The clinical significance of asymmetric dimethylarginine

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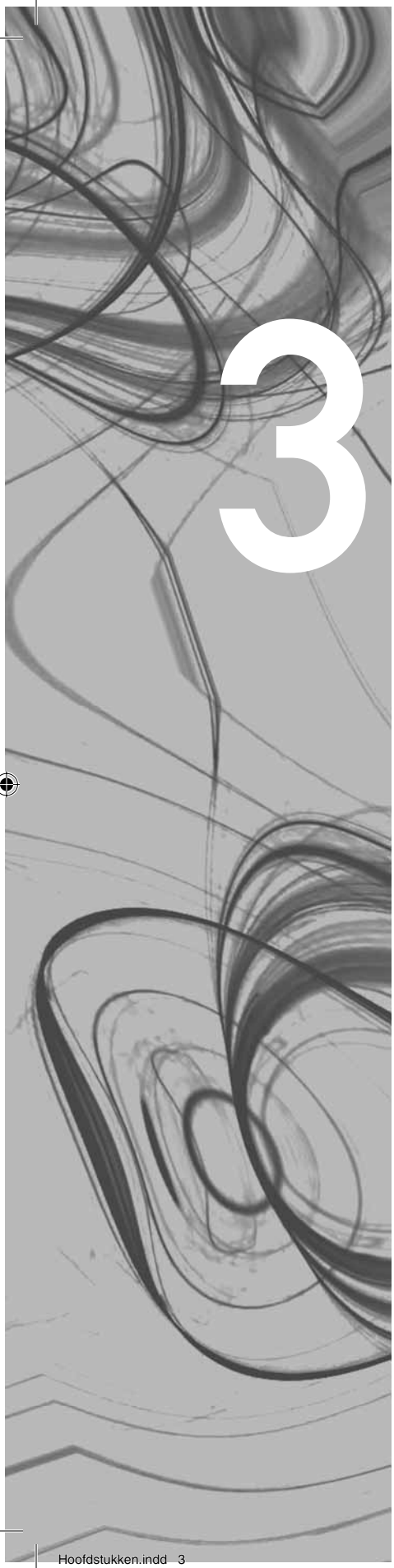

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The transplanted liver graft is capable of clearing asymmetric dimethylarginine

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ABSTRACT

Asymmetric dimethylarginine (ADMA) has been recognised as an endogenous inhibitor of the arginine-nitric oxide (NO) pathway. Its concentration is tightly regulated by urinary excretion and degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which is highly expressed in the liver. Considering the liver as a crucial organ in the clearing of ADMA, we hypothesised increased ADMA levels during hepatic failure and, consequently, a decline of ADMA concentrations after successful liver transplantation. The aim of the present study was to investigate the role of the liver in the metabolism of ADMA in patients undergoing liver transplantation. In this prospective study, we investigated the course of ADMA concentrations in 42 patients undergoing liver transplantation and results showed that preoperative ADMA concentrations were higher in patients with acute (1.26 μM, $p<0.001$) and in patients with chronic (0.69 μM, $p<0.001$) hepatic failure compared with healthy volunteers (0.41 μM). In addition, ADMA concentrations decreased from the preoperative day to the first postoperative day in both the acute ((Δ_{ADMA}: -0.63 μM, $p=0.005$) and the chronic hepatic failure group (Δ_{ADMA}: -0.15 μM, $p<0.001$). Furthermore, in patients who experienced acute rejection, ADMA concentrations were higher during the whole first postoperative month compared with non-rejectors ($p=0.012$). Moreover, in 11 of 13 rejectors (85%) a clear increase in ADMA concentration preceded the onset of the first episode of rejection which was confirmed by liver biopsy. In conclusion, our results indicate that the transplanted liver graft is quickly capable of clearing ADMA, suggesting preservation of DDAH. In addition, increased ADMA concentrations in the posttransplantation period reflect serious dysfunction of the liver graft during acute rejection.

INTRODUCTION

Raised concentrations of asymmetric dimethylarginine (ADMA) have been reported in patients with conditions characterised by endothelial dysfunction and the role of ADMA as a novel cardiovascular risk factor is emerging.[1] Therefore, it is of great importance to understand which organs are of relevance in the metabolism of ADMA. ADMA reduces the bioavailabilty of nitric oxide (NO) both by inhibiting the enzyme NO synthase and by competing with arginine for cellular transport across cationic amino acid transporters (CAT) of system y^+.[2] The elimination of ADMA occurs partially via urinary excretion.[3] However, the most important metabolic pathway for ADMA is degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) which is highly expressed in the liver, but is also present in the pancreas, the kidney, and in endothelial cells.[4,5]

The role of the kidney in the metabolism of ADMA has been extensively studied. Several reports have shown elevated concentrations in patients with renal failure and ADMA has emerged as a predictor of mortality and cardiovascular outcome in these patients.[6-11] Unfortunately, the role of the liver, which contains large amounts of the ADMA degrading enzyme DDAH, has been underexposed. Carnegie and coworkers[12] for the first time pointed out the potential role of the liver in the metabolism of ADMA by reporting an increased urinary excretion of ADMA in patients with liver disease. However, from this study no precise data on the hepatic metabolism of ADMA can be derived, as only urinary concentrations were measured. Recently, in a rat model, we[13] found a very high uptake of ADMA by the liver, suggesting a crucial role for the liver in regulating systemic ADMA concentrations. Moreover, in critically ill patients, ADMA concentrations were seriously elevated and proved to be significantly related to hepatic dysfunction.[14] The results of these studies support the concept that the role of ADMA is not only confined to chronic diseases as cardiovascular disorders and renal failure. Considering the human liver as a crucial organ in the clearing of ADMA, increased ADMA levels in patients eligible for liver transplantation may be expected. Hepatic function improves after a successful transplantation procedure and, consequently, ADMA levels may decrease. To investigate the course of ADMA levels after a liver transplantation procedure, we determined ADMA plasma concentrations before the operation, during the first postoperative month, and one year after the transplantation procedure.

PATIENTS and METHODS

Patients

In this prospective study, blood samples were drawn from 42 patients who underwent liver transplantation between June 1998 and April 2000 at the Erasmus University Medical Center, Rotterdam, The Netherlands. The protocol was approved by the institutional review boards, and informed consent was obtained before participation in the study.

Immunosuppression and antimicrobial prophylaxis

Immunosuppression was started as triple therapy (n=16), i.e. prednisone, cyclosporine A, and azathioprine, or as double therapy (n=26), i.e. prednisone with cyclosporine A or tacrolimus (Prograft, Fujisawa, Deerfield, IL).

For selective bowel decontamination, all patients received cefotaxime 1 g four times a day until cultures of throat and rectum were negative for Gram-negative bacteria, amphotericin B suspension 500 mg four times per day, and colistin/norfloxacin capsule 200/50 mg four times per day during 30 days posttransplantation.

Acute liver allograft rejection

Liver biopsies were obtained when an episode of clinical signs compatible with rejection (malaise, fever, jaundice, decreased bile volume, and decreased bilirubin concentration in bile) was present in combination with deteriorating hepatic function (elevated serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT), and alkaline phosphatase (AP)). Acute liver allograft graft rejection was diagnosed by histological characteristics of acute rejection according to the Banff criteria.[15] Patients were treated when Rejection Activity Index (RAI) $\geq$ 6. For treatment of acute rejection, patients received high dose methylprednisolone (1000 mg per day for 3 days) intravenously. The onset of acute rejection was defined as the first day of treatment.

Blood sampling and laboratory procedures

One day before, and on day 1, 3, 7, 14, 21, 28, and 1 year after the transplantation procedure, blood was drawn for the determination of ADMA, which was measured by high-performance liquid chromatography with fluorescence detection, as recently

described.[16] Briefly, 0.1 mL of plasma was mixed with 0.1 mL of a 40 µmol/L solution of the internal standard MMA and 0.8 mL phosphate buffered saline (PBS). This mixture was applied to Oasis MCX solid-phase extraction columns (Waters) for extraction of basic amino acids. The columns were consecutively washed with 1.0 mL of 100 mM HCl and 1.0 mL methanol. Analytes were eluted with 1.0 mL of concentrated ammonia/water/methanol (10/40/50). After evaporation of the solvent under nitrogen, the amino acids were derivatised with ortho-phthaldialdehyde reagent containing 3-mercaptopropionic acid. The derivatives were separated by isocratic reversed-phase chromatography on a Symmetry C18 column (3.9 x 150 mm; 5µm particle size; Waters). Potassium phosphate buffer (50 mM; pH 6.5), containing 8.7% acetonitrile was used as mobile phase at a flow-rate of 1.1 mL/min and a column temperature of 30°C. Fluorescence detection was performed at excitation and emission wavelengths of 340 and 455 nm, respectively. After elution of the last analyte, strongly retained compounds were quickly eluted by a strong solvent flush with 50% acetonitrile, resulting in a total analysis time of 30 min. The coefficients of variation were 1.2% within-assay and 2.0% between-assay. Reference values for ADMA have been obtained from plasma of healthy laboratory personnel and medical students.[16] Laboratory parameters indicating hepatic function (bilirubin, ALT, AST, γ-GT, and AP) and renal function (creatinine and urea) were measured by standard laboratory methods.

Statistical analyses

Logarithmic transformation was performed when data where not normally distributed. Differences among various timepoints between two groups were tested using independent-samples t-test. Within-group differences between timepoints were tested by paired-samples t-test. When more appropriate, Mann-Whitney U test was used to investigate differences among various timepoints between two groups and Wilcoxon signed-ranks test was used to investigate within-group differences between timepoints. Transformed data are presented as geometric means and 95% confidence intervals (95% CI). Non-parametric data are presented as medians and interquartile ranges (IQR). For the assessment of differences during the first postoperative month (day 1 to day 28) between groups, general linear model (GLM) for repeated measurements was used. Relations between variables during the first postoperative month were investigated by computing cross-product deviations and

covariances. As a result of the way the correlation coefficients were constructed, only approximations of p-values and confidence intervals could be computed. For this, Fisher's Z-transformation was used where the variance was adjusted with the correct number of degrees of freedom. P<0.05 was considered statistically significant. Statistical analyses were performed using SPSS (SPSS 11.0 for Windows).

RESULTS

Patients

Indications for liver transplantation were chronic hepatic failure (n=35) caused by: hepatitis B and hepatits C virus-related cirrhosis (n=11), primary sclerosing cholangitis or primary biliary cirrhosis (n=8), cryptogenic cirrhosis (n=5), alcoholic cirrhosis (n=5), Wilson's disease (n=3), and other diseases (n=3), and acute hepatic failure (n=7).

Thirteen patients (31%) were treated with high-dose methylprednisolone for one or more episodes of biopsy-proven acute rejection during the first postoperative month.

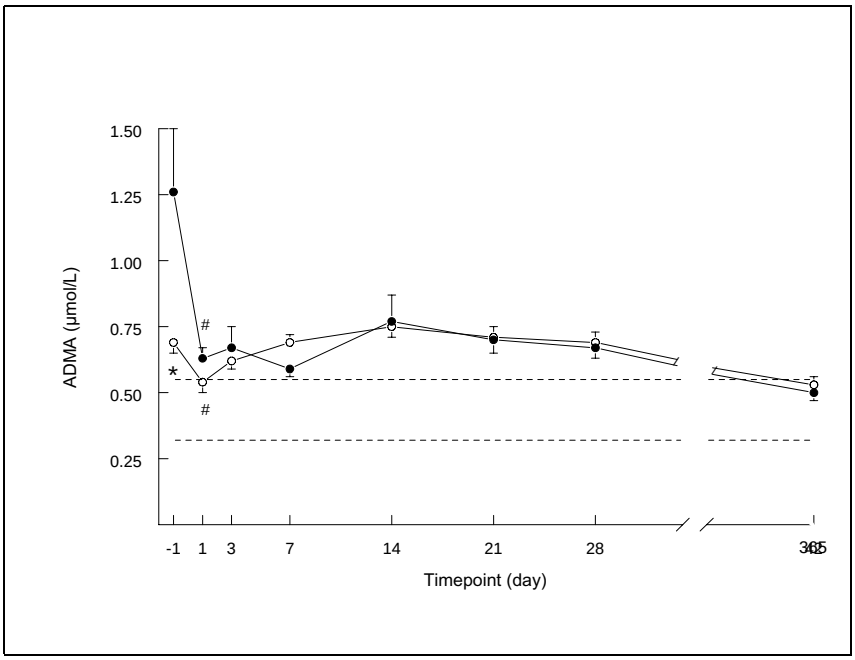
Course of ADMA concentration

In **Figure 1**, the course of ADMA concentrations in patients with acute and patients with chronic hepatic failure is shown. Compared with the mean ADMA concentration of healthy volunteers (0.41 μmol/L, 95% CI: 0.32-0.55), preoperative ADMA levels were significantly higher in patients with acute hepatic failure (1.26 μmol/L, 95% CI: 0.54-2.89 μmol/L, p<0.001) and in patients who underwent liver transplantation because of chronic hepatic failure (0.69 μmol/L, 95% CI: 0.35-1.39, p<0.001). In addition, the preoperative ADMA concentration of the acute hepatic failure group (1.26 μmol/L) was higher compared with the preoperative ADMA concentration of the chronic hepatic failure group (0.69 μmol/L, p=0.003). Furthermore, concentrations of ADMA were significantly lower one day after the transplantation procedure in comparison with the preoperative day in both the acute (0.63 μmol/L, 95% CI: 0.47-0.84, p=0.005) and the chronic group (0.54 μmol/L, 95% CI: 0.29-1.00, p<0.001). One day after the operation and during the whole postoperative course, ADMA concentrations did not differ between both groups. One year after the transplantation

procedure, mean ADMA concentrations of both the acute (0.50 μmol/L, 95% CI: 0.44-0.56) and the chronic hepatic failure group (0.53 μmol/L, 95% CI: 0.45-0.63) were below the upper limit of the reference range (0.32-0.55 μmol/L).

Figure 1:

The course of ADMA concentrations in patients with acute or chronic hepatic failure undergoing liver transplantation



Data represent geometric means. Scattered line: upper + lower limit of reference range.
Black dots: acute hepatic failure (n=7). White dots: chronic hepatic failure (n=35).
* indicates statistically significant difference between groups.
# indicates statistically significant difference with respect to previous timepoint.

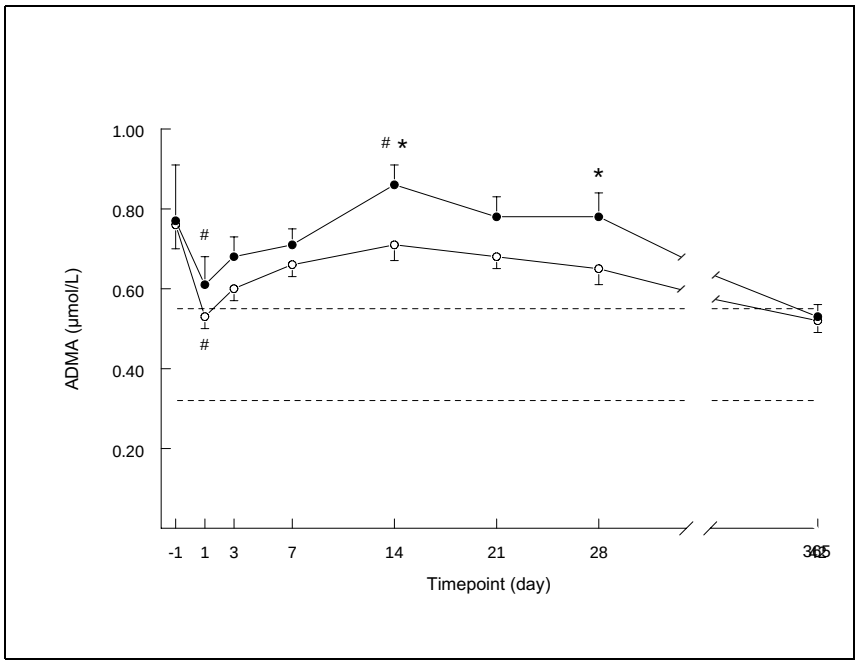
ADMA concentrations during rejection

The course of ADMA concentrations in patients who experienced acute rejection (rejectors: n=13) and patients without episodes of rejection (non-rejectors: n=29) is shown in **Figure 2**. General linear model for repeated measurements (corrected for hepatic failure) showed that during the first postoperative month, ADMA levels were significantly higher in rejectors compared with non-rejectors (p=0.013). In rejectors, ADMA increased significantly from day 7 (0.71 μmol/L, 95% CI: 0.48-1.04) to day 14

(0.86 μmol/L, 95% CI: 0.78-1.28, p=0.032). Furthermore, ADMA levels were significantly higher in the rejectors on day 14 (p=0.012) and day 28 (p=0.027) compared with non-rejectors.

Figure 2:

The course of ADMA concentrations in patients with biopsy proven acute liver allograft rejection and patients without episodes of rejection

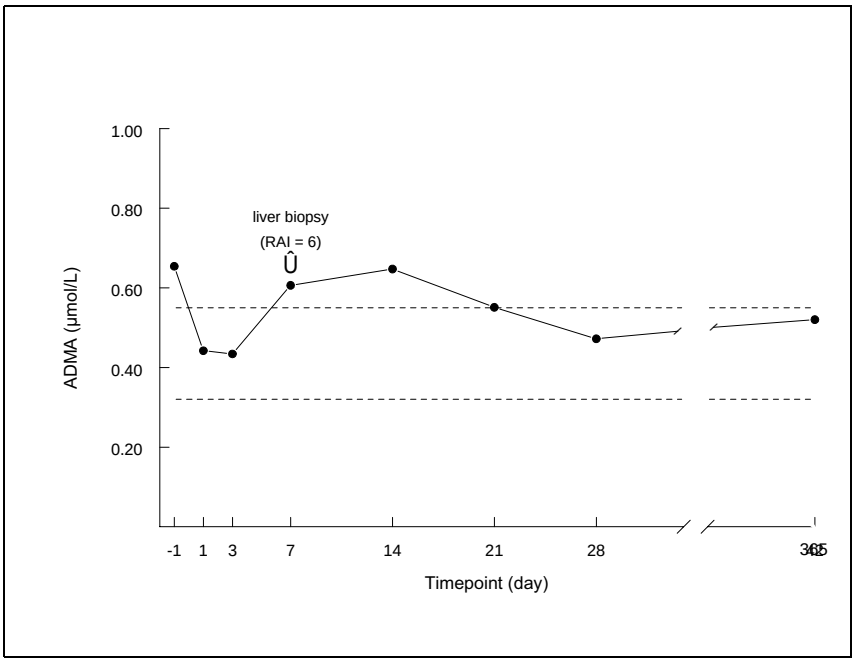


Data represent geometric means. Scattered line: upper + lower limit of reference range.
Black dots: rejectors (n=13). White dots: non-rejectors (n=29).
* indicates statistically significant difference between groups.
# indicates statistically significant difference with respect to previous timepoint.

Liver biopsies were taken in 26 of 42 patients. From this group, the course of ADMA prior to the first biopsy clearly predicted whether rejection occurred or not in 13 cases. Furthermore, in 11 of 13 rejectors (85%), an obvious increment in ADMA concentration preceded the onset of the first episode of rejection as proven by liver biopsy (example in **Figure 3**).

Figure 3:

The course of ADMA concentrations in one patient with biopsy proven acute liver allograft rejection



RAI = Rejection Activity Index according to the Banff criteria.[15]

Course of biochemical markers of hepatic function

Mean concentrations of ALT and AST of the whole patient group significantly increased from the day before transplantation (226 U/L, 95% CI: 218-234 and 195 U/L, 95% CI: 186-203, respectively) to the first postoperative day (725 U/L, 95% CI: 720-730, $p<0.001$ and 666 U/L, 95% CI: 661-670, $p<0.001$, respectively). In both rejectors and non-rejectors, a decrease of ALT and AST was seen during the first postoperative month. Values for each parameter returned to normal values within one month. General linear model for repeated measurements showed that the concentrations of ALT and AST during the first postoperative month were higher ($p=0.049$ and $p=0.04$, respectively) in rejectors compared with non-rejectors.

Median AP concentration of the whole patient group decreased from the day before transplantation (177 U/L, IQR: 110-204) to the first postoperative day (82 U/L, IQR:

64-110, p<0.001). Mean concentration of γ-GT also decreased in the whole patient group. However, this decline was not statistically significant (p=0.055). The postoperative course of γ-GT and AP showed no significant differences between rejectors and non-rejectors.

In the whole patient group, mean bilirubin concentration decreased following the transplantation procedure from 91 μmol/L (95% CI: 86-97) to 64 μmol/L (95% CI: 60-68, p=0.006). During the whole first postoperative month, bilirubin concentrations were higher (p<0.001) in rejectors compared with non-rejectors.

Correlations between ADMA and biochemical markers of hepatic function of all patients are shown in the **Table** below.

Table: Correlations of ADMA with biochemical markers

	ADMA		
	r	95% CI	p
Markers of hepatic function			
ALT	0.205	0.033 – 0.365	0.020
AST	0.207	0.035 – 0.367	0.018
AP	0.175	0.002 – 0.338	0.047
γ-GT	0.043	-0.131 – 0.214	0.629
Bilirubin	0.392	0.235 – 0.529	<0.001
Markers of renal function			
Creatinine	0.192	0.020 – 0.353	0.029
Urea	0.390	0.233 – 0.527	<0.001

Course of biochemical markers of renal function

Median creatinine concentration of the whole patient group increased from the day before transplantation (74 μmol/L, IQR: 60-95) to the first postoperative day (78 μmol/L, IQR: 65-126, p=0.005). The postoperative course of creatinine levels did not show a statistically significant difference between rejectors and non-rejectors. In addition, there were no differences between creatinine concentrations on corresponding timepoints of rejectors and non-rejectors.

In the whole patient group, mean urea concentration increased from the day before transplantation (5.8 mmol/L, 95% CI: 2.2-9.4) to the first postoperative day (10.3 mmol/L, 95% CI: 6.9-13.8, p<0.001). Concentrations of urea during the entire first postoperative month were higher (p=0.014) in rejectors compared with non-rejectors.

Correlations between ADMA and biochemical markers of renal function of all patients are shown in the **Table** above.

DISCUSSION

The fact that ADMA concentrations are elevated during hepatic failure in combination with the significant decline on the first postoperative day after liver transplantation points to a crucial role of the human liver as ADMA eliminating organ and suggests that DDAH activity is preserved during the transplantation procedure. These results confirm our finding of an organ balance study in rats in which a high net uptake of ADMA by the liver was calculated from the determination of arteriovenous concentration differences and organ blood flow across the liver, indicating an important role for the liver in the clearing of ADMA.[13]

Dimethylarginines are synthesised by post-translational modification, involving addition of methyl groups to arginine residues in proteins by enzymes called protein arginine methyltransferases. These specific proteins are predominantly found in the nucleus and play a role in RNA processing and transcriptional control.[17] Both dimethylarginines are released when these proteins are hydrolysed, thereby being an obligatory product of protein turnover. Besides ADMA, the stereo-isomer symmetric dimethylarginine (SDMA) is found in human plasma. In contrast to ADMA, SDMA does not directly inhibit NO synthase but is able to interfere with NO synthesis by competing with arginine and ADMA for cellular transport across cationic amino acid transporters (CAT) of system y^+.[2] ADMA can be regarded as the predominant endogenous inhibitor of NO biosynthesis and has been proven to be a marker of endothelial dysfunction. Both ADMA and SDMA are removed from the body by urinary excretion. Therefore, many studies in the field of dimethylarginines have focussed on the elevation of both dimethylarginines during renal failure.[6,7,10,11] The liver may also be important in the clearing of ADMA because this organ contains large amounts of the enzyme DDAH which breaks down ADMA into dimethylamine and citrulline.[5] Surprisingly, the role of the liver as ADMA clearing organ has hardly received attention up till now. The potential role of the liver in the metabolism of dimethylarginines has for the first time been described in 1977.[12] In this study, the investigators found a decreased urinary excretion ratio of SDMA to ADMA in patients with chronic active hepatitis, owing to an increased output of ADMA. It could be hypothesised that the ADMA eliminating capacity of the liver in these patients is impaired and that the body balanced ADMA plasma concentration via the kidney by increasing urinary excretion of ADMA. Because hepatic function slowly deteriorates

during chronic hepatic failure, another potential compensatory pathway may be upregulation of the ADMA degrading enzyme DDAH at locations outside the liver, e.g. kidney, endothelium, and pancreas. In contrast, during acute liver failure, hepatic dysfunction develops quicker and the body may not be able to compensate for the loss of the ADMA eliminating capability of the liver. Moreover, the severity of hepatic dysfunction on the day before the transplantation procedure is often worse in patients who suffer from acute hepatic failure. Therefore, higher preoperative ADMA levels and a more dramatic decline in ADMA clearing capacity could be expected during acute hepatic failure. These hypotheses are substantiated by the present study which showed significantly higher concentrations of ADMA in patients with acute hepatic failure compared with patients who suffered from chronic hepatic failure. Probably, future experiments measuring DDAH activity in the livers removed at the time of transplantation will give more insight in the underlying mechanism of the difference in preoperative ADMA levels between patients with acute and with chronic liver failure.

Up till now, no single laboratory parameter has been found to sensitively mirror hepatic function in the posttransplantation period. Nowadays, acute liver graft rejection is suspected when biochemical markers of hepatic function deteriorate in combination with the presence of an episode of clinical signs. In the present study, significantly higher concentrations of ALT and AST were seen during the postoperative course in patients with episodes of rejection. This coincides with the finding that hepatocellular damage is greater during rejection, leading to leakage of intracellular enzymes. Interestingly, urea levels were also significantly higher in rejectors. Since renal function, as determined by creatinine concentrations, did not differ between rejectors and non-rejectors, the most likely explanation for higher urea concentrations is an increased liberation of the enzyme arginase which is abundantly present in the liver and breaks down arginine into ornithine and urea.[18] Arginase levels have been shown to fluctuate slightly beyond the normal range in successful liver recipients, while higher levels were observed in unsuccessful recipients.[19] Efflux of arginase from the graft causes a significant increase of ornithine and a significant drop of arginine after liver transplantation.[20] Theoretically, arginine deficiency caused by arginase may limit synthesis of NO in these patients. NO deficiency has been reported to play an important role in several models of liver injury. Very recently, Yagnik and coworkers[21] investigated the arginine-NO pathway in liver transplant preservation injury. They performed orthotopic liver transplantation in syngeneic rats

and also measured an increase of arginase with a concomitant increase in the concentration of ornithine and a decrease in arginine levels. In addition, they showed that the administration of arginine significantly decreased levels of ALT and AST compared with control grafts, while nonselective (blocking both constitutively expressed endothelial NO synthase (cNOS) and inducible NO synthase (iNOS)) blockade of NO synthesis significantly increased serum transaminase levels and necrotic and apoptotic cell death in the transplanted graft. Interestingly, addition of an iNOS selective inhibitor only mildly increased liver transaminase levels but also increased apoptosis in the liver graft.

It has been shown that potent inhibitors of NOS, identified as monomethylarginine and ADMA, are produced in human livers during the cold ischaemia period of the graft and that a longer ischaemia time caused significantly greater concentrations of these inhibitors in the preservation solution.[22] Moreover, a significant relationship was found between the extent of NO synthase inhibition and early graft function. Therefore, the authors suggested that measurement of methylarginines in the graft preservation solution could be a useful predictor of early liver function. However, no plasma concentrations of methylated arginines were measured in the postoperative course and the graft function was only evaluated for the first 3 days after the transplantation procedure. In the present study, we measured plasma concentrations of ADMA during the first postoperative month and showed that ADMA levels were higher in rejectors compared with non-rejectors in this period. Raised ADMA concentrations may result from both increased production and decreased elimination. Production of dimethylarginines could theoretically arise from increased protein methylation or increased turnover (e.g. catabolism) of these specific proteins that play a role in protein-nucleic acid interactions.[23] Because only ADMA, and not SDMA (data not shown), proved to be significantly higher in rejectors compared with non-rejectors, higher catabolic rate of body proteins in rejectors is not a likely cause of the higher ADMA concentrations in this group. Conversely, a rise in ADMA is seen when renal function is inadequate. However, in this study there was no significant difference between creatinine levels in rejectors and in non-rejectors. This indicates that the sole significant rise of plasma ADMA levels in rejectors can not be explained by a worse function of the kidney in this group. Therefore, the most likely explanation for the difference between ADMA in rejectors and non-rejectors is impaired graft function, as reflected by higher bilirubin levels in the rejectors of our patient

population, leading to a reduced activity of the ADMA degrading enzyme DDAH in the liver. Measuring DDAH activity in liver tissue of rejectors and non-rejectors after liver transplantation would have provided more information on impairment of DDAH. However, these measurements were not feasible because it is not ethical to routinely obtain liver biopsies from all studied patients. In addition, it should be realised that a small biopsy specimen may not be representative of overall hepatic DDAH activity because there may be areas with high and areas with low activity in the liver. Interestingly, it has recently been revealed that DDAH activity is inhibited by several factors such as oxidative stress, inflammation, and hyperglycaemia.[24-26] Immunosuppressive and anti-microbial medications given to the patients could have influenced activity of DDAH. However, both agents focus on reduction of the inflammatory state, thereby most likely attenuating instead of enhancing DDAH impairment caused by cytokines and microbial products.

In conclusion, our results point to a crucial role of the liver in the elimination of ADMA from the systemic circulation and show that increased concentrations of ADMA during the posttransplantation period reflect impaired hepatic function of the graft during rejection.

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