Research Article
Volume 11:1, 2021

Journal of
Transplantation Technologies & Research

ISSN: 2161-0991 Open Access

Analytical and Clinical Validation of a Novel Blood-Based Biomarker of Liver Transplant Rejection

Juston C Weems*, Deirdre Pierry, John Holman, Manoj Kandpal, Sunil M Kurian, Peter Meintjes, Neal Beswick and Josh Levitsky

Transplant Genomics Inc, Mansfield, MA 02048, USA; Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA; Scripps Research Institute, La Jolla, CA 92037

Abstract

Background: We have discovered and validated a microarray-based test that analyzes blood gene expression profiles (GEP) as an indicator of immune status in liver transplant recipients with stable liver function.

Methods: Analytical performance studies to characterize stability of RNA in blood during collection and shipment, analytical sensitivity (input RNA concentration), analytical specificity (interfering substances) and assay performance (clinical validity, and intra-assay, inter-assay, inter- laboratory reproducibility).

Results: Total RNA extracted from whole blood specimens collected in PAXgene Blood RNA tubes was stable up to 3 days at room temperature (stable RNA yield). Under routine ambient shipping conditions, storage and shipping temperatures did not affect results. However, specimen shipments exposed to temperatures >400°C or to ambient temperatures for >3 days were unacceptable for processing. Analytical sensitivity studies demonstrated tolerance to variation in RNA input (50 to 400 ng per 3' IVT (in vitro transcript] labeling reaction). Specificity studies using genomic Jurkat DNA spiked into 3 'IVT reactions at 10-20% demonstrated negligible assay interference. The test was reproducible across operators, runs, reagent lots, and laboratories. External validation demonstrated that the TruGraf Liver blood test accurately classified patients in 84% of 155 samples.

Conclusions: The previously published biomarker is the first non-invasive test to be demonstrated to have clinical utility in assessing immune status of LT recipients with stable liver function and shows promise as a reasonable and necessary tool supporting personalizing immunosuppressive therapy.

Keywords: Liver transplant recipients • Gene expression signatures• Immunosuppression • TruGraf blood test

Introduction

The survival benefits of organ transplants are well documented [1]. Improvements in immunosuppression and other aspects of ancillary care have led to significant improvement in outcomes. At the present time, despite considerable research efforts, there are only a few proven tests aimed at measuring or monitoring the adequacy of immunosuppression, the failure of which may result in over-immunosuppression and opportunistic infections and kidney dysfunction, or under-immunosuppression and acute rejection (AR) [2].

The TruGraf® blood test for kidney transplant recipients (Transplant Genomics Inc., Mansfield, MA) is a commercially available test provided exclusively through the Clinical Laboratory Improvement Amendments (CLIA) certified laboratory at Transplant Genomics Inc. (Freemont, CA). This test relies on a specific gene expression signature in the peripheral blood to enable proactive non-invasive serial testing of kidney transplant recipients with stable renal function. The gene expression profile (GEP) was first reported in 2014 [3] and then subsequently modified to distinguish healthy transplant (TX = Transplant excellent) from non-TX, specifically sub-clinical acute rejection (subAR) [4-6].

We have developed a similar GEP in liver transplant (LT) recipients that can potentially distinguish healthy transplant (TX) from acute rejection (AR) [7], such that modifications to lower or increase immunosuppressive therapy could be guided by the test. This could personalize the treatment of LT recipients to improve outcomes. However, before the test (TruGraf Liver) is further developed and moved into commercial use, additional validation with external samples beyond the clinical trial is needed. In this study, we analyzed additional samples using the locked gene expression profile to demonstrate its effectiveness in assessing the clinical phenotype (AR or TX) (Summary Table 1). Overall, we performed analytical validation for a microarray assay that analyzes blood gene expression profiles (GEP) as an indicator of immune status in liver transplant recipients with stable liver function.

***Address for Correspondence:** Weems Juston, Transplant Genomics Inc, 905 South Main Street, Suite 102, Mansfield, MA 02048, USA, Tel: 8168722379; E-mail: justonweems@eurofins-tgi.com*

Received 22 December 2020; **Accepted** 12 January 2021; **Published** 19 January 2021

Materials and Methods

The multiple steps necessary for a TruGraf® blood test have been previously published and validated (First MR, 2017). A brief overview of the process includes obtaining blood samples from patients in nucleic acid stabilizing tubes, and subsequently taking the RNA through a series of steps for extracting, amplifying and hybridization to the DNA microarray plate. Microarray plates are then washed, stained and scanned in order to determine intensity of hybridization of patient RNA to predesigned specific oligonucleotide probes. A proprietary classification algorithm is then used to analyze the pattern of hybridization while being compared to a reference dataset that ultimately generates a qualitative result of "TX" or "AR". These results of a TruGraf® Liver blood test may be used by clinicians and physicians in combination with other relevant clinical information, to determine whether or not a liver transplant recipient is avoiding adverse graft function from either under- and over- immunosuppression regiments.

RNA extraction, amplification and hybridization

As previously described, total RNA was collected from PAXgene Blood RNA (IVD) tubes (Qiagen, Valencia, CA) (First MR, 2017). RNA extraction was performed from PAXgene tubes that were processed using PAXgene Blood microRNA (miRNA) reagents on the QIAcube instrument (Qiagen, Valencia, CA). Total RNA concentrations and yield were determined using the Nanodrop 8000 (Thermo Fisher Scientific, Carlsbad, CA) and samples were subjected to a globin-reducing step using the Ambion GLOBIN clear Human kit (Thermo Fisher Scientific, Carlsbad, CA). Concentrations of globin-reduced RNA were determined via the Nanodrop 8000 and further subjected to the Bioanalyzer RNA Nano system to assess quality and to generate an

Table 1. Summary of analytical validation studies.

Study	Sample Source	Design Summary	Data Evaluated
Analytical Sensitivity – LOD	HeLa/WAC Control RNA	LOD testing was performed on a dilution series (4 dilutions of 3' IVT labeling reaction input concentrations and 4 Hybridization reaction input concentrations) of HeLa and WACcontrol RNAsamples. Sample data analysis was performed on the Affymetrix Expression Console software.	In-process QC - NanoDrop and RIN values Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb control acceptability, GAPDH signal Intensity, GAPDH 3'-5' ratios, Pearson correlations. LOD – 4 sample input concentrations for both IVT labeling and hybridization reactions
Analytical Specificity Interference	HeLa/WAC Control RNA	RNA from Hela/WAC control supplied with the 3' IVT was spiked with Jurkat gDNA and processed through array Hyb on the GeneTitan. Sample data analysis was performed on the Affymetrix Expression Console software.	In-process QC - NanoDrop and RIN values Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb control acceptability, GAPDH signal Intensity, GAPDH 3'-5' ratios, Pearson correlations. Array CEL file data was analyzed on theAffymetrix Expression ConsoleSoftware. Resulting information about probeset intensity variation was used to evaluate effects of gDNA contaminationon RNA specimenhybridization.
Accuracy (vs biopsy results)	PAXgene Blood RNA from kidney transplant subjects	A total of 221 Transplant Subjects in 2 cohorts (129 NU and 92 CTOT14 samples). PAXgene RNA Blood tubes from samples across reporting rangewere received at the TGI CLIA Lab for processing through the complete Affymetrix GeneTitanworkflow. Sample data analysis was performed on the Affymetrix Expression Console software.	In-process QC - NanoDrop and RIN values Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb control acceptability, GAPDH signal Intensity, GAPDH 3'-5' ratios, Pearson correlations. Array CEL file data was analyzed on the Affymetrix Expression Console software to generate Hyb QCdata and on the TruGraf® Liver Classifier algorithm to generate IQ/IAscores.
Preanalytical Factors	PAXgene Blood RNA derived from non-transplant subjects (sufficient blood was collected to allow for replicates for samples to be run).	Normal subject blood specimens in PAXgene tubes were obtained by the TGI CLIA Lab. Specimens were subjected to varying preanalytic conditions and extracted. Downstream GLOBINclear, 3' IVT and array hybridization processing were performed on a single run.	In-process QC - NanoDrop and RIN values Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb control acceptability, GAPDH signal Intensity, GAPDH 3'-5' ratios, Pearson correlations. Array CEL file data was analyzed on the Affymetrix Expression Console Software.
Reproducibility – Inta- assay	PAXgene Blood RNA derived from non-transplant subjects (sufficient blood was collected to allow for replicates for samples to be run).	Normal subject blood specimens in PAXgene tubes were obtained by the TGI CLIA Lab. Multiple replicates of 4 patient samples were processed on a single run.	In-process QC - NanoDrop and RIN values Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb control acceptability, GAPDH signal Intensity, GAPDH 3'-5' ratios, Pearson correlations. Array CEL file data was analyzed using RMA data from the Affymetrix Expression Console to generate hybridization metrics. Descriptive statistics were evaluated for reproducibility and precision.
Reproducibility – Intermediate Precision	PAXgene Blood RNA derived from non-transplant subjects (sufficient blood was collected to allow for replicates for samples to be run).	Normal subject blood specimens in PAXgene tubes were obtained by the TGI CLIA Lab. Replicates of 4 patient samples were run in duplicate on each of 8 separate runs. New reagent lots were rotated into the run schedule while holding the remaining reagent lots constant so that reagent effects could be pinpointed to the new reagent lot. At least 2 different lots of GeneTitan HGU133+ GLOBINclear reagents,Qiagen PAXgene RNA (IVD), and 3' IVT Plusand Hyb/Wash/Stain reagents were usedfor this cohort.	In-process QC - NanoDrop and RIN values Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb control acceptability, GAPDH signal Intensity, GAPDH 3'-5' ratios, Pearson correlations. Array CEL file data was analyzed using RMA data from the Affymetrix Expression Console to generate hybridization metrics. Descriptive statistics were evaluated for reproducibility and precision.
TruGraf® Liver Classifier Bioinformatics	Raw data files (.CEL)	Internal validation performed on ~129 data files from the original discovery data set. External/Independent Validation - ~92 data files processed independent of the discovery set	Array CEL files were analyzed using the TruGraf® Liver Classifier

RNA integrity number (RIN). Thresholds were set of RNA yield and quality in order to be used for downstream processing. In vitro transcription and labeling reactions (3' IVT) on globin- reduced RNA (input of 200ng) were performed on the Affymetrix 3' IVT (in vitro transcript) PLUS labeling system (Affymetrix, Santa Clara, CA). After IVT, samples were fragmented and a final pre-hybridization RNA quality check was performed on labeled cRNA in addition to a check on the fragmented final cRNA product. Using an input 7.5 µg input of biotin-labeled cDNA, we prepared a hybridization cocktail for use in array hybridization. All hybridization, washing, staining and array scanning steps were performed on Affymetrix HG-U133+ arrays using the standard GeneTitan Gene Expression array workflow (Affymetrix, Santa Clara, CA). A whole assay control (WAC) RNA from a subject with a known TruGraf® Liver response processed from RNA extraction to Gene Titan processing was used in addition to a HeLa control RNA sample, no-template control and Affymetrix External RNA controls (Poly A RNA, B2 Oligo and 20X hybridization controls) as in-process controls for both RNA labeling and hybridization reactions. Raw expression data files in the form of CEL files (an ASCII text file used for Affymetrix software) was generated by the Gene Titan and checked for quality control metrics using Affymetrix Expression Console (build 1.4.1.46, Affymetrix). Predefined specifications for yield, array data quality and control sample classifier results were used as acceptance criteria prior to sample data being analyzed by the TruGraf® Liver classifier.

The TruGraf® Liver Classifier

The TruGraf® Liver classifier uses coefficient of variance to identify the top 5,000 variables probes from the discover NU dataset. Using a random-forest based algorithm with 10,000 trees, feature selection was performed, while using an out-of-bag error as minimization criterion and carry-out variable elimination from random-forest by successively eliminating the least important variables [8]. The most informative genes were identified using random- forests and Gini importance providing a relative ranking of classifier features from which a final model was selected to distinguish AR from TX. A performance threshold was selected favoring NPV over PPV (above the threshold = AR), and the model and threshold were locked for validation of the CTOT-14 cohort, an independent sample set that was not used in training.

Blood samples from patients with biopsy-confirmed TX or AR were used to perform the analysis.

Preanalytic Conditions

Due to the nucleic acid stabilizing components of the PAXgene tubes, samples can be shipped overnight at ambient temperatures without compromising the RNA quality. We therefore wanted to examine the effects of different shipping scenarios during the validation to establish acceptable specimen criteria. WAC control PAXgene blood specimens and numerous PAXgene tubes were collected at the same time from the same donor, and individual tubes were subject to varying Preanalytic conditions prior to RNA extraction (Table 2).

Downstream GLOBIN clear and 3' IVT processing were performed on a single run, while in- process QC metrics (Concentration, OD 260/280 ratio, RIN value and BA electropherogram data as applicable) in addition to Hyb QC metrics were obtained to determine the effects of Preanalytic sample handling. Resultant .CEL file raw data was subsequently analyzed with the TruGraf® Liver Classifier algorithm.

Table 2. Preanalytic Conditions.

Sample ID	PreAnalytic Condition	Condition Duration
6908	PAX tube held @ 40^{0}C	36 hours
6909	PAX tube held @ 2-8^{0}C	48 hours
6910	PAX tube held @ ambient temp	48 hours
6911	PAX tube held @ ambient temp	72 hours
6912	PAX tube held @ 2-8^{0}C	72 hours
6913	PAX tube held @ ambient temp	96 hours
6914	PAX tube held @ 2-8^{0}C	96 hours
6915	PAX tube held @ 2-8^{0}C	144 hours

As expected, samples held at 40°C for 36 hours yielded RNA too degraded to analyze (Table 3). Of the remaining samples in this cohort, all samples met in-process QC metrics and were hybridized. Hybridization QC data was generated from analysis of raw .CEL file data with GeneChip® Expression Console.

Table 3. TruGraf® Liver Results – Preanalytic conditions.

Sample ID / Condition	TruGraf® Liver Result	TruGraf® Liver Score
6908 / 40^{0}C 36 hr	n/a - degraded	n/a - degraded
6909 / Fridge 48 hr	AR	0.572
6910 / RT 48 hr	TX	0.339
6911 / RT 72 hr	TX	0.418
6912 / Fridge 72 hr	AR	0.515
6913 / RT 96 hr	TX	0.234
6914 / Fridge 96 hr	TX	0.431
6915 /Fridge 144 hr	TX	0.376

BA analysis of GLOBIN-reduced RNA from each of the specimens shows noticeable degradation of samples held beyond 72 hours post collection at ambient temperature, and beyond 96 hours at 2-8°C. These results were used to establish rejection criteria as samples held at ambient temperature for >72 hours, or specimens subjected to elevated temperatures (>40°C) prior to RNA extraction yield degraded or poor quality RNA unsuitable for downstream applications. Figure 1 shows the Actin and GAPDH ratio metrics for samples subjected to elevated preanalytic temperatures or samples held at ambient temperatures for the indicated times. Along with GAPDH ratio for data, we also determined the relative logarithmic expression (RLE) values as a relative measure of the deviation of a single array signal when compared to a group average (Figure 2). This is routinely used to assess variation within microarray studies.

Analytical sensitivity: RNA input

Limit of detection or LOD studies were performed utilizing dilutions of HeLa Control RNA (included with the GeneChip® 3' IVT Plus reagents) as well as WAC Control RNA and a liver transplant subject. LOD testing was performed on a dilution series (5 dilutions of 3' IVT Labeling reaction input concentrations and 3-4 Hybridization reaction input concentrations) of HeLa & WAC Control RNA samples. Data analysis for this cohort was performed on the GeneChip® Expression Console software. CEL files were also analyzed with the TruGraf® Liver Classifier (v1.1.0) to obtain result data.

In-process and hybridization QC Metrics were reviewed for all samples in this cohort. All samples met in-process QC criteria (criteria, criteria) and Hyb QC criteria (GAPDH Ratio <4; Poly A Labeling Control intensities; HYB Control intensities, All probes RLE Mean). A trend was seen in the hybridization QC metrics (Tables 4a & 4b, Figures 3 and 4). While the HeLa Control showed consistent intensity data for the RLE Mean, Nonspecific

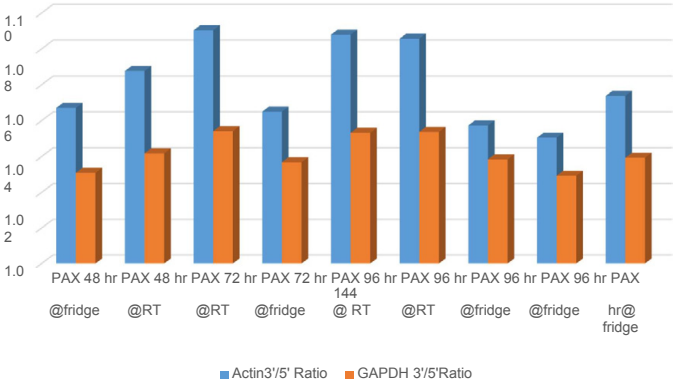

Figure 1. Actin and GAPDH 3'/5' Ratios for samples with at different storage conditions.

Table 4a. Hybridization QC results – LODSamples.

	RLE mean	Nonspecific binding	PolyA - Lys	PolyA - Dap	HYB – bio B	HYB – bio C	HYB – bio D	HYB - Cre	GAPDH 3'-5' Ratio
HeLa Control -% CV	0.41%	2.4%	33.6%	39.4%	16.2%	17.5%	12.5%	7.2%	3.1%
Liver Transplant Pt - % CV	18.9%	5.1%	41.8%	27.4%	29.5%	29.0%	17.6%	8.2%	9.9%
WAC Control -% CV	17.7%	2.9%	45.7%	28.0%	55.1%	43.1%	22.6%	12.7%	7.5%

Table 4b. Hybridization QC results – LODSamples.

Sample ID	RLE Mean	Nonspecific Binding	Poly A Lys (low) Control
HeLa – 25 ng	0.42	90	453
HeLa – 50 ng	0.42	95	418
HeLa – 100 ng	0.42	91	310
HeLa – 200 ng	0.42	87	212
Liver TX – 25 ng	0.19	105	1265
Liver TX – 50 ng	0.13	89	759
Liver TX – 100 ng	0.12	96	628
Liver TX – 200 ng	0.12	91	387
Liver TX – 500 ng	0.12	88	379
WAC – 25 ng	0.16	101	1331
WAC – 50 ng	0.14	96	757
WAC – 100 ng	0.09	97	473
WAC – 200 ng	0.13	91	275
WAC – 500 ng	0.15	93	336

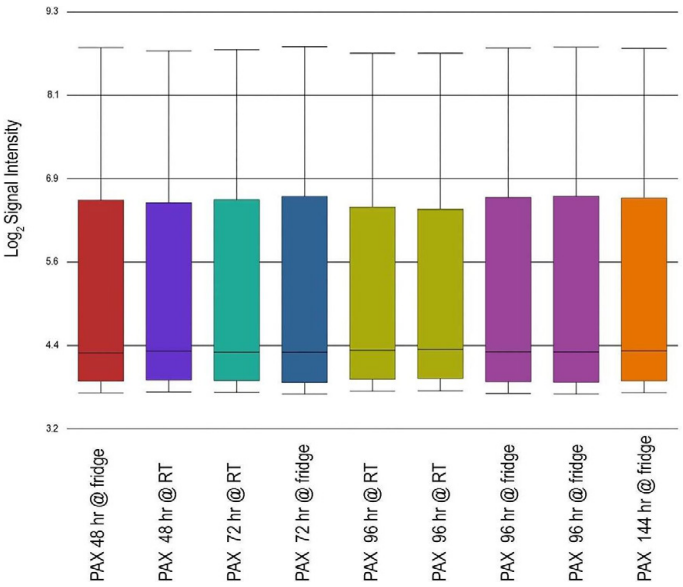

Figure 2. Boxplots of Normalized Signal Intensities for PAXgene tubes at different storage conditions.

Binding and Poly A Lys Control, the 2 patient – derived samples in this cohort demonstrated outlier intensity data for the lowest dilution of the LOD series (25 ng IVT reaction input). This IVT input concentration demonstrates labeling reaction inconsistencies not seen for the samples with 50-500 ng IVT reaction input, consistent with Manufacturer data. This data re-validates the TruGraf® sample processing workflow that utilizes a 200 ng IVT reaction RNA input.

Analytical specificity: genomic DNA

As previously reported, the Affymetrix Gene Expression analysis workflow has many distinct steps designed for purification and elimination of interfering molecules. The RNA extraction process removes DNA and heme particles during the purification; during globin clearing, the globin RNA is removed and a final purification step during 3' IVT labeling removes unlabeled and excess reagents, ultimately yielding a purified labeled cRNA.

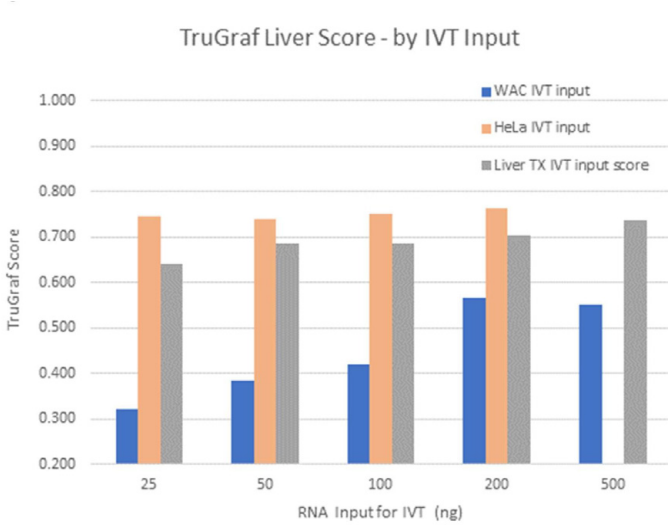

Figure 3. TruGraf® Liver score by IVT input.

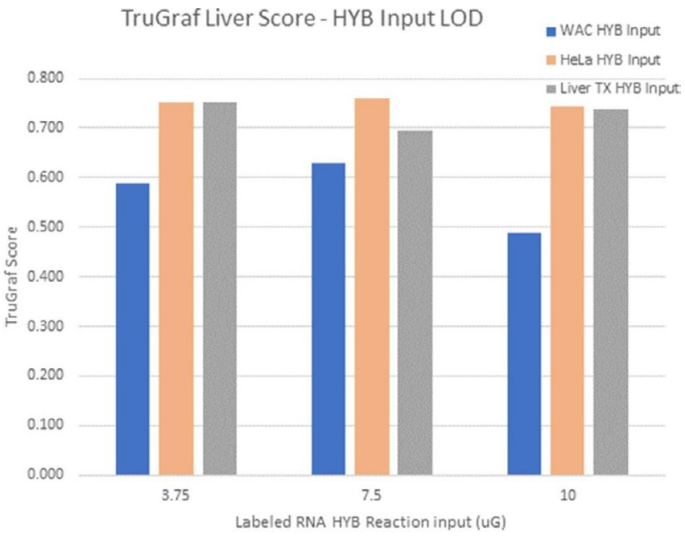

Figure 4. TruGraf® Liver score by Hybridization reaction input.

However, additional experiments were designed to determine what, if any, effect genomic DNA might have as a potential interfering substance.

Therefore, RNA from HeLa & WAC Controls were spiked with Jurkat genomic DNA (Life Technologies Cat # SD1111) and processed thru array Hyb on the GeneTitan (Table 5, Figures 5 & 6). A 2nd set of 5 samples with elevated bilirubin levels was also processed through the TruGraf® workflow to assess the effect of hyperbilirubinemia on assay performance (Table 6, Figures 7, 8). In-Process QC data was obtained (RNA concentration, OD 260/280 ratio, RIN value and BA electropherogram data for intermediate steps) and compared to current processing results.

Resultant gene expression data from .CEL files had QC metric data analysis performed on the GeneChip® Expression Console software. Raw CEL file data analysis was completed on the TruGraf® Liver Classifier under validation in order to determine effects on classifier results.

In-process QC metric results were unremarkable for samples tested as part

Table 5. TruGraf Liver score from Jurkat gDNA spiked samples.

Sample (% Jurkat)	TruGraf Liver Result	TruGraf Liver Score
HeLa-Jurkat (10%) - A	AR	0.7792
HeLa-Jurkat (10%) - B	AR	0.7597
HeLa-Jurkat (20%) - A	AR	0.7761
HeLa-Jurkat (20%) - B	AR	0.7430
HeLa-Jurkat (30%) - A	AR	0.7546
HeLa-Jurkat (30%) - B	AR	0.7705
WAC-Jurkat (10%) - A	AR	0.8216
WAC-Jurkat (10%) - B	AR	0.8088
WAC-Jurkat (20%) - A	AR	0.7891
WAC-Jurkat (20%) - B	AR	0.8236
WAC-Jurkat (30%) - A	AR	0.7102
WAC-Jurkat (30%) - B	AR	0.7394

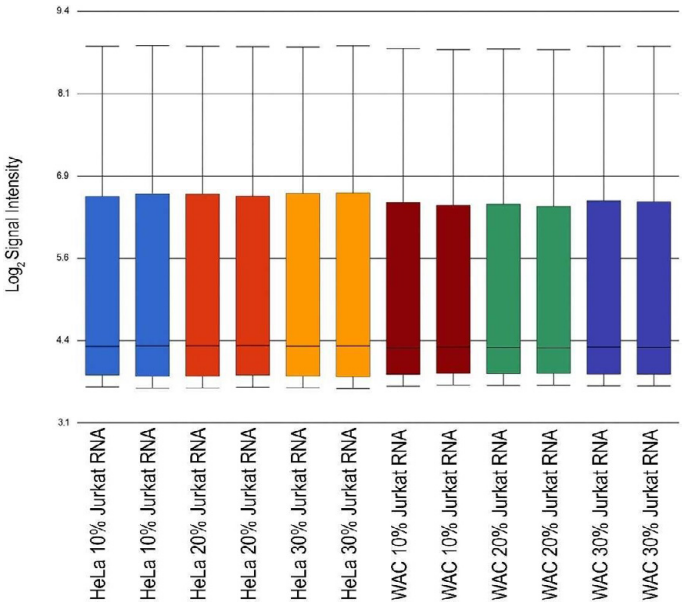

Figure 5. Boxplots of Normalized Signal intensities for HeLa/WAC samples with spiked Jurkat gDNA.

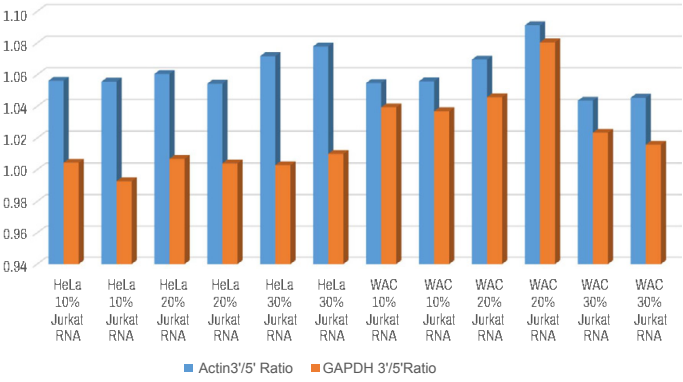

Figure 6. Actin and GAPDH 3'/5' Ratios for HeLa/WAC samples with spiked Jurkat gDNA.

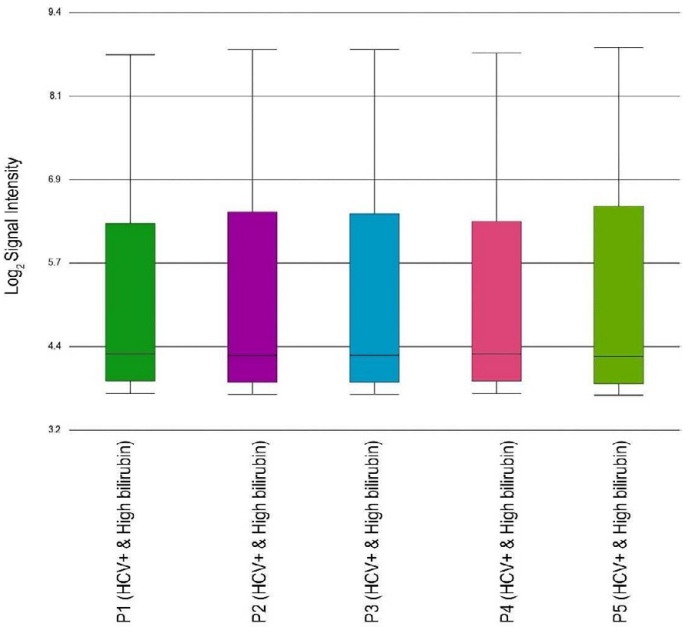

Figure 7. Boxplots of Normalized Signal intensities for HVC+ Patients with high bilirubin.

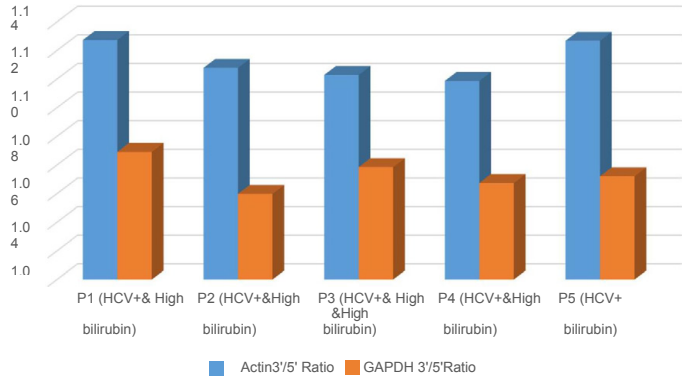

Figure 8. Actin and GAPDH 3'/5' Ratios for HVC+ Patients with high bilirubin.

of the genomic DNA cohort (Figures 5 and 6). GAPDH 3':5' ratios were < 2, and lower signal intensities for the PolyA Labeling Controls (as compared to results from "non-spiked" samples) were noted for all samples processed in this cohort.

Review of probeset signal intensities generated by .CEL file normalization and processing illustrated that signal intensities decrease subtly as the concentration of Jurkat gDNA increases as a percentage of total RNA.

While there did not appear to be an effect on the TruGraf® score for the HeLa Control samples spiked with Jurkat gDNA, and the expected "AR" classification was achieved with the HeLa Control; there was an impact seen within the WAC control at the highest percentages of Jurkat gDNA.

Table 6. Hyperbilirubinemic Sample QC metrics.

	RLE_mean	Nonspecific GC12-signal	PolyA-lys_avg-signal	PolyA-phe_avg-signal	PolyA-thr_avg-signal	PolyA-dap_avg-signal	HYB-bioB_avg-signal	HYB-bioC_avg-signal	HYB-bioD_avg-signal	HYB-cre_avg-signal	GAPDH_3-5-ratio
S06710	0.18	131	682	1092	2022	3780	872	1841	5686	10272	1.59
S06711	0.15	126	481	778	1529	3192	779	1738	5810	10559	1.37
S06712	0.19	127	450	698	1223	2794	718	1431	5202	10746	1.56
S06713	0.17	131	379	668	1042	2762	1102	2258	7116	12304	1.41
S06714	0.18	128	375	649	1049	2677	749	1529	5824	11034	1.44
Avg	0.17	129	473	777	1373	3041	844	1759	5927	10983	1.47
SD	0.01	2	102	149	338	374	127	263	581	644	0.08
CV%	7.28%	1.37%	21.60%	19.20%	24.59%	12.31%	15.02%	14.98%	9.79%	5.86%	5.21%

As the percentage of Jurkat gDNA increased the TruGraf® Liver score decreased slightly, particularly with the 30% Jurkat gDNA replicates, though still reliably produced the same TruGraf® phenotype.

Additionally, possible sample contamination from hyperbilirubinemia was also tested (Figures 7 and 8). We used a set of five HCV+ / hyperbilirubinemic samples and processed the samples through the TruGraf workflow in order to assess the impact of hyperbilirubinemia on TruGraf® results. These samples passed all in-process QC metrics and their Hybridization QC metric results were all within limit as well. Array probeset intensities were found to be similar to "routine" samples. TruGraf® Classifier analysis of these 5 samples yielded 'AR' results for all samples, with unremarkable scores (range = 0.66 to 0.81). These subjects were not given a phenotype as they all had a Hepatitis C diagnosis and were excluded from biomarker studies.

Accuracy and reportable range

Independent clinical validation of the performance of the TruGraf® Liver assay was performed on a total of 221 Transplant Subjects in 2 cohorts (129 NU and 92 CTOT14 samples), with 66 samples biopsy identified as ADNR and therefore excluded from this analysis (Table 7). PAXgene RNA Blood tubes from samples across reporting range were received at the TGI CLIA Lab for processing through the complete Affymetrix GeneTitan workflow. This Cohort included representative samples of TX and AR as determined by paired biopsy sample histology. Samples were randomized to one of several arrays in order to minimize processing bias. Molecular phenotype was compared to original histology results. Data analysis was performed on the GeneChip Expression Console software to generate Hyb QC data and on the TruGraf® Liver Classifier algorithm. Cumulative results are shown in Table 7 and Figure 9. Accuracy of the TruGraf® Liver blood test was 84% (95% CI, ± 0.01%), specificity at 87%, sensitivity at 64% and positive predictive and negative predictive values at 61% and 88%, respectively. Our NPV and specificity indicate that a "true TX" will be correctly identified as such in a high proportion of the intended clinical patient population using TruGraf® Liver testing.

Assay Reproducibility

CEL file data for samples tested on the precision cohorts were analyzed using the Affymetrix Gene Console software, and PLIER (probe logarithmic intensity error) analysis was performed to generate QC metric data. Technical performance of sample processing was accessed by using data for the internal and external RNA controls. Descriptive statistics for external RNA controls were used to assess precision for hybridization (Hyb, 20X Hyb controls) and labeling (PolyA IVT controls) while distribution stats for

Table 7. Accuracy Statistic.

Raw Data			
True Phenotype	**N**	**TruGraf ®TX**	**TruGraf® AR**
TX	95	78	17
AR	60	26	34
Total	155	104	51
Statistics (n = 155)			
Accuracy	77%		
Sensitivity	57%		
Specificity	82%		
PPV	47%		
NPV	87%		

	Clinical Phenotype	
TruGraf® Liver Result	AR	TX
AR	9	3
TX	10	15
Sensitivity / PPA	47.4%	
Specificity / PNA	83.3%	

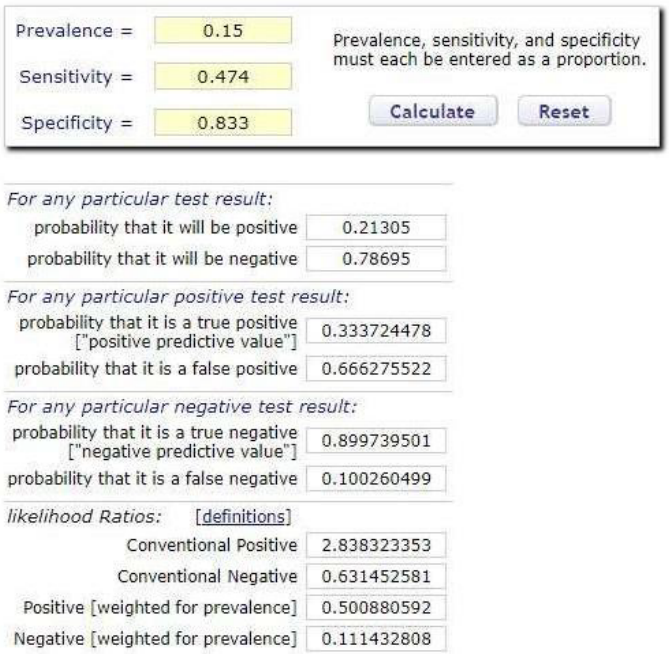

Figure 9. Inferential Statistics.

average GAPDH signal intensity along with the GAPDH 3' to 5' ratio were used as internal sample metrics (Table 8).

All samples used in Intra-run precision experiments were processed with the same reagent lot numbers throughout the workflow (Figures 10 & 11). Furthermore, the samples were hybridized on the same Affymetrix HG-U133 Plus array plate creating a group of samples to use in acquiring baseline statistics on within run variability (Tables 9, 10 and 11 a,b).

TruGraf® classifier development and validation

The TruGraf classifier algorithm (version 0.6) is a proprietary software package developed for use in the TGI automated bioinformatics pipeline. The TruGraf classifier is based upon previously published data (7). The current algorithm version has been locked, validated and implemented in TGI's CLIA laboratory workflow in the R statistical computing environment (version 3.1.2). The input for the software is an individual .CEL file generated by the Affymetrix GeneTitan instrument. Within the software, the data from the .CEL file is converted to a list of normalized gene expression values (signals) which correlates with the amount of RNA detected by each probeset on the Affymetrix GeneChip DNA microarray for the sample being analyzed. The values generated for a specific group of probesets present in the locked classifier are used by a locked Support Vector Machine (SVM) model (implemented from the e1071 R package version 1.6-6) which was trained on a discovery dataset (129 samples total) to generate a phenotypic classification / interpretation of Transplant excellence (TX) or AR for the sample.

Bioinformatics Validation – Internal and External Validation

The top 5000 variables probes were selected based on coefficient of variance. Features selection was performed using a random-forest based algorithm with 10000 trees. The variable selection algorithm uses out of bag error as minimization criterion and carry out variable elimination from random forest, by successively eliminating the least important variables. The most informative genes were identified using random forests and Gini importance providing a relative ranking of the classifier features from which a final model was selected distinguishing AR vs. TX. A performance threshold was selected favoring NPV over PPV (above the threshold = AR), and the model and threshold were then locked for validation (CTOT-14 cohort). The locked model and threshold were also used on pre–AR, pre–TX and pre–non-AR samples as well as post-AR. As each subject had

Table 8. Average coefficient of variation (CV) for external RNA controls.

	RLE_mean	Nonspecific GC12-signal	PolyA-lys_avg-signal	PolyA-phe_avg-signal	PolyA-thr_avg-signal	PolyA-dap_avg-signal	HYB-bioB_avg-signal	HYB-bioC_avg-signal	HYB-bioD_avg-signal	HYB-cre_avg-signal	GAPDH_3-5-ratio
S06710	0.18	131	682	1092	2022	3780	872	1841	5686	10272	1.59
S06711	0.15	126	481	778	1529	3192	779	1738	5810	10559	1.37
S06712	0.19	127	450	698	1223	2794	718	1431	5202	10746	1.56
S06713	0.17	131	379	668	1042	2762	1102	2258	7116	12304	1.41
S06714	0.18	128	375	649	1049	2677	749	1529	5824	11034	1.44
Avg	0.17	129	473	777	1373	3041	844	1759	5927	10983	1.47
SD	0.01	2	102	149	338	374	127	263	581	644	0.08
CV%	7.28%	1.37%	21.60%	19.20%	24.59%	12.31%	15.02%	14.98%	9.79%	5.86%	5.21%

Validation Study	Qiagen RNA Kit	Ambion GLOBINclear	Affy 3' IVT Kit	Affy H/W/S Kit	HG-U133 Array	Affy Wash A	Affy Wash B	Operator
L5	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #1	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 1
L6A	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #2	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 1
L6B	Qiagen PAXgene RNA Lot #2	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #3	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 2
L6C	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #2	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 1 & 2
L6D	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #2	GeneChip 3' IVT PLUS Lot #2	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #3	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 2
L6E	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	Held @ grRNA for later testing	Held @ grRNA for later testing	Held @ grRNA for later testing	Held @ grRNA for later testing	Held @ grRNA for later testing	Held @ grRNA for later testing
L6F	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #2	GeneChip HT Array Lot #1	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 3
L6G	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #1	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 1 & 2
L6H	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #2	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #2	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 1 & 2
L6I	Qiagen PAXgene RNA Lot #1	GLOBINclear Lot #1	GeneChip 3' IVT PLUS Lot #1	GeneChip H/W/S Lot #1	GeneChip HT Array Lot #2	GeneChip Wash Buffer A Lot #1	GeneChip Wash Buffer B Lot #1	Operator 2

Figure 10. Intermediate Precision Reagent Rotation Schedule.

Probeset_id	P_142 PS CV%	P_303 PS CV%	P_305 PS CV%	P_306 PS CV%
200878_PM_at	9.0%	7.1%	3.5%	6.9%
202016_PM_at	9.1%	5.3%	4.9%	10.2%
205654_PM_at	4.8%	9.5%	10.4%	5.5%
206486_PM_at	5.0%	13.5%	9.5%	6.3%
209773_PM_s_at	8.3%	4.3%	8.9%	11.1%
210358_PM_x_at	5.4%	3.9%	7.4%	9.0%
212478_PM_at	9.2%	9.8%	11.8%	9.6%
213060_PM_s_at	8.9%	18.0%	16.8%	4.5%
217714_PM_x_at	6.0%	5.2%	6.4%	5.9%
218350_PM_s_at	5.6%	8.4%	10.1%	7.0%
218782_PM_s_at	7.7%	9.4%	10.8%	6.4%
219859_PM_at	9.3%	14.7%	9.0%	6.5%
221874_PM_at	9.4%	7.5%	9.4%	7.9%
227530_PM_at	7.3%	6.4%	7.4%	13.4%
227671_PM_at	18.2%	39.8%	6.1%	20.0%
231034_PM_s_at	8.4%	11.7%	10.7%	5.0%
232229_PM_at	9.9%	13.4%	14.5%	15.1%
233263_PM_at	12.0%	6.9%	9.2%	12.8%
233700_PM_at	17.8%	13.2%	14.9%	17.5%
233957_PM_at	12.8%	16.4%	9.1%	13.0%
234431_PM_at	7.5%	4.9%	7.3%	8.6%
236216_PM_at	17.3%	5.4%	4.8%	9.1%
236409_PM_at	12.4%	19.1%	15.8%	14.7%
237376_PM_at	5.7%	8.6%	6.1%	10.2%
238281_PM_at	11.0%	9.0%	10.1%	8.8%
238446_PM_at	11.0%	19.1%	37.3%	12.8%
240765_PM_at	12.4%	8.7%	7.1%	11.6%
241391_PM_at	9.6%	5.7%	7.8%	10.1%
242800_PM_at	7.1%	7.6%	7.3%	11.7%
242854_PM_x_at	8.7%	8.2%	8.6%	11.3%
243874_PM_at	15.5%	7.2%	10.8%	16.2%
243954_PM_at	15.0%	7.7%	7.1%	13.4%
244578_PM_at	14.5%	17.4%	14.7%	14.1%
1554696_PM_s_at	5.9%	8.5%	8.1%	8.8%
1557685_PM_at	8.7%	12.7%	13.3%	10.4%
1560552_PM_a_at	13.0%	12.6%	11.1%	18.8%

Figure 11. TruGraf® Liver -specific probeset correlation.

serial samples collected, a linear mixed effect model with random intercept was used to estimate the pre-biopsy (or virtual TX biopsy) slope for each phenotype to account for within patient correlation. Data first stratified by phenotypes and coefficients were estimated and compared via linear mixed effect model. We used bootstrap method (n=1000) to generate the 95% CI for estimation of the earliest day prior to diagnosis of detecting differences between groups. Another linear mixed effect model was fitted to compare the pre and post-AR slopes. Analysis was performed using R version 3.5.1 (R Studio).

Probes from the final locked models were then fed to Ingenuity Core Analysis (Qiagen, Inc., Hilden, Germany) that provides information about enriched pathways and allows comparison to literature data. Enriched pathways were selected based on Fisher's exact test (p-value <0.05 statistically significant)

Discussion

The development of acute rejection after LT can significantly impact patient and graft survival. To date, there are no established non-invasive tests for serially detecting pre-clinical or clinical signs of rejection or healthy graft function. In a recent similar publication, we described the validity of kidney transplant biomarker (TruGraf) in detecting silent rejection on biopsy in patients with stable graft function [5] Similarly in LT, there is a great need for non-invasive serial monitoring of patients undergoing key immunosuppression modifications post-operatively [9-17]. To address this, we have discovered and validated a blood biomarker profile diagnostic for AR that can be detected prior to AR. This paper serves as additional clinical validation in the Transplant Genomics Inc. laboratory. Prior studies have demonstrated genetic polymorphisms, blood/hepatic gene expression profiles, microRNAs, blood lymphocyte populations, chemokines and

Table 9. Summary of Intermediate Precision – whole array signal correlation R^2.

R2 Values - Patient 142										
	P142_5a	P142_5b	P142_6a	P142_6b	P142_6c	P142_6d	P142_6f	P142_6g	P142_6h	P142_6i
P142_5b	0.995	n/a	0.995	0.976	0.985	0.983	0.980	0.980	0.979	0.981
R2 Values - Patient 303										
	P303_5a	P303_5b	P303_6a	P303_6b	P303_6c	P303_6d	P303_6f	P303_6g	P303_6h	P303_6i
P303_5b	0.999	n/a	0.996	0.983	0.988	0.989	0.967	0.989	0.884	0.982
R2 Values - Patient 305										
	P305_5a	P305_5b	P305_6a	P305_6b	P305_6c	P305_6d	P305_6f	P305_6g	P305_6h	P305_6i
P305_5b	0.998	n/a	0.994	0.985	0.985	0.985	0.987	--	0.903	0.983
R2 Values - Patient 306										
	P306_5a	P306_5b	P306_6a	P306_6b	P306_6c	P306_6d	P306_6f	P306_6g	P306_6h	P306_6i
P306_5b	0.998	n/a	0.995	0.971	0.980	0.975	0.980	0.975	0.979	0.981

Table 10. Hybridization Metrics (% CV) for Intermediate Precision Sample Replicates.

Sample ID	Cohort 6 RLE Mean (CV%)	Cohort 6 GAPDH3'-5' Ratio(CV%)
P_142	8.2%	12.9%
P_303	11.6%	8.7%
P_305	7.5%	7.6%
P_306	5.1%	7.0%

Table 11a. Hybridization Metrics (% CV) for Within-run Sample Replicates.

Sample ID	PM Mean (CV%)	RLE Mean (CV%)	GAPDH3'-5' Ratio(CV%)
P_142	2.1%	2.7%	0.4%
P_303	2.3%	0.3%	1.7%
P_305	7.1%	1.0%	2.5%
P_306	3.2%	1.3%	1.3%

Table 11b. Summary of Intra-run Precision – whole array signal correlation regressions.

Subject	R2 ("a' replicate vs"b" replicate)
142	0.995
303	0.999
305	0.998
306	0.998

complement proteins associated with AR in the LT population [18-33] These were not performed in LT patients in standard management and mainly in specific studies involving immunosuppression withdrawal. Blood-based tests validated in routine serial monitoring, such as our model, may be more useful and generalizable. This study has limitations that need to be addressed. In summary, the development of biomarkers in LT could transform the field, particularly with the focus on avoiding adverse events from both under- and over-immunosuppression. Our data represent an advance toward the development of clinically serviceable, blood-based GEP tests for use in liver transplantation, similar to other organs [3, 5, 34] . We are rapidly moving toward conducting biomarker-based interventional studies to proactively detect and reduce deleterious LT complications.

References

1. Abbas Rana, Angelika Gruessner, Vatche G Agopian and Zain Khalpey, et al. "Survival benefit of solid-organ transplant in the United States." *JAMA Surg* 150(2015):252-259.
2. Waldmann Herman. "Drug minimization in transplantation: an opinion." *Curr Opin Organ Transplant* 19(2014): 331-333.
3. SM Kurian, AN Williams, T Gelbart and TS Mondala, et al. "Molecular classifiers for acute kidney transplant rejection in peripheral blood by whole genome gene expression profiling."*Am J Transplant* 14(2014):1164-1172.
4. MR First, VR Peddi, R Mannon and R Knight, et al. "Investigator Assessment of the Utility of the TruGraf Molecular Diagnostic Test in Clinical Practice." *Transplant Proc* 51(2019):729-733.
5. JJ Friedewald, SM Kurian, RL Heilman and TC Whisenant, et al."Clinical Trials in Organ T. Development and clinical validity of a novel blood-based molecular biomarker for subclinical acute rejection following kidney transplant." *Am J Transplant* 19(2019):98-109.
6. CL Marsh, SM Kurian, JC Rice and TC Whisenant, et al."Application of TruGraf v1: A Novel Molecular Biomarker for Managing Kidney Transplant Recipients with Stable Renal Function."*Transplant Proc* 51(2019):722-728.
7. J Levitsky, SK Asrani, T Schiano and A Moss, et al. Clinical Trials in Organ Transplantation C. Discovery and validation of a novel blood-based molecular biomarker of rejection following liver transplantation. *Am J Transplant* 20(2020):2173-2183.
8. R Diaz-Uriarte and Alvarez de Andres S. "Gene selection and classification of microarray data using random forest." *BMC Bioinformatics* 3(2006).
9. Levitsky J, Feng S. "Tolerance in clinical liver transplantation". *Hum Immunol.* 79(2018):283-7.
10. Levitsky J, O'Leary JG, Asrani S, and Sharma P, et al. "Protecting the Kidney in Liver Transplant Recipients: Practice-Based Recommendations from the American Society of Transplantation Liver and Intestine Community of Practice." *American J of transpl: J American Society of Transpl America Society Trans Surg* 16(2016):2532-44.
11. Benitez C, Londono MC, Miquel R, and Manzia TM et al. Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. Hepatology. 58(2013):1824-35.
12. Feng S, Ekong UD, Lobritto SJ, and Demetris AJ, et al. "Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants." *Jama*. 307(2012):283-93.
13. De Simone P, Nevens F, De Carlis L, and Metselaar HJ, et al. "Everolimus with reduced tacrolimus improves renal function in de novo liver transplant recipients: a randomized controlled trial." *Am J Transplant* 12(2012):3008-20.
14. Teperman L, Moonka D, Sebastian A, and Sher L, et al. "Calcineurin inhibitor-free mycophenolate mofetil/sirolimus maintenance in liver transplantation: the randomized spare-the-nephron trial." *Liver Transpl* 19(2013):67589.

15. Shaked A, DesMarais MR, Kopetskie H, and Feng S, et al. "Outcomes of immunosuppression minimization and withdrawal early after liver transplantation." *Am J Transplant.* 19(2019):1397-409.

16. Banff Working Group on Liver Allograft P. "Importance of liver biopsy findings in immunosuppression management: biopsy monitoring and working criteria for patients with operational tolerance." *Liver Transpl.* 18(2012):1154-70.

17. AJ, Demetris and Isse K. "Tissue biopsy monitoring of operational tolerance in liver allograft recipients." *Curr Opin Organ Transplant.* 18(2013):345-53.

18. H, Kamei. Masuda S, Nakamura T, and Fujimoto Y, et al. "Impact of glutathione S-transferase T1 gene polymorphisms on acute cellular rejection in living donor liver transplantation." *Transpl Immunol.* 28(2013):14-7.

19. J, Gomez-Mateo. Marin L, Lopez-Alvarez MR, and Moya-Quiles MR, et al. "TGF-beta1 gene polymorphism in liver graft recipients." *Transpl Immunol.* 17(2006):55-7.

20. F, Bohne. Martinez-Llordella M, Lozano JJ, and Miquel R, 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):368-82.

21. MH, Karimi. Daneshmandi S, Pourfathollah AA, and Geramizadeh B, et al. "Association of IL-6 promoter and IFN-gamma gene polymorphisms with acute rejection of liver transplantation." *Mol Biol Rep.* 38(2011):4437-43.

22. MR, Moya-Quiles. Alvarez R, Miras M, and Gomez-Mateo J, et al. "Impact of recipient HLA-C in liver transplant: a protective effect of HLA-Cw*07 on acute rejection." *Hum Immunol* 68(2007):51-8.

23. R, Sindhi. Higgs BW, Weeks DE, and Ashokkumar C, et al. "Genetic variants in major histocompatibility complex-linked genes associate with pediatric liver transplant rejection. Gastroenterology." 135(2008):830-9, 9 e1-10.

24. PC, Evans. Smith S, Hirschfield G, and Rigopoulou E, et al. "Recipient HLA-DR3, tumour necrosis factor-alpha promoter allele-2 (tumour necrosis factor-2) and cytomegalovirus infection are interrelated risk factors for chronic rejection of liver grafts." *J Hepatol.* 34(2001):711-5.

25. R, Hanvesakul. Spencer N, Cook M, and Gunson B, et al. "Donor HLA-C genotype has a profound impact on the clinical outcome following liver transplantation." *Am J Transplant* 8(2008):1931-41.

26. TK, Toby. Abecassis M, Kim K, and Thomas PM et al. "Proteoforms in Peripheral Blood Mononuclear Cells as Novel Rejection Biomarkers in Liver Transplant Recipients." *Am J Transplant.* 17(2017):2458-67.

27. D, Joshi. Salehi S, Brereton H, and Arno M. et al. "Distinct microRNA profiles are associated with the severity of hepatitis C virus recurrence and acute cellular rejection after liver transplantation." *Liver Transpl.* 19(2013):38394.

28. H, Fan. Li LX, Han DD, and Kou JT et al. "Increase of peripheral Th17 lymphocytes during acute cellular rejection in liver transplant recipients." *Hepatobiliary Pancreat Dis Int*. 11(2012):606-11.

29. WR, Farid. Pan Q, van der Meer AJ, and de Ruiter PE et al. "Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation." *Liver Transpl.* 18(2012):290-7.

30. O, Massoud. Heimbach J, Viker K, and Krishnan A, Poter et al. "Noninvasive diagnosis of acute cellular rejection in liver transplant recipients: a proteomic signature validated by enzyme-linked immunosorbent assay." *Liver Transpl.* 17(2011):723-32.

31. A, Shaked. Chang BL, Barnes MR, and Sayre P, et al. "An ectopically expressed serum miRNA signature is prognostic, diagnostic, and biologically related to liver allograft rejection." *Hepatology.* 65(2017):269-80.

32. V, Jucaud. Shaked A, DesMarais M, and Sayre P, et al. "Prevalence and Impact of De Novo Donor-Specific Antibodies during a Multicenter Immunosuppression Withdrawal Trial in Adult Liver Transplant Recipients." *Hepatology.* 69(2019):1273-86.

33. E, Bonaccorsi-Riani. Pennycuick A, Londono MC, and Lozano JJ, et al. "Molecular Characterization of Acute Cellular Rejection Occurring During Intentional Immunosuppression Withdrawal in Liver Transplantation." *Am J Transplant.* 16(2016):484-96.

34. BD, Modena. Kurian SM, Gaber LW, and Waalen J, et al. Gene Expression in Biopsies of Acute Rejection and Interstitial Fibrosis/Tubular Atrophy Reveals Highly Shared Mechanisms That Correlate With Worse Long Term Outcomes. *Am J Transplant.* 16(2016):1982-98.

How to cite this article: Weems JC, Pierry D, Holman J, Kandpal M, Kurian SM, Meintjes P, Beswick N, and Levitsky J. "Analytical and Clinical Validation of a Novel Blood-Based Biomarker of Liver Transplant Rejection." *J Transplant Technol Res* 11 (2021): 180.