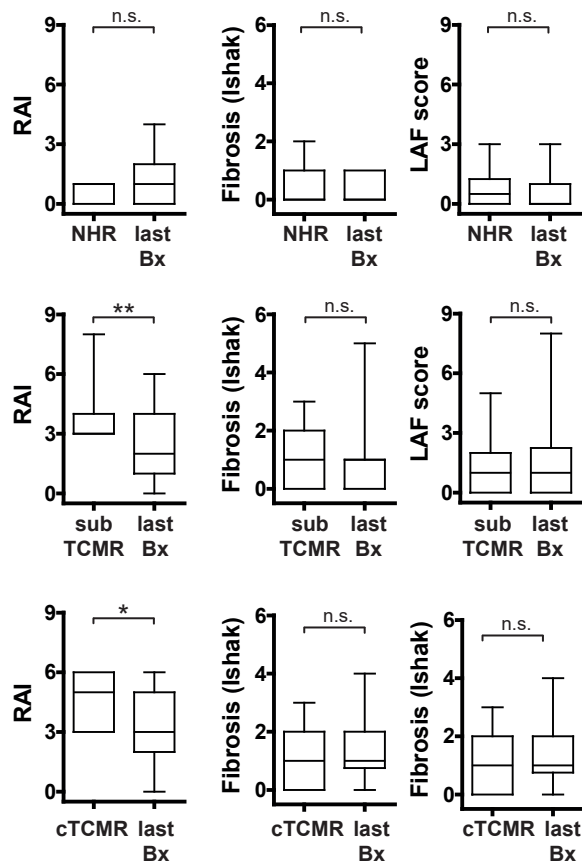


Figure S3

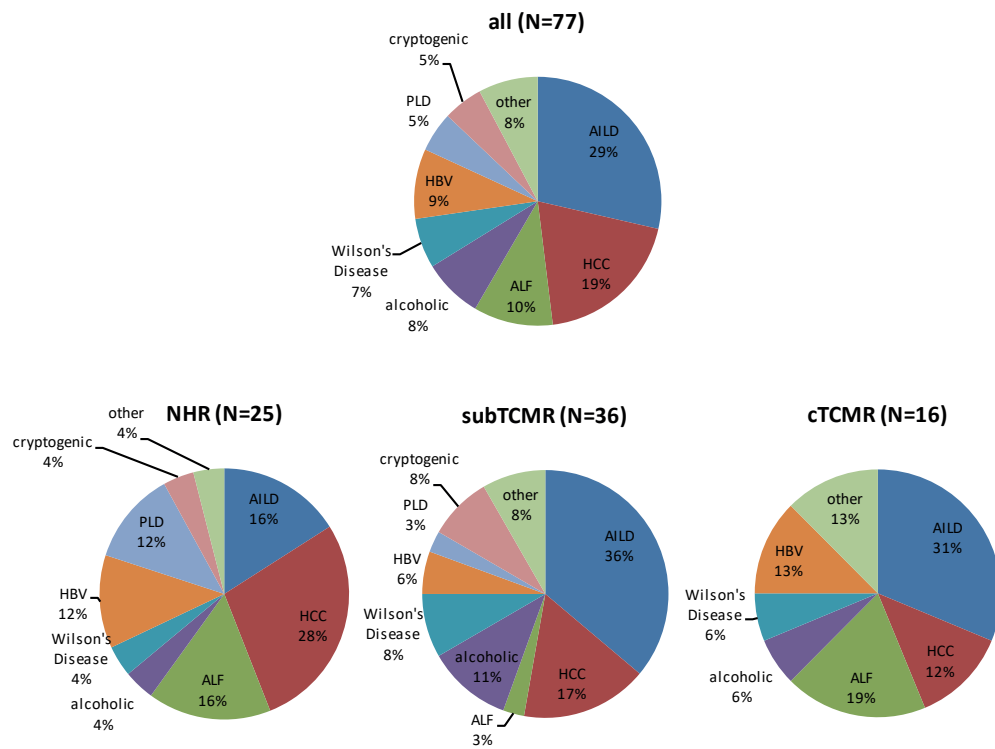


Figure S4

