

# World Journal of *Hepatology*

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2014-2017

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**ORIGINAL ARTICLE****Retrospective Study**

- 1115** T-cell allorecognition of donor glutathione S-transferase T1 in plasma cell-rich rejection

*Martínez-Bravo JM, Sánchez B, Sousa JM, Acevedo MJ, Gómez-Bravo MA, Núñez-Roldán A, Aguilera I*

**Prospective Study**

- 1125** Evaluation of Doppler-ultrasonography in the diagnosis of transjugular intrahepatic portosystemic shunt dysfunction: A prospective study

*Nicolas C, Le Gouge A, d'Alteroche L, Ayoub J, Georgescu M, Vidal V, Castaing D, Cercueil JP, Chevallier P, Roumy J, Trillaud H, Boyer L, Le Pennec V, Perret C, Giraudeau B, Perarnau JM; STIC-TIPS group*

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Retrospective Study

# T-cell allorecognition of donor glutathione S-transferase T1 in plasma cell-rich rejection

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## Abstract

### AIM

To investigate the role of glutathione S-transferase T1 donor-specific T lymphocytes in plasma cell-rich rejection of liver allografts.

### METHODS

The study group included 22 liver transplant patients. Among them, 18 patients were mismatched for the glutathione S-transferase T1 (GSTT1) alleles (don+/rec-), and 4 were matched (don+/rec+). Seven of the mismatched patients produced anti-GSTT1 antibodies and developed plasma cell-rich rejection (former *de novo* immune hepatitis). For the detection of specific T

lymphocytes, peripheral blood mononuclear cells were collected and stored in liquid nitrogen. The memory T cell response was studied by adding to the cell cultures to a mix of 39 custom-made, 15-mer overlapping peptides, which covered the entire GSTT1 amino acid sequence. The specific cellular response to peptides was analyzed by flow cytometry using the markers CD8, CD4, IL-4 and IFN $\gamma$ .

## RESULTS

Activation of CD8<sup>+</sup> T cells with different peptides was observed exclusively in the group of patients with plasma-cell rich rejection (3 out of 7), with production of IL-4 and/or IFN $\gamma$  at a rate of 1%-4.92% depending on the peptides. The CD4<sup>+</sup> response was most common and not exclusive for patients with the disease, where 5 out of 7 showed percentages of activated cells from 1.24% to 31.34%. Additionally, two patients without the disease but with the mismatch had cells that became stimulated with some peptides (1.45%-5.18%). Highly unexpected was the finding of a double positive CD4<sup>+</sup>CD8<sup>low</sup> T cell population that showed the highest degree of activation with some of the peptides in 7 patients with the mismatch, in 4 patients with plasma cell-rich rejection and in 3 patients without the disease. Unfortunately, CD4<sup>+</sup>CD8<sup>low</sup> cells represent 1% of the total number of lymphocytes, and stimulation could not be analyzed in 9 patients due to the low number of gated cells. Cells from the 4 patients included as controls did not show activation with any of the peptides.

## CONCLUSION

Patients with GSTT1 mismatch can develop a specific T-cell response, but the potential role of this response in the pathogenesis of plasma cell-rich rejection is unknown.

**Key words:** Donor-specific glutathione S-transferase T1 antibodies; Indirect presentation; Glutathione S-transferase T1-memory T cells; *De novo* immune hepatitis; Donor/recipient mismatch

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**Core tip:** In solid organ transplants, donor recipient mismatch of glutathione S-transferase T1 (GSTT1) alleles triggers a specific immune response with the production of IgG antibodies. In a proportion of mismatched liver and kidney transplants, the clinical outcome is rejection. However, detection of GSTT1-specific T lymphocytes has not been documented. We provide the first evidence of T cells able to become activated by GSTT1 peptides in patients who develop plasma cell-rich (PC-rich) rejection after GSTT1-mismatch liver transplantation. Interestingly, not only CD8<sup>+</sup> or CD4<sup>+</sup> cells but also double positive CD4<sup>+</sup>CD8<sup>low</sup> cells reacted to the antigenic stimulation *in vitro*.

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## INTRODUCTION

In the context of liver transplantation, both glutathione S-transferase T1 (GSTT1) mismatch and the presence of GSTT1 antibodies have been associated with the development of *de novo* immune hepatitis<sup>[1-3]</sup>, recently accepted as a rejection of the liver allograft in which allogeneic hepatocytes that express GSTT1 constitutively in their cytoplasm are the main targets of the immune response. The Banff Working Group on Liver Allograft Pathology has recently updated the terminologies of post-transplant complications and encourages the use of "plasma cell-rich rejection" instead of the former "*de novo* autoimmune hepatitis"<sup>[4]</sup>. Therefore, in this manuscript, we will use the new terminology.

Plasma cell-rich (PC-rich) rejection is a liver disorder of unclear pathogenesis that is usually diagnosed within the first two years after liver transplantation. A common feature of all the patients diagnosed in our hospital is the presence of GSTT1 antibodies due to the recognition of GSTT1 as a foreign antigen expressed in the graft when the recipient lacks this gene. Although it is a very specific anti-donor response, it is unclear whether these antibodies have a pathogenic effect since some patients with sustained antibody-titers will never develop PC-rich rejection.

Pregnancy, transfusion and transplantation are circumstances where the host immune system is able to recognize foreign major and minor histocompatibility antigens. This is the case for GSTT1, a drug metabolizing enzyme that is abundantly expressed in the liver and kidney. Recipients who lack this gene (*GSTT1*\*0/0) might generate antibodies against GSTT1 after blood transfusion and/or organ transplantation from GSTT1-positive donors (*GSTT1*\*A/0 or \*A/A)<sup>[5,6]</sup>. It has been reported that the GSTT1 protein is able to induce a memory B cell response in *GSTT1*\*0/0 women after pregnancy with GSTT1-positive offspring<sup>[6]</sup>. Moreover, it has been demonstrated that GSTT1-specific plasma cells are quickly activated when a GSTT1-positive patient receives an infusion of hematopoietic cells from an HLA-identical sensitized donor<sup>[7]</sup>.

The liver is a very special organ with a variety of important cell types able to function as APCs. Hepatocytes, which represent 60% of the liver cells, express MHC class I at low levels and have the ability to serve as antigen presenting cells (APCs). Furthermore, under some pathological circumstances in a pro-inflammatory environment, parenchymal cells and biliary epithelial cells can express MHC class II antigens<sup>[8]</sup>. Some studies in mouse models

have indicated that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells can independently initiate hepatocyte rejection, more rapidly in the case of CD8<sup>+</sup> cells, somehow preceding the CD4<sup>+</sup> mediated response<sup>[9]</sup>. In humans, patients with chronic allograft failure of kidney grafts have significantly higher frequencies of CD4<sup>+</sup> T cells indirectly activated by allogeneic peptides when compared with controls, whereas CD4<sup>+</sup> T cells activated in a direct manner reduced the cytotoxic T cell response<sup>[10]</sup>. However, there are variables such as immunosuppression therapy that can alter the immunological response in different ways.

In this study, we aim to explore the role of T cells in the context of PC-rich rejection. We have compared the T cell response in PBMCs collected from 18 GSTT1-mismatched liver transplant patients, 7 of which had a diagnosis of PC-rich rejection, with 4 GSTT1-matched transplanted patients after re-stimulation *in vitro* with the whole set of GSTT1 peptides. In summary, we have the first evidence of GSTT1-specific memory T cells ready to become activated after recall with the antigen, but further studies will be needed to test the potential role of these cells in the pathogenesis of PC-rich rejection.

## MATERIALS AND METHODS

### Patients

The study group included 22 liver transplant patients, 10 females and 12 males, who had transplants between June 1996 and April 2011. Eighteen of the patients lacked the GSTT1 gene and received a liver from a GSTT1 positive donor (rec-/don+). Consequently, all of them were candidates to develop a specific immune response against this foreign antigen. Four additional patients without the GSTT1 mismatch (rec+/don+) were included as a control group. Within the mismatched patients, we observed three different types of immune and clinical responses regarding the GSTT1 antigen. Group 1 consisted of 7 patients who produced anti-GSTT1 antibodies and developed PC-rich rejection. Group 2 included 2 patients who produced anti-GSTT1 antibodies but did not develop PC-rich rejection. Group 3 included 9 patients who did not produce anti-GSTT1 antibodies (which always precede clinical manifestations) and consequently did not develop the disease. Written informed consent was obtained from all of the participants, and the procedures were in accordance with the Helsinki Declaration. The study protocol was approved by the Ethics Committee of the University Hospital Virgen del Rocío, Seville, Spain. Patient characteristics are described in Table 1. Baseline immunosuppression was cyclosporine in 13 cases and tacrolimus in 9 cases, either alone or combined with mycophenolate mofetil and steroids during the first months. Cells were obtained at a mean time of 6.68 years after the transplant (1-16). Changes in the immunosuppression therapy at the time of cell extraction are described in Table 1. Six of the patients with PC-rich rejection were

also receiving prednisone as a specific treatment, and one patient was not adequately diagnosed and died in 2014.

### GSTT1 genotyping

Peripheral blood samples from the patients and their donors were collected, and genomic DNA was purified using the QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primers and conditions for the GSTT1 PCR reaction have been described in detail elsewhere<sup>[11]</sup>.

### Detection of GSTT1 antibodies

Following the manufacturer's protocol, total IgG antibodies in sera were analyzed using a commercially available ELISA, which contains the human GSTT1 recombinant protein (Biomedal, Seville, Spain).

### GSTT1 peptides

We selected 15-mer peptides that overlapped by 9 amino acids and spanned the GSTT1 protein. In total, there were 39 peptides (Table 2). Peptides were synthesized by Innovative Peptide Solutions, JPT (Berlin, Germany). Peptide purity was higher than 80%, as assayed by HPLC, and the peptide composition was verified by mass spectrometry. Lyophilized peptides were dissolved at 10 mg/mL in DMSO, aliquoted, and stored at -20 °C.

### Cell isolation and culture with the GSTT1 peptides

Post-transplant PBMCs were isolated using BD Vacutainer CPT ficoll tubes (BD Biosciences, CA, United States), frozen in FCS containing 10% DMSO, and stored in liquid nitrogen. For stimulation experiments, 3-4 × 10<sup>5</sup> cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany), Penicillin/Streptomycin (100 U penicillin/mL, 100 µg streptomycin/mL), 1 mmol/L Na-Pyruvate (Sigma Aldrich, MI, EEUU) and L-Glutamine (2 mmol/L, Irvine Scientific, Wicklow, Ireland) in the presence of 8 pools, each one containing 5 peptides and the last one containing only 4 (10 µg/mL each peptide). Next, 10 µg/mL anti-CD28/CD49d (BD Biosciences, CA, United States) was added for 48 h at 37 °C 5% CO<sub>2</sub>, and 10 µg/mL Brefeldin A was added to the samples during the last four hours (Golgi Plug: BD Biosciences). A negative control (without peptide but with the proportional amount of DMSO) and a positive activation control with 10 ng/mL PMA + 1 µg/mL ionomycin (Sigma Aldrich) were included in each assay. Pre-transplant samples were not available.

### In silico analysis of MHC-peptide binding affinity

HLA class I and II binding affinity to GSTT1 peptides was analyzed by the Immune Epitope Database (IEDB) and Analysis Resources NetMHCII/IIpan.

### Flow cytometry

Immunofluorescence staining was performed after

Table 1 Patient characteristics

Group	Patient	Gender	LT date	Original disease	Baseline IS	PBMC ex- traction date	Years after Tx	Treatment at PBMC extraction
1	1	M	06-05-99	Alcoholic cirrhosis	CYA (N), MMF, ST	12-04-12	13	CYA (N), MMF, ST
	2	F	07-05-07	Cirrhosis probably autoimmune	CYA, MMF, ST	16-04-12	5	CYA (N), MMF, ST
	3	F	02-07-00	HCV cirrhosis	CYA (N), ST, BASILISIMAB	13-03-12	12	TAC, AZA, ST
	4	F	18-09-03	Alcoholic cirrhosis	CYA, MMF, ST	09-05-12	9	TAC, MMF, ST
	5	M	02-11-01	HCV + alcoholic cirrhosis	CYA (N), ST, BASILISIMAB	14-06-12	11	MMF, ST
	6	F	27-03-09	Primary biliary cirrhosis	CYA, MMF	12-04-12	3	MMF, ST
	7	F	18-11-06	Secondary biliary cirrhosis	CYA (N), MMF, ST	08-05-12	6	CYA, MMF
2	8	M	23-11-96	HBV cirrhosis	CYA (N), MMF, ST	19-06-12	16	CYA (N), MMF
	9	F	03-06-96	Agenesis of the bile ducts	CYA, ST	21-05-12	16	TAC
3	10	M	12-7-06	Alcoholic cirrhosis + hepatocarcinoma	TAC, MMF, ST	16-04-12	6	MMF, SIR
	11	M	12-02-09	HBV cirrhosis	TAC, MMF, ST	16-04-12	3	MMF, SIR
	12	M	06-07-10	Non-alcoholic steatohepatitis	TAC (10 d) CYA, RAPA	17-04-12	2	MMF, SIR
	13	M	19-04-11	HCV cirrhosis+ hepatocarcinoma	CYA, ST	18-04-12	1	CYA, ST
	14	M	18-01-09	HCV cirrhosis	TAC, MMF, ST	02-05-12	3	TAC
	15	F	18-06-04	Alcoholic cirrhosis	TAC	07-05-12	8	MMF, EVE
	16	M	30-07-08	HCV cirrhosis	TAC, MMF, ST	08-05-12	4	MMF, EVE
	17	M	20-12-04	HCV cirrhosis	TAC, MMF, ST	22-05-12	7	TAC, MMF
	18	F	20-09-99	HCV cirrhosis	CYA, ST	18-06-12	13	CYA
4	E	F	09-03-09	Alcoholic cirrhosis	CYA, ST	21-03-12	3	CYA, MMF
	G	M	16-11-08	HCV cirrhosis	TAC, DACLIZUMAB	30-04-12	3	CYA
	J	F	22-05-10	Hepatocarcinoma	CYA, ST	02-05-12	2	CYA
	L	M	28-07-11	Primary biliary cirrhosis	TAC, MMF, ST	19-06-12	1	TAC

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

fixation and permeabilization using lysing solution (BD Biosciences, CA, United States) with the following surface and intracellular markers: Anti-human CD4-PerCP/CD8-APC/IFN $\gamma$ -FITC/IL-4-PE (Becton Dickinson BD Biosciences, CA, United States). Lymphocyte cytokine release patterns were analyzed by flow cytometry (FACSsort; BD Biosciences) using CELLQuest software. The specific cellular response to the different pools was calculated by subtracting the percentage of activation of T cells cultured without GSTT1 peptides (negative control). Typically, 50000 events were collected using FL3 (CD4PerCP-Cy5) or FL4 (CD8-APC) as a fluorescent trigger. A second set of gating was drawn to include CD8<sup>+</sup> or CD8<sup>low</sup> and IFN $\gamma$  and IL-4 expression.

## RESULTS

### Identification of memory T cell subsets specific for the GSTT1 antigen

We have categorized as positive the populations with more than 1% of activated cells. All of the patients who showed stimulation revealed a polyclonal T cell response since we observed stimulation with more than one peptide. For simplicity reasons, we have represented the highest percentage of cell activation among the positive values obtained with each peptide (Figure 1 and Table 3). The group of patients with PC-rich rejection (group 1) was the only group in which activation of CD8<sup>+</sup> T cells was detected in 3 patients, expressing IL-4, IFN $\gamma$  or both cytokines simultaneously. This group also shows the most abundant and diverse patterns of CD4<sup>+</sup> T cell activation exhibiting Th0 (IL-4/IFN $\gamma$ ), Th1 (IFN $\gamma$ ) and Th2 (IL-4) pathways, although

cellular activation is not exclusive of group 1 and was also observed in two patients included in group 3 (Table 3). The most striking result was the presence of CD4<sup>+</sup>CD8<sup>low</sup> double positive (DP) cells that seem to be enriched in GSTT1-specific cells, especially cells with a secretion profile of both cytokines tested (3.44% patient 2, 78.95% patient 3, 9.54% patient 4, 4.56% patient 5). Unfortunately, DP cells are not abundant, and only 4 of the 7 patients with PC-rich rejection could be analyzed due to the low number of double positive cells gated in the remaining 3 cases.

The patients with antibodies but without PC-rich rejection (group 2) did not show CD4<sup>+</sup> or CD8<sup>+</sup> T cell activation, whereas five of the 9 patients included in group 3, without antibodies and therefore without disease, exhibited stimulation with some peptides. Again, the higher percentages of activation occurred in the double positive CD4<sup>+</sup>CD8<sup>low</sup> cells (6.63% patient 10, 29.58% patient 17 and 43.05% patient 18), although in some cases the number of double positive CD4<sup>+</sup>CD8<sup>low</sup> cells was too low to perform further analysis (Table 3). The four patients included as the control group with recipients and donors that were matched for the GSTT1 positive allele did not become activated with any of the peptides assayed.

In summary, 12 out of 18 liver transplant recipients with the GSTT1 mismatch showed different degrees of T lymphocyte activation upon exposure to the GSTT1 peptides. Although we could not test for memory markers, the short time of stimulation (48 h) indicates that this is not a primary response but a reactivation of pre-existing GSTT1-specific lymphocytes. There are 3 cell types involved, including CD4<sup>+</sup>, CD8<sup>+</sup> and

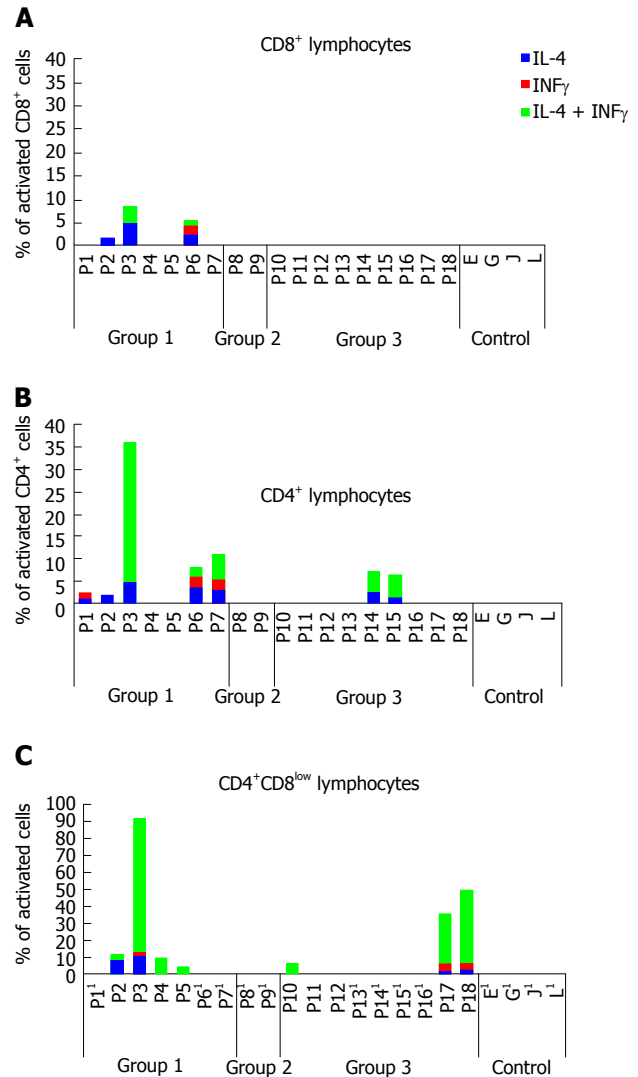
**Table 2** Glutathione S-transferase T1 peptides and amino acid position and sequence

Pool	Amino acid #	Amino acid sequence
1	1-15	MGLEYLDLLSQPCR
	7-21	LDLLSQPCRAVYIFA
	13-27	PCRAVYIFAKKNDIP
	19-33	IFAKKNDIPFELRIV
	25-39	DIPFELRIVDLIKGQ
2	31-45	RIVDLIKGQHLSDAF
	37-51	KGQHLSDAFAQVNPL
	43-57	DAFAQVNPLKKVPAL
	49-63	NPLKKVPALKDGDFT
	55-69	PALKDGDFTLTESVA
3	61-75	DFTLTESVAIILYLT
	67-81	SVAIILYLTRKYKVP
	73-87	YLTRKYKVPDYWYPQ
	79-93	KVPDYWYPQDLQARA
	85-99	YPQDLQARARVDEYL
4	91-105	ARARVDEYLAWQHTT
	97-111	EYLAWQHTTLRRSCL
	103-117	HTTLRRSCLRALWHK
	109-123	SCLRALWHKVMFPVF
	115-129	WHKVMFPVFLGEPVS
5	119-133	MFPVFLGEPVSPQTL
	125-139	GEPVSPQTLAATLAE
	131-145	QTLAATLAEALDVTILQ
	137-151	LAELDVTILQLLEDKF
	143-157	TLQLLEDKFLQNKAF
6	149-163	DKFLQNKAFITGPHI
	155-169	KAFLTGPHISLADLV
	161-175	PHISLADLVAITELM
	167-181	DLVAITELMHVPVAG
	173-187	ELMHVPVAGCQVFEG
7	179-193	GAGCQVFEGRPKLAT
	185-199	FEGRPKLATWRQRVE
	191-205	LATWRQRVEAAVGED
	197-211	RVEAAVGEDLFQEAH
	203-217	GEDLFQEAHEVILKA
8	209-223	EAHEVILKAKDFPPA
	215-229	LKAKDFPPADPTIKQ
	221-235	PPADPTIKQKLMWPV
	226-240	TIKQKLMWPVLAMIR

CD4<sup>+</sup>CD8<sup>low</sup> cells, all of them with diverse cytokine expression patterns whose role is not easy to interpret, although DP cells are known to appear in situations of long-term exposure to antigens.

#### Antigenic areas of the GSTT1 protein

When we analyzed the relative contribution of each pool to the activation of T lymphocytes in each patient, we found that pools 3 and 4 seemed especially antigenic for the DP cells, whereas pool 4 did not stimulate any of the single CD4<sup>+</sup> or CD8<sup>+</sup> T cells of any patient in which other pools seem to have a more relevant role (Figure 2). A representative plot of flow cytometry data with cells gated on CD4<sup>+</sup> first and then CD8, selecting those cells with a low expression of CD8, is shown in Figure 3. We have selected 3 patients with different degrees of activation after stimulation with the pools of peptides; the negative control (without peptide) is also shown and was subtracted to obtain the final values (Figure 3).



**Figure 1** Polyclonal response of T cells upon recall with peptides of the glutathione S-transferase T1 protein. Percentages of activation upon exposure to the different pools are represented. Three cell types showed activation at different levels. A: Only CD8<sup>+</sup> lymphocytes of patients included in group 1 showed activation. The numbers in the figure represent the highest value obtained for each pathway when activation was induced by different pools of peptides; B: Activation of CD4<sup>+</sup> lymphocytes was observed in patients of groups 1 and 3; C: CD4<sup>+</sup>CD8<sup>low</sup> cells showed the highest percentage of activation. †Indicate that the analysis could not be performed in these patients.

#### Indirect presentation pathway

These results have to be interpreted in the context of an indirect allo-recognition pathway since the experiments were performed only in the presence of recipient cells. The recipients' HLA genotypes are described in Table 4, highlighting in bold HLA class I and II alleles with the best percentile ranks for presentation of GSTT1 peptides, as concluded from the *in silico* analysis. We found a good correlation with part of the experimental results of T cell activation measured in terms of IL-4 and/or INF<sub>γ</sub> production by CD8<sup>+</sup>, CD4<sup>+</sup> and CD4<sup>+</sup>CD8<sup>low</sup> T cells upon exposure to GSTT1 peptides. However, the fact that HLA genotyping was performed by low resolution methods constitutes a limitation of the analysis. When we placed the *in silico*-proposed

**Table 3** Specific immune response after stimulation of T lymphocytes in culture with glutathione S-transferase T1 peptides<sup>1</sup>

Group	Pat #	CD8 <sup>+</sup>			CD4 <sup>+</sup>			CD4 <sup>+</sup> CD8 <sup>low</sup>		
		IL-4	IFN $\gamma$	IL-4/IFN $\gamma$	IL-4	IFN $\gamma$	IL-4/IFN $\gamma$	IL-4	IFN $\gamma$	IL-4/IFN $\gamma$
1	1	-	-	-	1.24%	1.41%	-	$\Delta$	$\Delta$	$\Delta$
	2	1.7%	-	-	2.04%	-	-	8.23%	-	3.44%
	3	4.92%	-	3.54%	4.93%	-	31.34%	10.77%	2.25%	78.95%
	4	-	-	-	-	-	-	-	-	9.54%
	5	-	-	-	-	-	-	-	-	4.56%
	6	2.36%	2.03%	1.15%	3.71%	2.45%	2.14%	$\Delta$	$\Delta$	$\Delta$
	7	-	-	-	3.19%	2.35%	5.63%	$\Delta$	$\Delta$	$\Delta$
2	8	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
	9	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
3	10	-	-	-	-	-	-	-	-	6.63%
	11	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
	14	-	-	-	2.65%	-	4.71%	$\Delta$	$\Delta$	$\Delta$
	15	-	-	-	1.45%	-	5.18%	$\Delta$	$\Delta$	$\Delta$
	16	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
	17	-	-	-	-	-	-	1.68%	4.6%	29.58%
	18	-	-	-	-	-	-	2.51%	4.13%	43.05%
	E	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
Control	G	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
	J	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$
	L	-	-	-	-	-	-	$\Delta$	$\Delta$	$\Delta$

<sup>1</sup>Pathways of activation are defined by the production of IL-4, IFN $\gamma$  or both cytokines simultaneously. “-”: No activation; “ $\Delta$ ”: Analysis was not possible due to the low number of gated cells. Group 1: don+/rec-, with Abs, with PC-rich rejection; Group 2: don+/rec-, with Abs, without PC-rich rejection; Group 3: don+/rec-, without Abs, without PC-rich rejection; Control group: don+/rec+.

**Table 4** Class I and class II human leukocyte antigen genotypes of the patients

Pat #	HLA-A*		HLA-B*		DRB1*	
1	01	03	07	57	11	15
2	01	66	08	41	03	13
3	11	29	07	35	07	13
4	02	11	51	60	04	13
5	30	-	13	18	03	07
6	02	11	35	44	07	08
7	26	29	38	44	01	03
8	23	24	14	52	01	11
9	11	68	35	44	01	14
10	02	33	14	35	01	07
11	01	29	57	61	01	04
12	01	33	44	64	01	07
13	01	-	08	18	04	07
14	29	-	44	-	07	-
15	01	30	08	51	03	07
16	02	29	39	44	07	11
17	03	32	37	44	03	12
18	03	11	14	49	07	-

peptides along the GSTT1 amino acid sequence, we were able to define very clearly a highly antigenic zone of the protein that basically shared amino acids from positions 60 to 80 (Table 5). Interestingly, the selected peptides are long, not only for HLA class II, as expected, but also for HLA class I alleles.

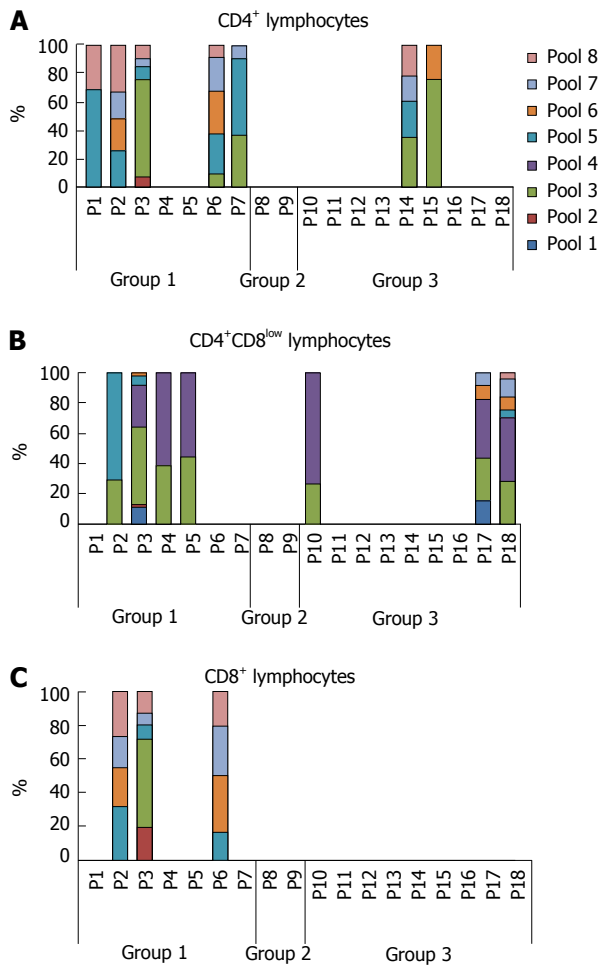
## DISCUSSION

In this study, we have demonstrated the existence of memory T cells specific for the GSTT1 antigen in

patients with PC-rich rejection after GSTT1-mismatched liver transplants. The results support our initial hypothesis in which both specific B and T cells are required to function simultaneously in the development of the immune response leading to PC-rich rejection. In fact, only patients diagnosed with the disease showed a combined T and B cell response, whereas those patients with specific T cells but lacking the humoral response never experienced this type of rejection.

The fact that GSTT1 is a drug metabolizing enzyme found in the cytoplasm of hepatocytes and cholangiocytes makes it difficult to explain a pathogenic role of anti-GSTT1 antibodies. Although it cannot be assumed that cytosolic antigens are never expressed on the cell surface<sup>[12]</sup>, the presence of antibodies in all of the patients with a diagnosis of PC-rich rejection is evidence of specific B cells capable of presentation of GSTT1 to specific T cells. B cells are known to be critical for alloreactive T cells to differentiate into memory T cells<sup>[13,14]</sup>. In fact, a very interesting report by Zeng *et al.*<sup>[15]</sup> demonstrated in an animal model of chronic allograft vasculopathy (CAV) that mice deficient in both B cells and antibodies were protected from CAV, while mice that were deficient for antibodies but not for B cells developed CAV. The conclusion was that B cells contributed to CAV by enhancing T-cell responses<sup>[15]</sup>. Very recently, Shiu *et al.*<sup>[16]</sup> demonstrated that B cells are involved in supporting T-cell responses in patients with antibody-mediated rejection in a B-cell-dependent indirect T-cell alloreactivity.

CD4<sup>+</sup> cells seem to have a predominant role in the



**Figure 2** Different areas of the glutathione S-transferase T1 protein induce different degrees of activation in patients' cells. A: The majority of CD4<sup>+</sup> T cells that showed some degree of activation recognized peptides in pools 5 (blue) and 3 (green) but never in pool 4 (purple); B: CD4<sup>+</sup>CD8<sup>low</sup> cells became stimulated almost homogeneously with in pools 3 (green) and 4 (purple); C: CD8<sup>+</sup> lymphocytes that showed activation recognized peptides in different pools but never in pool 4 (purple).

context of GSTT1 mismatch in the patients described in this study. Mouse models have provided evidence of the role of CD4<sup>+</sup> T cells acting as effectors that directly mediate injury in renal allografts, while CD8<sup>+</sup> T cells had very little influence in promoting graft dysfunction<sup>[17]</sup>. Similarly, CD4<sup>+</sup> cells were sufficient to mediate rapid rejection of a cardiac allograft through the indirect pathway of alloantigen recognition<sup>[18]</sup>. Hence, CD4<sup>+</sup> specific T cells are key elements for the progression of allograft immunity, especially within the CD4<sup>+</sup> T cell indirect response. In the liver of mice with clinical manifestations of hepatitis, MHC class II -expressing hepatocytes are able to act as APCs and activate specific CD4<sup>+</sup> T lymphocytes<sup>[19]</sup>.

The pathogenic role of GSTT1-specific CD8<sup>+</sup> T cells in PC-rich rejection has not been explored. The results obtained in this study reveal the existence of reactive CD8<sup>+</sup> cells in the group of patients with PC-rich rejection, with percentages of activation that range from 1.1% to 8.46%, which is not as

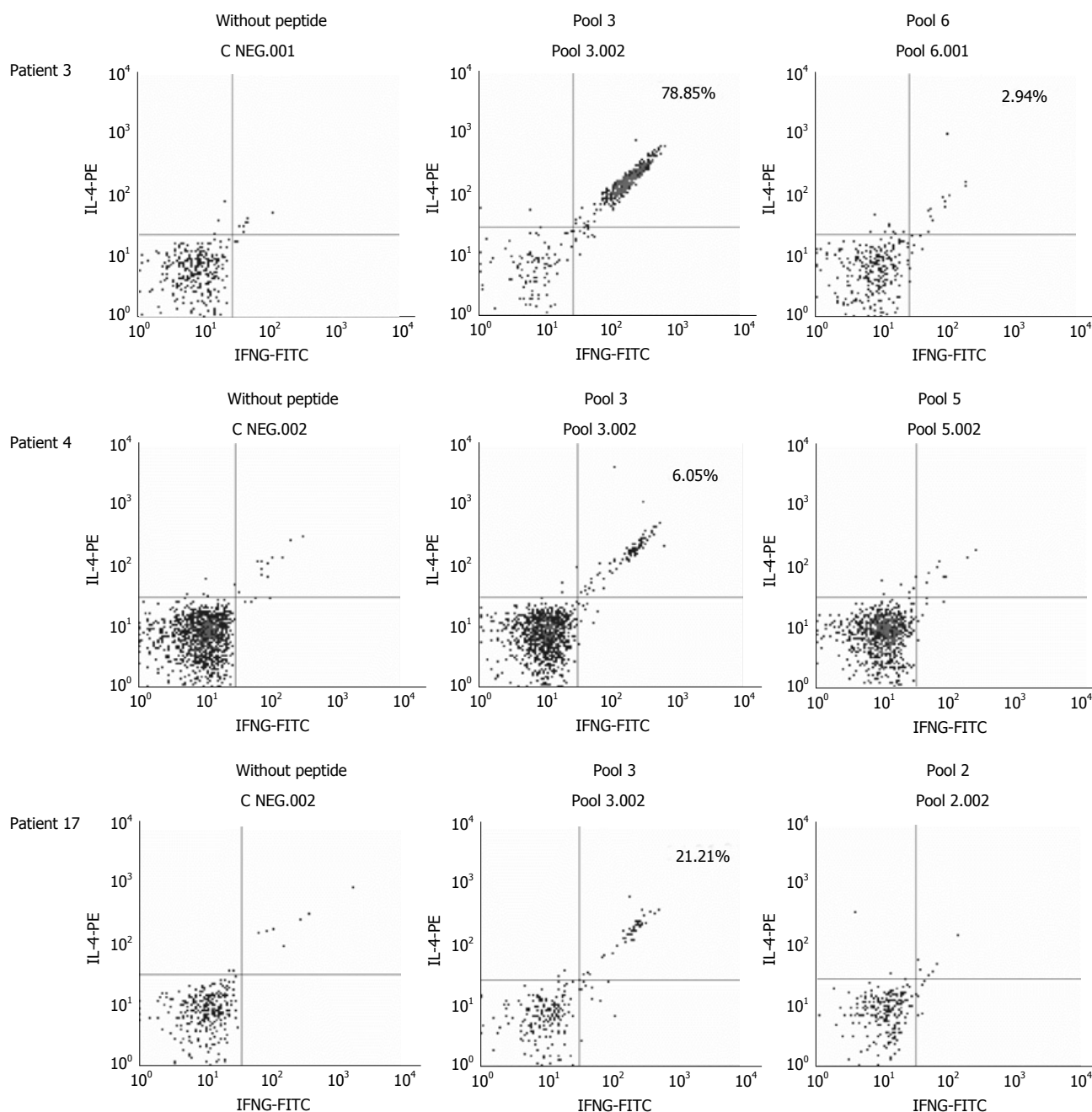
**Table 5** Identification of several regions of the glutathione S-transferase T1 protein, whose peptides would be suitable to be presented by human leukocyte antigen class I and II alleles of the patients based on *in silico* predictions

Recipient's HLA	Peptide sequence	Aa position	Length	Percentile rank
Class I				
A*01:01	FTLTESVAILLY <sup>1</sup>	62-73	12	0.1
A*02:01	YIFAKKNDIPFEL	18-30	13	0.1
	CLRALWHKVMFPV	110-122	13	0.1
	IKQKLMPWVFLAMI	227-239	13	0.1
A*03:01	SVAIIYLTRKYK <sup>1</sup>	67-79	13	0.2
A*11:01	ESVAILLYLTRK <sup>1</sup>	66-77	12	0.1
A*29:02	FTLTESVAILLY <sup>1</sup>	62-73	12	0.2
B*07:02	SPQTLAATLAEL	129-140	12	0.1
	RPKLATWRQRVEAA	188-201	14	0.2
B*08:01	FAQVNPLKKVPAL	45-57	13	0.2
	LAWQHITTLRRSCL	99-111	13	0.2
	DPTIKQKLMPWVL	224-236	13	0.2
B*35:01	YPQDLQARARVDEY	85-98	14	0.1
B*44:02	TESVAILLY <sup>1</sup>	65-73	9	0.15
	AELDVTILQL	138-146	9	0.15
Class II				
DRB1*01:03	GDFTLTESVAILLYL <sup>1</sup>	60-74	15	0.6
DRB1*07:01	ALKDGDFTLTESVAI <sup>1</sup>	56-70	15	0.4
DRB1*11:01	AIIYLTRKYKVPDY <sup>1</sup>	69-83	15	0.5
DRB1*12:01	SVAIIYLTRKYKVP <sup>1</sup>	67-81	15	0.72
DRB1*13:01	LTESVAILLYLTRKY <sup>1</sup>	64-78	15	0.17
DRB1*14:01	SVAIIYLTRKYKVP <sup>1</sup>	67-81	15	0.48
DRB1*15:01	ESVAILLYLTRKYKVP <sup>1</sup>	66-80	15	0.41

<sup>1</sup>Highly antigenic areas of the GSTT1 protein whose peptides are shared by HLA class I and II alleles. GSTT1: Glutathione S-transferase T1; HLA: Human leukocyte antigen.

low as expected in immunosuppressed patients. A substantial difference between the percentages of IFN $\gamma$ -producing CD8<sup>+</sup> T cells at diagnosis and during treatment with prednisolone has been demonstrated in patients with type 2 autoimmune hepatitis<sup>[20]</sup>. We should keep in mind that the patients with PC-rich rejection described in this study are under successful treatment with prednisone that has to be maintained throughout life. It would be very interesting to know the level of stimulation of cells obtained at diagnosis, before initiation of the treatment, since cells from immunosuppressed patients exhibit much lower levels of activation than immunocompetent cells. For that reason, it is even more remarkable that certain types of T lymphocytes from the patients with PC-rich rejection showed high percentages of activation.

The results of this study leave many questions about the function of GSTT1-specific CD4<sup>+</sup>CD8<sup>low</sup> T cells in the context of transplantation. Subgroups of CD4<sup>+</sup>CD8<sup>low</sup> T cells have been described in chronic viral infections, with antigen specificity and memory phenotype<sup>[21,22]</sup>, or in parasitic infections where the frequency of CD4<sup>+</sup>CD8<sup>low</sup> T cells was higher in Chagasic patients than in healthy donors<sup>[23]</sup>. In a study performed with human cells from CMV-seropositive patients, the CD4<sup>+</sup>CD8<sup>low</sup> population contained a two- to eight-fold higher frequency of antigen-specific IFN $\gamma$ <sup>+</sup> cells than



**Figure 3** Representative plots showing CD4<sup>+</sup>CD8<sup>low</sup> T lymphocyte activation in terms of cytokine production. Production of IL-4 and/or IFN $\gamma$  was analyzed after exposure of the cells to the glutathione S-transferase T1 peptides. As an example, patient 3 had 78.85% of the double positive cells activated with pool 3 and 2.9% with pool 6. Patient 4 had 6.05% of cells activated with pool 3, whereas pool 5 did not have any effect. Patient 17 showed 21.21% of cell activation with pool 3, but no response was observed when the cells were cultured with pool 2. In all the cases, the cells produced both cytokines, indicating a Th0 type of response.

the CD4<sup>+</sup>CD8<sup>-</sup> population<sup>[24]</sup>. It seems that this type of cell appears in chronic processes, mainly in viral infections, but this scenario could also be extended to the transplant setting where sustained expression of a foreign antigen, such as GSTT1, might lead to chronic rejection.

The terminologies used to describe post-transplant clinical situations with overlapping manifestations might be confusing. Late rejection, *de novo* autoimmune/alloimmune hepatitis or idiopathic post-transplant hepatitis may all be part of immune-mediated injury<sup>[25]</sup>. The underlying pathology of the formerly called *de*

*novo* autoimmune hepatitis was poorly understood, and diagnoses were based mainly on histological findings such as the presence of plasma cell rich infiltrates or hepatocyte rosette formation; however, because rosettes are poorly reproducible, some groups do not consider them a diagnostic feature<sup>[26]</sup>.

Although we did not have enough samples to check for memory markers, based on the short time of stimulation *in vitro* (48 h), we can say that GSTT1-specific lymphocytes are memory cells. It is still too soon to propose a model, as we have not yet tested what would be the response when recipients' cells are

confronted with GSTT1 peptides presented *via* the direct pathway. Apparently, there is not a predominant HLA class I or II allele among the donors of the patients with PC-rich rejection that could explain why some patients develop rejection and others do not. Given that donor cells are not available, in future studies we will have to design strategies to demonstrate the existence of donor HLA-restricted GSTT1-specific T lymphocytes through the use of artificial molecules such as pentamers, as well as cytotoxicity assays on “donor-like” target cells.

## COMMENTS

### Background

Antibody-mediated rejection of the liver allografts has never been considered a main problem after liver transplantation until now. The Banff Working Group on Liver Allograft pathologies published last year a new report in which the role of HLA as well as glutathione S-transferase T1 (GSTT1) donor specific antibodies is discussed. In this report, they have included new criteria and have suggested changes in the terminology of post-transplant complications. The process termed *de novo* autoimmune hepatitis is now defined as plasma cell-rich rejection.

### Research frontiers

The authors' group has studied *de novo* immune hepatitis for years. The authors identified the target antigen as a donor protein expressed in the graft but absent from the donor. A genetic mismatch between a GSTT1+ donor and a GSTT1- recipient constitutes a risk factor to produce GSTT1 antibodies and to develop PC-rich rejection (former *de novo* immune hepatitis) but the pathogenic mechanisms leading to this type of rejection are still unknown.

### Innovations and breakthroughs

The existence of T lymphocytes specific for GSTT1 in patients with PC-rich rejection has never been explored. The immune response requires collaboration between GSTT1-specific B and T lymphocytes. The hypothesis contemplated that the patients might have memory T cells able to become activated after recall with the antigenic stimulus. This is the first study in which GSTT1-specific T cells have been found in patients with PC-rich rejection in conjunction with anti-GSTT1 antibodies.

### Applications

Although ultimate diagnosis of PC-rich rejection relies on histological examination, the fact that some histological features are common to different post-transplant outcomes makes a reliable diagnosis a complicated task. Understanding the mechanisms leading to PC-rich rejection would contribute to a correct diagnosis and appropriate therapy.

### Terminology

Glutathione S-transferase T1 is a drug metabolizing enzyme highly expressed in liver and kidney.

### Peer-review

This manuscript investigated the role of GSTT1 donor-specific T lymphocytes in plasma cell-rich rejection of liver allografts in patients, and found that T cells were able to become activated by GSTT1 peptides in patients who develop plasma cell-rich rejection after GSTT1-mismatch liver transplantation. The research design and detecting methods are reasonable, data analysis is correct, writing is fluent, written informed consent was obtained from all of the participants, and the study protocol was approved.

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Prospective Study

# Evaluation of Doppler-ultrasonography in the diagnosis of transjugular intrahepatic portosystemic shunt dysfunction: A prospective study

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**Clinical trial registration statement:** This study is registered with ClinicalTrials.com. The registration identification number is 00593528.

**Informed consent statement:** All study participants provided written consent prior to study enrollment.

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**Data sharing statement:** There is no additional data available.

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## Abstract

### AIM

To prospectively evaluate the performance of Doppler-ultrasonography (US) for the detection of transjugular intrahepatic portosystemic shunt (TIPS) dysfunction within a multicenter cohort of cirrhotic patients.

### METHODS

This study was conducted in 10 french teaching hospitals. After TIPS insertion, angiography and liver Doppler-US were carried out every six months to detect dysfunction (defined by a portosystemic gradient  $\geq 12$  mmHg and/or a stent stenosis  $\geq 50\%$ ). The association between ultrasonographic signs and dysfunction was studied by logistic random-effects models, and the diagnostic performance of each Doppler criterion was estimated by the bootstrap method. This study was approved by the ethics committee of Tours.

### RESULTS

Two hundred and eighteen pairs of examinations performed on 87 cirrhotic patients were analyzed. Variables significantly associated with dysfunction were: The speed of flow in the portal vein ( $P = 0.008$ ), the reversal of flow in the right ( $P = 0.038$ ) and left ( $P = 0.049$ ) portal branch, the loss of modulation of portal flow by the right atrium ( $P = 0.0005$ ), ascites ( $P = 0.001$ ) and the overall impression of the operator ( $P = 0.0001$ ). The diagnostic performances of these variables were low; sensitivity was  $< 58\%$  and negative predictive value was  $< 73\%$ . Therefore, dysfunction cannot be ruled out from Doppler-US.

### CONCLUSION

The performance of Doppler-US for the detection of TIPS dysfunction is poor compared to angiography. New tools are needed to improve diagnosis of TIPS dysfunction.

**Key words:** Transjugular intrahepatic portosystemic

shunt; Dysfunction; Doppler-ultrasonography

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**Core tip:** This large multicentric prospective study evaluates the performance of Doppler-ultrasonography (US) for the detection of transjugular intrahepatic portosystemic shunt dysfunction within a cohort of cirrhotic patients. Although many Doppler-US variables were significantly associated with dysfunction, the diagnostic performances of these variables were low compared to angiography.

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## INTRODUCTION

Transjugular intrahepatic porto-systemic shunt (TIPS) is now routinely used for the treatment of complications of portal hypertension<sup>[1-4]</sup>. One of the main disadvantages of this technique is the frequent occurrence of stent dysfunction. Indeed, with bare-stents, a reintervention is necessary in more than half of the cases at 1 year<sup>[5-8]</sup>. Thus, strict and scheduled monitoring to search for dysfunction is usually recommended. However, the use of polytetrafluoroethylene covered stents (e-PTFE) since 2000 has improved shunt patency<sup>[9-13]</sup>. Nevertheless, shunt dysfunction can still arise in more than 25% of cases after one year with covered stents<sup>[13]</sup>.

Portography to measure portal pressure gradient is the gold-standard for the detection of TIPS dysfunction<sup>[5]</sup>; however, it is an invasive procedure which cannot be conducted routinely. Doppler ultrasonography (Doppler-US) has been proposed as an alternative to angiography. Many studies have tried to define valid criteria for shunt dysfunction<sup>[8,12,14-17]</sup> but sensitivity and specificity are very different from one study to another. Among these criteria, the velocity of the portal flow, the direction of the intrahepatic portal flow and the velocity of the flow in the shunt were the most studied, but no threshold was defined. Given the inter-individual variability of portal velocity, some authors preferred an individual criterion such as the decrease of baseline value<sup>[17]</sup>. An association of many criteria may also be more relevant<sup>[14-18]</sup> but none has been properly validated so far.

The aim of our study was to evaluate prospectively the performance of Doppler-US for the detection of

shunt dysfunction assessed by portography, in a multicentric cohort of cirrhotic patients.

## MATERIALS AND METHODS

The study protocol was approved by the ethics committee of Tours and each patient gave written consent. This study was funded by the French Ministry of Health and by the Société Nationale Française de Gastroentérologie. This study has been registered on ClinicalTrials.com under # 00593528.

### Patients

This is an ancillary study from a randomized trial comparing covered and bare stents<sup>[13]</sup>. Patients were prospectively included in the cohort between February 2008 and July 2009. Cirrhotic patients who needed a TIPS for refractory ascites, hydrothorax or to prevent variceal rebleeding and were treated in 10 French tertiary teaching hospitals were included. The inclusion criteria were: (1) age between 18 and 75 years; (2) cirrhosis previously documented on histological or typical clinical signs; (3) Child-Pugh score < C12 at inclusion; (4) affiliation to the social security system; and (5) provision of informed consent to participate in the study. The exclusion criteria were total portal thrombosis, known hepatocellular carcinoma, cardiac failure, pulmonary hypertension (MAP > 40 mmHg), hepatic polycystosis, dilatation of intrahepatic bile ducts, history of recurrent spontaneous hepatic encephalopathy (HE), hepatic arterial insufficiency, pregnancy, breastfeeding, inadequate contraception for patients of childbearing age.

### TIPS procedure

The TIPS procedure was performed with covered or bare stent randomly assigned.

**Protocol:** For each patient, a Doppler-US was performed by a radiologist working in the center which included the patient. Doppler-US was carried out before the TIPS procedure, during the days following TIPS insertion, at 1 mo, and every 3 mo thereafter up to 2 years. During this follow-up, portography with portosystemic pressure gradient measurements was scheduled every six months and was performed if dysfunction was suspected from clinical signs or ultrasound. Only Doppler-US performed the day of portography, or during the 15 d before, were compared with portography in this study.

**Dysfunction:** Shunt dysfunction was defined as an increase of portosystemic gradient  $\geq 12$  mmHg and/or a stent stenosis  $\geq 50\%$  of the lumen, during angiography. Cases of shunt stenosis without portal hypertension were examined by two independent radiologists. These radiologists were not aware of the Doppler-US results and had no practice at all with

vascular stents.

**Doppler-US variables:** Different Doppler-US variables were collected for each patient, every three months, by the same operator, on the same ultrasound unit. Patients were fasted for four hours at the time of examination: (1) flow velocity in the main portal vein and within the stent. Patients were asked to have a quiet and regular respiration, and velocities were recorded during a blockpnea. Reported result was the mean of three measurements (cm/s); (2) direction of blood flow in the intrahepatic portal vein branches. The flow was characterized as hepatopetal or hepatofugal in the left branch and the right branch; (3) portal flow modulation induced by the right atrium. The phasicity of portal blood flow was recorded and was classified as demodulated when absent vs modulated; (4) stent filling in color Doppler. The wall to wall color flow within the stent was classified as incomplete vs complete; (5) presence of ascites. Ascites was quantified as absent or moderate (peritoneal effusion in the pouch of Douglas and/or in perihepatic area) vs severe (peritoneal effusion in the abdominal cavity); (6) the relative change of the flow velocity in the main portal vein. The portal velocity was compared to the one measured at one month (considered as the baseline value). Indeed, at one month after TIPS insertion, hemodynamic disturbances are stabilized and neointimal hyperplasia within the stent is not yet significant; and (7) the conclusion of the operator. The conclusion of the physician performing the examination was also recorded (suspected dysfunction; yes or no).

**Blinding:** The Doppler-US examination was performed before the portography; therefore, it could not be influenced by it. Furthermore, angiography and Doppler-US were performed by different operators and the operator who performed angiography was unaware of the results of Doppler-US.

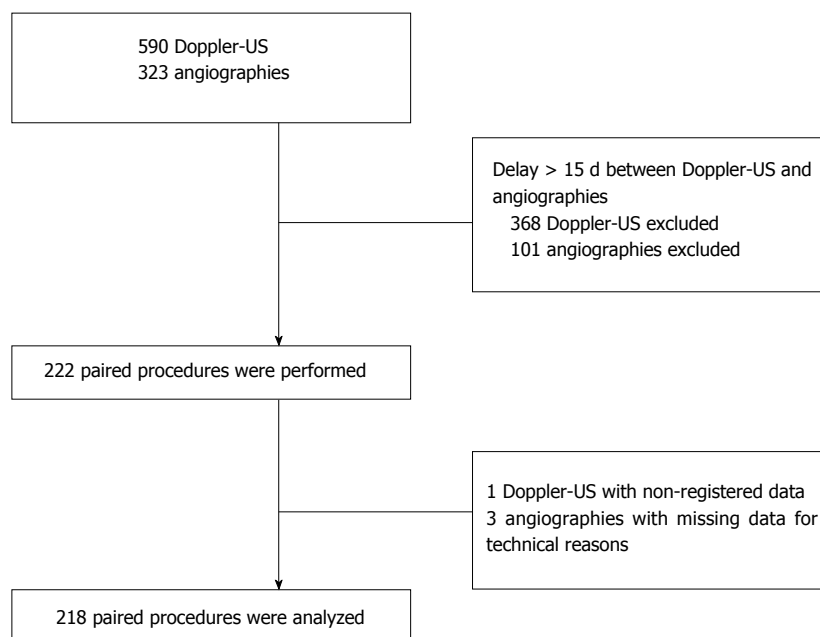
### Statistical analysis

Associations between shunt dysfunction defined by angiography and Doppler-US variables were analyzed with logistic random-effects models to account for the correlation of data (each patient had several measures).

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of binary criteria were estimated by a bootstrapping method with 95%CI. This non-parametric method uses the patient as a unit of resampling, to account for the correlation of data and avoid cluster effect.

For quantitative variables (flow velocities), the areas under the curves were estimated punctually and with a bootstrapping method<sup>[19]</sup>, with 95%CI.

Analyses were performed with SAS, version 9.2 (SAS Institute, Cary, North Carolina, United States) and R 2.12.1 (R Development Core Team. R: A language and environment for statistical computing R) by a



**Figure 1** Flowchart describing pairing procedure of Doppler-ultrasonography and angiography among the 129 patients of the initial STIC-transjugular intrahepatic portosystemic shunt cohort. Doppler and angiography were paired if they were carried out with an interval of 15 d or less. US: Ultrasonography.

biomedical statistician.

## RESULTS

### Patients

In the original study, 137 patients were included and 129 were finally analyzed<sup>[20]</sup>. Forty one patients were excluded because Doppler-US was not performed within the 15 d before portography. From these 88 patients, 222 paired Doppler-US and angiographies were selected. Some Doppler and angiography data were not registered for technical reasons. Therefore, we analyzed 218 paired Doppler-US and angiographies from 87 patients (Figure 1).

The main characteristics of the patients are presented in Table 1. The number of patients for each listed characteristic varies because of missing data. In our cohort, causes of cirrhosis were largely dominated by alcohol (81.6% vs 13.8% for viruses and 8% for NASH) and the patients were predominantly classified as Child B (68.8% vs 18.8% Child A and 12.5% Child C).

### Dysfunction

Among the 218 angiographies analyzed, 79 revealed a TIPS dysfunction in 51 patients.

Among these 79 dysfunction events, only 31 were suspected from Doppler-US, based on operator conclusions. Patency problems were detected for the first time with a median delay of 7.5 mo (6.2-18.3). The first event of dysfunction occurred in almost half of the cases (22/51)  $6 \pm 1$  mo after TIPS insertion. Among these cases, less than half (10/22) were suspected from Doppler-US.

During portography, stenosis was located in the

lower part of the stent in eight cases (20.5%), in the middle part of the stent in eight cases (20.5%), in the upper part of the stent in 16 cases (41%) and in the hepatic vein in seven cases (17.9%). Stenosis was suspected on Doppler-US in 62.5% cases (10/16) when the stenosis was located in the low or middle part of the stent, whereas it was suspected in 50% cases (8/16) when located in the upper part of the stent (Table 2).

### Doppler-US variables

The performances of each Doppler-US criterion to discriminate TIPS dysfunction are summarized in Table 3.

### Portal flow modulation

Loss of portal flow modulation induced by the right atrium was more frequent in case of dysfunction 29/65 (44.6%) vs 23/112 (20.5%) ( $P = 0.0005$ ). The sensitivity of this variable was 44.4% (Table 3).

**Intra hepatic portal flow direction:** In the right portal vein, the flow was hepatopedal in 35/61 cases of dysfunction (57.3%), whereas it was hepatopedal in 43/112 cases (38.3%) in the absence of dysfunction. This variable (hepatopedal vs hepatofugal flow) was associated with TIPS dysfunction for both right ( $P = 0.038$ ) and left branches ( $P = 0.049$ ). The sensitivity and specificity of this variable were 57.2% and 61.7%, respectively for the right branch, and 54.7% and 66.8%, respectively for the left branch (Table 3).

**Stent filling:** Stent filling was incomplete in 18/57 cases of dysfunction (31.5%) and in 15/83 cases in the absence of TIPS dysfunction (18%) ( $P = 0.155$ ).

**Table 1** Baseline characteristics of the 87 patients at enrollment

Characteristic	
Age (yr)	58.1 ± 7.6
Male	68 (78.2)
Etiology of cirrhosis	
Alcohol	71 (81.6)
Viruses	12 (13.8)
NASH	7 (8.0)
Others	2 (2.3)
TIPS indication	
Recurrent bleeding	30 (34.5)
Refractory ascites	59 (67.8)
Hydrothorax	4 (4.6)
Child-Pugh score, <i>n</i> = 86	7.8 ± 1.6
Child-Pugh score, <i>n</i> = 86	
A	18 (20.9)
B	58 (67.4)
C	10 (11.6)
MELD score	11.6 ± 3.4

Data are mean ± SD or *n* (%). MELD: Model for end-stage liver disease.

**Ascites:** Ascites was severe in 22/79 cases of dysfunction (27.8%) vs 13/139 cases in the absence of dysfunction (9.3%) ( $P = 0.001$ ). The sensitivity and specificity of this variable were 27.9% and 90.6%, respectively (Table 3).

**Stent velocity:** The mean velocity within the stent was  $76.6 \pm 52.5$  cm/s in cases of dysfunction and  $76.8 \pm 35.8$  cm/s in the absence of dysfunction ( $P = 0.753$ ).

**Portal vein velocity:** The mean portal vein velocity was  $25.1 \pm 14.9$  cm/s in cases of dysfunction and  $34.3 \pm 19.9$  cm/s in the absence of dysfunction ( $P = 0.008$ ). AUC is presented in Table 4.

The mean change of portal velocity relative to that measured 1 mo after TIPS insertion, called portal velocity delta, was  $-8.8 \pm 18.1$  cm/s in the dysfunction group, and  $-2.1 \pm 22.5$  cm/s in the absence of dysfunction group ( $P = 0.045$ ). However, AUC of this variable is 0.577 (Table 4).

**Portal velocity delta combined with right portal vein flow direction:** The AUC of this association was 0.626 (Table 4).

**Operator conclusion:** Dysfunction was suspected from Doppler-US in 31/79 patients with certified dysfunction (39.2%), and in 18/139 patients in the absence of dysfunction (12.9%) ( $P = 0.0001$ ). The sensitivity of this variable was 39.1% and its specificity was 87.1% (Table 3).

## DISCUSSION

In our study, low portal vein velocity, hepatopedal flow in portal vein branches, loss of portal flow modulation,

**Table 2** Suspicion of dysfunction based on the conclusion of the operator, according to the localization of the stenosis *n* (%)

	Dysfunction suspected ( <i>n</i> = 21 stenosis)	Not suspected ( <i>n</i> = 18 stenosis)
Lower part of the stent	6 (28.6)	2 (11.1)
Middle part of the stent	4 (19.1)	4 (22.2)
Upper part of the stent	8 (38.1)	8 (44.4)
Hepatic vein	3 (14.3)	4 (22.2)

severe ascites and operator conclusion were associated with TIPS dysfunction. Nevertheless, the performance of these Doppler-US criteria for the diagnosis of TIPS dysfunction was poor.

Many studies have shown that dysfunction is associated with low main portal vein velocity<sup>[8,15,20]</sup>. Some authors have tried to define a threshold value to discriminate patent from non-patent shunts; however, results were inconsistent<sup>[8,15,20]</sup>. In our study, the AUC of main portal vein velocity was 0.655, so we cannot propose a relevant cut-off value. These results underline the difficulties to obtain a reproducible cut-off value, possibly due to the inter-individual variability of this variable. However, in our study, temporal change in main portal vein velocity relative to its baseline value was not more relevant than main portal vein velocity itself. Similarly, other authors<sup>[15,17]</sup> have reported poor sensitivity for a decrease of 33% in portal vein velocity.

The change of flow direction in the portal vein branches was significantly associated with dysfunction, both in the right and left branch. However, the sensitivity and the specificity of these variables were insufficient (all below 70%). This association has been already reported<sup>[8,14,15,18]</sup> with variable results. Kanterman *et al.*<sup>[15]</sup> concluded this variable has a low sensitivity because intra hepatic flow reversal is a late sign of dysfunction.

Some authors have associated intrahepatic flow direction with another variable, portal vein velocity or stent velocity<sup>[14,18]</sup>. In our study, we evaluated the diagnostic accuracy of hepatopedal flow in the right portal branch combined with the decrease in portal vein velocity, but the AUC was mediocre. This is consistent with the low sensitivity we observed for each variable.

In our study, we did not find a significant modification of velocity within the shunt in cases of dysfunction. These results are consistent with some studies<sup>[16,18]</sup>, whereas other authors have reported intra-stent velocity as a predictive variable<sup>[15,17]</sup>. These differences can be explained by the poor reproducibility of this measurement. Indeed, the stent velocity increases from the portal extremity to the hepatic end<sup>[21]</sup> and consequently depends on the measurement site.

The lack of cardiac modulation of the portal flow was strongly associated with TIPS dysfunction. These

**Table 3** Performance of Doppler-ultrasonography binary criteria for the diagnosis of transjugular intrahepatic portosystemic shunt dysfunction

Variables	n paired	n patients	Sensitivity (95%CI)	Specificity (95%CI)	PPV	NPV (95%CI)
Portal flow modulation	177	73	44.4 (31.2-57.6)	79.6 (67.3-91.4)	56.4 (37.8-76.3)	71.1 (62.6-78.8)
Direction in right branch	173	76	57.2 (44.6-70.1)	61.7 (48.9-73.1)	45.2 (33.7-58.3)	72.4 (62.6-80.5)
Direction in left branch	171	77	54.7 (39.6-69.7)	66.8 (54.4-78.1)	48.5 (34.8-63.8)	72.1 (61.3-82.3)
Stent filling	140	64	31.3 (18.8-44.2)	81.8 (72.4-89.8)	54.1 (36.0-71.0)	63.4 (52.2-73.6)
Ascites	218	87	27.9 (16.9-39.7)	90.6 (84.0-95.4)	62.9 (43.7-79.1)	68.7 (61.0-76.1)
Conclusion	218	87	39.1 (27.6-51.4)	87.1 (79.3-93.3)	63.5 (45.8-79.8)	71.5 (63.6-78.5)

PPV: Positive predictive value; NPV: Negative predictive value.

**Table 4** Performance of Doppler-ultrasonography quantitative criteria for the diagnosis of transjugular intrahepatic porto-systemic shunt dysfunction

	Patients	Paired procedures	AUC	95%CI
Portal velocity	80	192	0.655	0.553-0.749
Stent velocity	80	195	0.536	0.454-0.634
Portal velocity delta	63	150	0.577	0.485-0.679
Delta + right direction	58	128	0.626	0.530-0.726

AUC: Area under the curve.

results are consistent with those reported by some authors<sup>[22,23]</sup>.

As others<sup>[5]</sup>, we observed that detection of ascites during Doppler-US examination was associated with shunt dysfunction with a high specificity (90.6%). This is consistent with the fact that ascites is a late sign of dysfunction and not a predictive one.

The conclusion of the operator was associated with dysfunction with high specificity but with low sensitivity. The negative predictive value of this variable was 71.5%, thus a dysfunction cannot be ruled out when Doppler-US examination does not suggest dysfunction. In other studies<sup>[8,15,17]</sup>, this variable predicted shunt dysfunction more accurately than in our study, probably because of the monocentric design of these studies. Indeed, Doppler-US is an operator-dependent examination<sup>[24]</sup> which explains differences observed from one study to another, and difficulties to identify objective and reproducible predictors of TIPS dysfunction. Moreover, this underlines the importance of the experience of the operator. Most of the Doppler-US were performed by experienced and specialized operators in this study. In only 2 centers, some examinations have been occasionally realized by residents.

In our study, we found lower sensitivities and specificities than those reported in literature, probably because we avoided institution bias. Indeed, this study was designed as a pragmatic study and represents the reality of current practice, with about half of the French centers realizing TIPS procedure included in this study.

Moreover, dysfunctions observed in our study were mostly located in the upper part of the stent and may be more difficult to diagnose in Doppler-US.

Other authors reported similar results to ours, and failed to identify Doppler-US variables relevant to diagnose shunt dysfunction<sup>[16,20]</sup>. Interestingly, these studies were also prospective and double-blinded but included fewer patients than our study.

In our study, some procedures were realized with bare stents and other with covered stent but this has no incidence on the results as we took in account only dysfunction. Shunt dysfunction occurs frequently, even with covered stents<sup>[13]</sup>; therefore, it is still necessary to monitor shunt patency, especially to avoid the recurrence of digestive bleeding as it is a life-threatening complication. Given its poor diagnostic performance, Doppler-US is not a good diagnostic tool for routine screening across centers. Clinical supervision may be sufficient for TIPS indications such as refractory ascites, whereas early detection of shunt dysfunction appears crucial for TIPS indications such as variceal bleeding. New tools, more efficient than Doppler-US and less invasive than angiography, are needed. Contrast-enhanced ultrasound<sup>[25]</sup>, as well as the measurement of azygos blood flow by magnetic resonance imaging<sup>[26]</sup> may be of interest but further studies are needed. In the meanwhile, an angiography should still be proposed, especially for bleeding indications of TIPS, sixth months after TIPS insertion because the first event of dysfunction occurs in almost half of cases at 6 mo.

In conclusion, this pragmatic study shows that the performance of Doppler-US for the detection of TIPS dysfunction is poor in current practice.

## COMMENTS

### Background

Angiography is the gold-standard procedure to evaluate transjugular intrahepatic porto-systemic shunt (TIPS) dysfunction. However, it is an invasive technic performed only in limited specialized centers. Thus, Doppler ultrasonography (Doppler-US) is frequently used for TIPS monitoring.

### Research frontiers

Despite frequent use of Doppler-US for TIPS monitoring, to date, no criterion of TIPS dysfunction have been prospectively evaluated.

### Innovations and breakthroughs

The authors conducted the first large prospective multicentric evaluation of the performance of Doppler-US for the detection of TIPS dysfunction.

## Applications

In routine practice, the performance of Doppler-US for the detection of TIPS dysfunction is insufficient. Thus, the gold standard remains angiography. Future researches have to focus on developing less invasive tools.

## Peer-review

The article is aimed to assess the factors related to the prognosis of intra-abdominal liposarcoma and find the optimal minimum duration for remnant tumor screening.

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