Blood Citrulline Level Is an Exclusionary Marker for Significant Acute Rejection After Intestinal Transplantation

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Background. Serum citrulline is a marker for acute cellular rejection (ACR) after intestinal transplantation; however, its clinical utility has not yet been established. The goal of this study was to determine clearcut serum levels beyond which the diagnosis of acute rejection could be supported or refuted, and predictors of citrulline levels posttransplant from which more accurate estimates of sensitivity and specificity could be obtained.

Methods. Since March 2004, we obtained 2135 dried blood spot (DBS) citrulline samples from 57 intestinal transplant recipients at or beyond 3 months posttransplant. Stepwise linear regression was performed to determine the most significant multivariable predictors of the patient's DBS citrulline level.

Results. Seven characteristics were associated with a significantly lower citrulline in multivariable analysis: presence of mild, moderate, or severe ACR; presence of bacteremia or respiratory infection; pediatric age; and time from transplant to DBS sample ($P<0.00001$ in each case). Using a <13 vs. ≥ 13 μmoles/L cutoff point, the sensitivity for detecting moderate or severe ACR and the negative predictive value were high (96.4% and >99% respectively). Specificity was 54% to 74% in children and 83% to 88% in adults.

Conclusions. Citrulline levels <13 μmoles/L should alert the clinical team that a serious problem (rejection or infection) could be looming in a previously stable intestinal recipient. Levels $\geq 13\mu$moles/L practically rule out moderate or severe rejection.

Keywords: Citrulline, Dried blood spot, Small intestine transplantation, Acute cellular rejection.

(*Transplantation* 2007;84: 1077–1081)

Short-term graft and patient survival after small intestinal transplantation have improved dramatically (*1–6*). Unfortunately, long-term survival is still impeded by late graft and patient losses. Typically, patients present unexpectedly with advanced rejection and other intestinal pathology, seemingly without warning.

In the absence of other markers, one has to depend on symptoms and clinical findings to monitor the intestinal graft, and once these signs occur there is often already advanced intestinal damage. This phenomenon is not unique to intestinal transplantation. For example, oliguria or jaundice only appear late in the course of renal or hepatic dysfunction. It is the presence of abnormal serum markers (kidney and liver function tests) that usually prompt the clinician to recognize early damage in such solid organ grafts and avoid the loss of the graft.

The intestinal graft, which is known to be highly immunogenic, is at an additional risk because the absorption of the necessary immunosuppressive agents is based on its function. Even mild intestinal dysfunction is associated with erratic absorption of immunosuppressive drugs, which can quickly destabilize even a previously well-functioning graft. As improved short-term results are mainly a result of improved immunosuppression protocols and graft monitoring (*3*, *6*, *7*) with frequent endoscopies and protocol biopsies, likewise, late failures are frequently due to a lack of adequate monitoring and imperfect immunosuppression.

Long-term monitoring with frequent endoscopies and biopsies (the current gold standard of intestinal monitoring) is all but impossible. Graft ileostomies, which provide easy access in the early posttransplant period, are usually closed

This work was supported in part by the National Institutes of Health (DK061445) and the Conselho Nacional Desenvolvimento Cientifico e Tecnologico, Brazil.

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Received 14 June 2007. Revision requested 13 July 2007.
Accepted 10 August 2007.

ISSN 0041-1337/07/8409-1077
DOI: 10.1097/01.tp.0000287186.04342.82

within 1 year from transplantation. Patients often return to their hometown, which can be located far from the specialized intestinal transplant centers.

This problem has been recognized by those engaged in intestinal transplants and has resulted in an intense effort to discover noninvasive markers to monitor the intestinal graft. Promising early results have been reported with the monitoring of stool calprotectin levels (*8*). In addition to the potential logistical problems that such measurements involve, sufficient data to make it clinically useful are missing at this time.

Serum citrulline is another attractive marker (*9–15*). Nearly all of the plasma concentration of this nonprotein amino acid derives from glutamine conversion within enterocytes (*16*). As a consequence, the plasma citrulline level has been invoked as a marker of functional enterocyte mass in patients with short bowel syndrome, villous atrophy-associated small bowel disease, and after radiation and myeloablative therapy (*17–22*). From studies in intestinal transplant patients, it is clear that citrulline levels decrease in the presence of intestinal graft dysfunction including rejection. Specifically, serum citrulline levels were found to decline with increasing grade of acute cellular rejection (ACR) (*10–12*, *14*, *15*).

In an effort to ease the logistics of blood sample collection (particularly in small babies) and shipping, we began a program in March 2004 to measure patients' citrulline levels from a dried blood spot (DBS). We recently demonstrated the accuracy and reliability of this method (*12*). The samples can be obtained with a finger or heel stick and mailed to the transplant center in a regular envelope using conventional filter paper strips (*23*).

In this study, we attempted to determine if a critical serum citrulline value exists that should alert the clinicians of a significant intestinal graft dysfunction in a previously stable graft, so as to trigger an appropriate response. We evaluated samples obtained longer than 90 days after transplantation, because only then do the citrulline levels achieve a plateau (*11*, *14*, *15*) after recovery of the graft from ischemia-reperfusion injury at the time of transplant.

MATERIALS AND METHODS

DBS samples were obtained as specified by the National Committee on Clinical Laboratory Standards (NCCLS) (*23*) and were analyzed by hydrophilic interaction chromatography tandem mass spectrometry (HILIC/MS/MS) using Thermo Finnegan Surveyor/TSQ7000 Instruments. Atmospheric pressure chemical ionization (APCI) was used for mass spectrometry, and quantitation with standard and internal standard ratios was performed utilizing the Xcalibur software. Citrulline determinations were usually obtained within 24 hr of receipt of the DBS sample.

This study was approved by our center's institutional review board; all patients who received a small intestine transplant at our center since March 2004 were eligible. All types of intestinal transplants were included: isolated intestine (I), liver-intestine (LI), multivisceral (MV), and modified multivisceral (MMV) (i.e., multivisceral but without the liver). Patients received immunosuppression, treatment of rejection, and antiviral prophylaxis as previously reported (*3* ,*4*, *6*). Citrulline samples were collected at specific time points posttransplant: biweekly during the first month, then weekly for 3 months, and monthly thereafter. In addition, measurements were obtained when intestinal biopsies were performed and/or if there was clinical suspicion of graft rejection.

Each sample was matched with the patient's clinical status on that sample date regarding the presence/absence of ACR and other complications. Diagnosis of ACR was confirmed with a biopsy, which was read by a transplant pathologist, according to current protocols (*24*, *25*). Diagnosis of ACR also required the presence of clinical symptoms as well as the initiation of treatment for the rejection. The maximum histopathologic grade observed during each ACR episode was recorded as the grade of rejection for that episode, and termination of the episode occurred once the patient had two consecutive negative biopsies coinciding with clinical resolution of symptoms, or when the graft was explanted or if the patient expired. Standard criteria for histopathologic grading of ACR were applied: no ACR, indeterminate for rejection (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3) (*24*, *25*).

Infectious episodes were defined by the presence of clinical symptoms and confirmed by positive cultures as to pathogen and location. Resolution of an infectious event was defined by the occurrence of a negative culture without further clinical symptoms (i.e., 14 days of appropriate therapy without further clinical signs of infection, or death). DBS citrulline and culture samples were matched according to the sample date.

Statistical Methods

Because the distribution of citrulline was skewed towards larger values, the statistical analysis was performed on the log scale (a concentration often follows a lognormal distribution) (*26*). The median is equal to the geometric mean in the log-normal distribution; thus, all means were expressed here as the geometric mean. Stepwise linear regression was performed in determining the most important predictors of log {DBS citrulline}. Factors that were considered included: recipient gender and age at transplant, type of transplant (I or LI vs. MV or MMV), time from date of transplant to DBS sample (in months), presence of mild, moderate, or severe ACR, presence of a blood, respiratory, urinary tract, or intestinal infection, and presence of posttransplant lymphoproliferative disorder. Only the significant univariable predictors ($P<0.05$) were considered in the multivariable stepwise regression analysis.

RESULTS

Fifty-seven (54 primary transplant and 3 retransplant) patients contributed 2,135 DBS citrulline samples that met the study entry criteria. Most of the citrulline samples were collected serially every time there was clinical suspicion of rejection (within 24 hr of the initiation of symptoms) and during the rejection episode, in addition to the surveillance samples that were routinely collected posttransplantation. Thirty-seven patients were children (median age at transplant: 1 year); 20 patients were adults (median age at transplant: 36 years). Twenty-one patients received an isolated intestinal or combined liver-intestinal transplant (9 children, 12 adults); 36 patients received a multivisceral or modified multivisceral trans-

TABLE 1. Citrulline values according to the presence of acute cellular rejection (ACR)

	DBS citrulline level					
	<5 μmoles/L	5–10 μmoles/L	10–15 μmoles/L	15–20 μmoles/L	≥20 μmoles/L	Total
ACR	77 (28.7)	122 (45.5)	46 (17.2)	13 (4.9)	10 (1.9)	268
No ACR	128 (6.9)	463 (24.8)	440 (23.6)	315 (16.9)	521 (27.9)	1,867
Total	205 (9.6)	585 (27.4)	486 (22.8)	328 (15.4)	531 (24.9)	2,135

Data are n (%).

plant (28 children, 8 adults). The median number of DBS citrulline samples contributed per patient was 26 (range: 1–142); the median time after transplant until DBS sample date was 18.8 months (range: 3.0–125.3 months). The median DBS citrulline was 12.7 μmoles/L (range: 1.4 to 91.8 μmoles/L). There were 25 ACR episodes observed among 15 patients (five patients had two episodes, one patient had three episodes, and one patient had four episodes); these ACR episodes were mild, moderate, and severe in 9, 11, and 5 cases, respectively.

Distribution of the 2,135 DBS citrulline values according to the presence/absence of ACR is shown in Table 1, using five categories for DBS citrulline: <5, 5–10, 10–15, 15–20, and ≥20 μmoles/L. As expected, there was a highly significant association of lower citrulline with the presence of ACR ($P<0.00001$). When rejection was present, 91.4% (245 of 268) of samples showed citrulline levels <15 μmoles/L vs. 55.2% (1031 of 1867) when ACR was absent. These results demonstrate a high sensitivity but poor specificity of citrulline levels <15 vs. ≥15 μmoles/L for detecting ACR.

Univariable analysis found eight factors to be associated with a significantly lower citrulline level (Table 2). These included the presence of any rejection, mild ($P<0.00001$), moderate ($P<0.00001$) or severe ($P<0.00001$). Other significant factors were: presence of bacteremia ($P<0.00001$), shorter time from date of transplant to DBS sample ($P<0.00001$), younger age at transplant ($P<0.00001$), presence of a MV or MMV transplant ($P<0.00001$), and presence of a respiratory infection ($P=0.00005$). Sex, urinary tract infection, localized intestinal infection, and posttransplant lymphoproliferative disorder were not associated with a lower citrulline level ($P>0.15$). Patients receiving a MV or MMV transplant had a shorter time from transplant to DBS sample (58.7% of samples were obtained 3 years or later from transplant in I/LI transplant recipients vs. 18.2% in MV/MMV transplant recipients ($P<0.00001$) because a greater percentage of MV/MMV transplants were performed more recently. Thus, the observed univariable association of MV/MMV transplant with lower citrulline levels was explained entirely by its association with a shorter time to DBS sample.

Stepwise linear regression analysis led to a seven-variable predictive model for citrulline level ($P<0.00001$ for each factor; Table 3). The presence of moderate or severe rejection was associated with the greatest decline in the expected mean for DBS

TABLE 2. Univariable linear regression predictors of log{DBS citrulline}

	Percent (n) or Median	Univariable F-test *P* value (corresponding F-test Statistic)
Male sex	36.9% (788/2,135)	0.94
Younger age at transplant (median years)	1.0	<0.00001 (151.5)
Shorter time to DBS sample (median months)	18.8	<0.00001 (212.6)
Received MV or MMV transplant	69.0% (1,474/2,135)	<0.00001 (50.7)
Mild rejection	3.3% (71/2,135)	<0.00001 (20.8)
Moderate rejection	6.3% (134/2,135)	<0.00001 (190.6)
Severe rejection	3.0% (63/2,135)	<0.00001 (41.4)
Bacteremia (blood infection)	31.7% (677/2,135)	<0.00001 (308.9)
Respiratory infection	2.5% (53/2,135)	0.00005 (16.6)
Urinary tract infection	2.5% (54/2,135)	0.19
Localized intestinal infection	3.7% (80/2,135)	0.37
Posttransplant lymphoproliferative disorder	2.9% (61/2,135)	0.20

Each *P* value was based on comparing the F-test statistic with the F distribution having 1 degree of freedom in the numerator and 2133 degrees of freedom in the denominator. A larger F-test statistic corresponds with a smaller *P* value.

TABLE 3. Multivariable linear regression model for log{DBS citrulline}

Characteristic	Model Coefficient± Standard error	Multivariable F-test *P* value
Severe rejection	−0.839±0.074	<0.00001
Moderate rejection	−0.729±0.052	<0.00001
Respiratory infection	−0.497±0.080	<0.00001
Bacteremia (blood infection)	−0.470±0.027	<0.00001
Mild rejection	−0.468±0.070	<0.00001
Time to DBS sample <36 months	−0.328±0.027	<0.00001
Pediatric patient	−0.289±0.028	<0.00001

All characteristics were dichotomous, with 0 and 1 representing the absence and presence of the characteristic. Characteristics were listed in order of magnitude of the regression coefficient. The magnitude of each variable's effect is represented by it model coefficient, and a negative coefficient implies a lowering of the citrulline level.

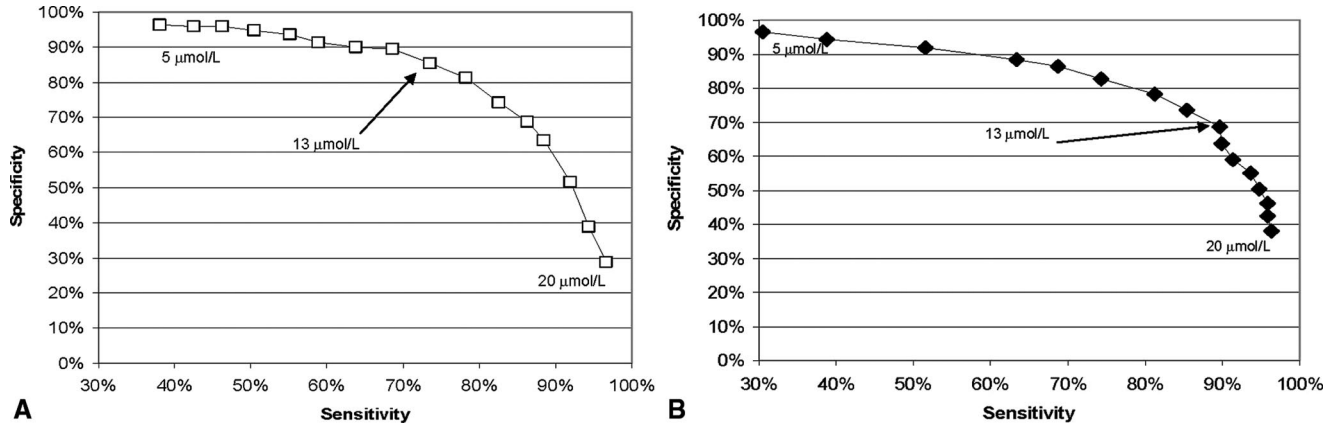

FIGURE 1. Calculated sensitivity and specificity for citrulline values ranging from 5 through 20 μmoles/L in detecting any type of rejection (A) or moderate/severe rejection (B). At 13 μmoles/L, the highest specificity/sensitivity combination is achieved.

citrulline, (52% to 57% decline), followed by the presence of a respiratory infection, bacteremia, and mild rejection (each predicting a 37% to 39% decline).

Analysis of the best value to use in clinical practice aiming at the highest possible sensitivity while maintaining reasonable specificity showed that the value of 13 μmoles/L was the best cutoff point (Fig. 1A, B). Calculations using this cutoff point of 13 μmoles/L showed a sensitivity of 96.4% (190 of 197) for detecting moderate or severe ACR. Specificity (percentage of DBS samples ≥13 μmoles/L in the absence of ACR and infection) was 68.6% (Table 4). Using the same parameter of <13 vs. ≥13 μmoles/L, the negative predictive value (NPV) was >99% (99.2% in children and 99.5% in adults). Contrary to this high NPV, the positive predictive value (PPV) was low: 14.4% in children and 28.7% in adults. In practical terms, levels of ≥13 μmoles/L exclude moderate or severe rejection with >99% certainty. Using the same value of 13 μmoles/L as a cutoff point, sensitivity for mild rejection was 70.4% (Table 4).

In the absence of ACR, a blood or respiratory infection resulted in a drop of citrulline levels below 13 μmoles/L, with an observed sensitivity of 75.6% (Table 4).

TABLE 4. Cross-tabulation of DBS citrulline values, using 13 μmoles/L as a cutoff

	DBS citrulline level		
	<13 μmoles/L	**≥13 μmoles/L**	**Total**
Any rejection	240 (89.5)	28 (10.5)	268
Moderate or severe rejection	190 (96.4)	7 (3.6)	197
Mild rejection	50 (70.4)	21 (29.6)	71
Infection (blood or respiratory) without rejection	476 (75.6)	154 (24.4)	630
No rejection, no infection	388 (31.4)	849 (68.6)	1,237

Data are n (%).

DISCUSSION

This is the largest study of serum citrulline levels after intestinal transplantation performed to date, and focuses on its use 3 months or longer after transplantation for two reasons. First, citrulline levels during the initial posttransplant period gradually increase due to recovery from the ischemia reperfusion injury and are impossible to correlate with rejection or other serious complications. Second, during this early posttransplant period, the need for noninvasive markers is less pressing because endoscopic surveillance and biopsies—the current gold standard of follow-up—can be used with relative ease. The patients usually remain in the vicinity of the transplant center and are followed closely. Access to the graft is easy through a standard graft ileostomy through which endoscopy is performed. As mentioned earlier, the major contribution from noninvasive studies is expected to be for long-term patients who need to be monitored at home.

The most important finding of our study is that citrulline levels have a very high negative predictive value for moderate or severe acute rejection in these patients. Indeed, there is only a less than 1% chance of such pathology to be found when the DBS is ≥13 μmoles/L. Thus, use of the <13 vs. ≥13 μmoles/L cutoff point appears to provide excellent sensitivity detecting for moderate or severe ACR. Sensitivity is 96.4%, with particularly high specificity in adult patients. A DBS value of <13 μmoles/L as measured at our laboratory should alert the clinical team that something might be seriously wrong with the patient. Although the occurrence of a rejection is a most likely possibility, the presence of a bacteremia or a respiratory infection needs to also be considered.

This simple guideline, combined with the simplicity of obtaining and shipping the samples by mail, could help detect "trouble" for the intestinal transplant recipient, prompt further studies, and hopefully avoid advanced and irreversible pathology as we now see it.

A limitation of our study is that it cannot assess the timeliness of this warning. This limitation is due to lack of a sufficiently large number of citrulline data immediately prior to the occurrence of those episodes, in addition to the rela-

tively small number of rejection episodes with corresponding citrulline samples that were available in the study.

This issue is currently being addressed with a systematic collection of more samples. A more complete panel of substances, perhaps including calprotectin and other assays, will be needed to improve the diagnostic power of serum citrulline as a monitoring marker of the intestinal transplant graft.

ACKNOWLEDGMENT

The authors gratefully acknowledge Ms. Wynn Howard for her expertise in preparing the figure for this manuscript.

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