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# Changes in Biliary Levels of Arginine and its Methylated Derivatives after Hepatic Ischaemia/Reperfusion

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*Abstract:* Arginine (Arg) can be methylated to form symmetrical dimethylarginine (SDMA) and asymmetrical dimethylarginine (ADMA), the latter an endogenous inhibitor of nitric oxide synthase (NOS). SDMA is excreted in the urine, while ADMA is mainly subjected to degradation in the liver. Arg competes with ADMA and SDMA for cellular transport across cationic amino-acid transporters (CATs). We evaluated the changes in serum, tissue and biliary levels of Arg, citrulline (Cit), ADMA and SDMA and the modifications in CATs after ischaemia-reperfusion (I/R). Male Wistar rats were subjected to 30-min. partial-hepatic ischaemia or sham-operated. After 60-min. reperfusion, the concentrations of ADMA, SDMA, Arg and Cit in serum, tissue and bile were measured. Serum levels of AST, ALT and alkaline phosphatase (AP) levels were determined. mRNA of cationic transporter 2A (CAT-2A) and 2B (CAT-2B) were also quantified. An increase in ADMA and a decrease in SDMA were observed in bile at the end of reperfusion. On the contrary, lower tissue ADMA levels and higher SDMA levels were quantified. No serum changes in ADMA and SDMA were found. A decrease in Arg and an increase of Cit were detected in serum, bile and tissue after I/R. A marked increase in AST, ALT and AP levels in serum confirmed I/R injury. A decrease in mRNA transporter CAT-2A but not in CAT-2B was detected. This study supported a biliary CAT-2B–dependent transport of ADMA and demonstrated, for the first time, that the liver is also responsible for the biliary excretion of SDMA into the bile.

There are three isoforms of the enzyme nitric oxide synthase (NOS) in mammals: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). All three isoforms oxidize arginine (Arg) to citrulline (Cit) in a reaction producing nitric oxide (NO), which regulates multiple signalling pathways and physiological functions in mammals [1]. Arg can be methylated to form asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA). The first step in the synthesis of these methylarginines is the methylation of protein arginine residues by intracellular protein methyltransferase (PRMTs); the second, the proteolytic degradation of the methylated protein, produces free ADMA and SDMA [2]. ADMA is an endogenous inhibitor of the NOS enzyme because it competes with L-arginine for each of the three isoforms of these enzymes, whereas SDMA is not biologically active.

Arg competes with ADMA and SDMA for cellular transport across cationic amino-acid transporters (CATs) [3]. CAT family members, CAT-1-3 mediate Na+-independent transport of cationic l-amino acids. Cotransport of Na+ is observed only for the CAT transporters when they carry neutral amino acids [4]. Interestingly, the liver expresses CATs abundantly, especially CAT-2A and CAT-2B, suggesting a higher uptake of ADMA in this organ as compared with the heart, lungs and kidneys [5]. CAT-2B are low-capacity transporters that have a high affinity for cationic amino acids and, in particular, present high affinity for ADMA. By contrast, CAT-2A, an alternate splice variant of CAT-2B, possesses low affinity but high transport capacity [3].

The liver and kidney represent the main sites of both ADMA and SDMA metabolism and excretion. The kidney plays an important role in the elimination of dimethylarginines from the body, since both ADMA and SDMA are found in human urine [6]. For ADMA, an additional pathway was found, namely the metabolic degradation by dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that is widely distributed in rats and human beings, but in particular in the liver, kidney and pancreas [7]. Nijveldt *et al.* provide a detailed insight into the liver's handling of dimethylarginine, showing how the liver plays a crucial role in the metabolism of ADMA with DDAH taking up a large amount of ADMA from the systemic circulation: this study showed that daily hepatic ADMA extraction is ~700 times more than the amount of plasma ADMA in plasma [8]. On the contrary, SDMA is not a substrate for DDAH; it has been reported that SDMA is predominantly disposed of by renal route (about 60%), whereas about 40% could be eliminated by metabolic degradation [6].

Several experimental and clinical studies have evaluated the handling of ADMA and SDMA by the body, but their metabolic fate is not fully understood. ADMA and SDMA can be detected in plasma, urine, cerebrospinal and bronchoalveolar fluids [2]; we recently reported that ADMA is also secreted in bile and a time-dependent increase of ADMA occurs during hepatic I/R injury [9].

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To further support our previous data, in this study we evaluated the role of liver in the handling of not only ADMA but also SDMA, Arg and Cit by quantifying their levels in samples of the hepatic tissue, bile and plasma obtained from rat liver subjected to I/R injury. Furthermore, the modifications in CATs, CAT-2A and CAT-2B were evaluated together with mRNA expression and DDAH-1 activity.

## Materials and Methods

*Materials.* ADMA and SDMA were obtained from Calbiochem. Arginine, o-phtalaldeheyde (OPA) and beta-mercaptoethanol (beta-ME) were obtained from Sigma. HPLC grade acetonitrile and methanol were obtained from Carlo-Erba. All other chemicals were of analytical grade.

*Animals, surgery and tissue processing.* Male Wistar rats (Harlan-Nossan, Italy) were used in this study. The animals were allowed free access to water and food in all the experiments. The use and care of animals in this experimental study was approved by the Italian Ministry of Health and by the University Commission for Animal Care (Document number 2-2010).

*Ischaemia-reperfusion (I/R) procedure.* The effects of I/R were studied *in vivo* in a partial normothermic hepatic I/R model (n = 8). Briefly, the rats were anaesthetized with sodium pentobarbital (40 mg/kg i.p.), the abdomen was opened via a midline incision and the bile duct was cannulated (PE-50). Ischaemia to the left and median lobe was induced for 30 min. with microvascular clips by clamping the branch of portal vein and the branch of the hepatic artery after the bifurcation to the right lobe, with the abdomen temporary closed with a suture [10]. After 30 min. of ischaemia, the abdomen was reopened, the clips were removed, the abdomen was closed again, and the liver was allowed to reperfuse for 60 min. By using partial, rather than total hepatic ischaemia, portal vein congestion and subsequent bacterial translocation into the portal venous blood was avoided. Sham animals were subjected to the same procedure without the clamping of the vessels (n = 7). To prevent postsurgical dehydration and hypotension, 1 ml of saline was injected into the inferior vena cava. All the animals were maintained on warm support to prevent heat loss: rectal temperature was maintained at 37 ± 0.1°C.

*Serum, bile and tissue sampling.* Bile was collected in obscured vials. Blood was drawn from vena cava in pre-heparinized syringes, immediately placed on ice and centrifuged at 3000 g for 10 min. at 4°C. Hepatic biopsies were quickly removed from the median lobe and immediately frozen in liquid nitrogen, as were bile and serum samples until analysis was undertaken.

*ADMA, SDMA, Arg and Cit analysis.* Quantitative analysis of ADMA, SDMA, Arg and Cit was performed in deproteinized rat serum, bile and homogenized hepatic biopsies by reversed phase-high performance liquid chromatography (RP-HPLC) with OPA/betaME and fluorescence detection, as previously described, with some modifications [11]. The chromatography was carried out on the HPLC/HT400E system (ESSECI-Group, CO, Italy), using equipment for amino acid analysis that allows automatic on-line mixing of all reagents for OPA derivatization. The OPA derivatives were separated on a Teknokroma-Mediterranea-Sea C18 column (4.6 × 150 mm; 3 μm particle size) by a binary gradient elution. Fluorescence was measured at an excitation and emission wavelength of 330 and 450 nm, respectively.

*Table 1.*
List of forward and reverse primers used in the experiments.

| Gene | Sequence |
|---|---|
| Rat DDAH-1 | Forward 5′-CAACGAGGTCCTGAGATCTTGGC-3′ |
| | Reverse 5′-GCATCAGTAGATGGTCCTTGAGC-3′ |
| Rat CAT-2A | Forward 5′-TACGTTGTCGCCGCAGGCTC-3′ |
| | Reverse 5′-TCGTGGCAGCAACGGGTGAC-3′ |
| Rat CAT-2B | Forward 5′-TACGTTGTCGCCGCAGGCTC-3′ |
| | Reverse 5′-GCTGCCACTGCACCCGATGA-3′ |
| Rat UBC | Forward 5′-CACCAAGAACGTCAAACAGGAA-3′ |
| | Reverse 3′-AAGACACCTCCCCATCAAACC-5′ |
| Rat Tub | Forward 5′-AGAAGCAACACCTCCTCCTGC-3′ |
| | Reverse 3′-ATACACTCACGCATGGTTGCTG-5′ |
| Rat RS9 | Forward 5′-CCCTTCGAGAAATCGCGTCT-3′ |
| | Reverse 3′-GCAGAGCGTTGCCTTCAAAC-5′ |

*Liver injury.* Liver injury was assessed by serum release of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (AP) by an automated Hitachi 747 analyzer (Roche/Hitachi, Indianapolis, IN, USA).

*DDAH activity.* DDAH activity was evaluated using the method proposed by Tain and Baylis [12]. Tissue samples were homogenized in a phosphate buffer 100 mM, pH 6.5 activity of urease (100 U/ml) was added and samples were incubated at 37°C for 15 min. ADMA 1 mM in a phosphate buffer was added (final ADMA concentration: 0.8 mM) and samples were incubated at 37°C for 60 min.; the reaction was stopped by mixing 1:1 with 4% sulphosalicilic acid and samples were centrifuged for 10 min. at 3000 × g. Finally, the supernatants were assayed for citrulline as follows: solution A (diacetilmonoxime 80 mM, tiosemicarbazide 2 mM) and solution B (H2PO4 3M, H2SO4 6M, NH4Fe(SO4)2 1.75 mM) were prepared, mixed 1:3 and added 1:1 to the samples. Samples were incubated at 60°C for 110 min. and read spectrophotometrically at 528 nm against citrulline standards.

Proteins were measured according to Lowry's method using albumin as standard [13].

*Nitrite/nitrate assay.* Total NO production was estimated by measurement of the tissue nitrite/nitrate (NOx) content. The levels of NOx in serum and liver were determined by Cayman Kit. The samples were filtered through a 30-kDa molecular mass cutoff filter for elimination of any proteins and mixed with an equal volume of Griess reagent, incubated for 10 min. at room temperature in reduced light, and measured at a wavelength of 540 nm.

*mRNA expression of DDAH-1, CAT-2A and CAT-2B.* DDAH-1, CAT-2A and CAT-2B mRNA were analysed by a real-time polymerase chain reaction (RT-PCR) (table 1): total RNA was isolated from the liver samples with Trizol reagent in accordance with the method of Chomczynski and Mackey [14]. RNA was quantified by measuring the absorbance at 260/280 nm. cDNA was generated using the iScript cDNA Synthesis kit (BIO-RAD, Segrate, MI, Italy) following the supplier's instructions. Gene expression was analysed using the SSO Advanced Sybr Green Supermix (BIO-RAD). Ubiquitin C (UBC), Tubulin (Tub) and Ribosomal Protein S9 (RS9) were used as housekeeping genes (table 1). DDAH-1, CAT-2A, CAT-2B, UBC, Tub and RS9 were subjected to 40 cycles of amplification. The expression of the house-keeping gene remained constant in all the experimental groups considered. The amplification was performed through two-step cycling (95–60°C) for 40 cycles in a CFX Connect RT-PCR Detection System (BIO-RAD) following the supplier's

instructions. All samples were assayed in triplicate. Gene expression was calculated using the ΔCt method. Comparison between groups was calculated using the ΔΔCt method.

*Statistical analysis.* Statistical analysis was performed using R software (R Development Core Team). Two independent samples were analysed by Student's *t*-test for normal distributions, or by the Mann–Whitney *U* test for data not normally distributed. The correlation between variables was analysed by means of Pearson's or Spearman's correlation coefficients. The value of $p < 0.05$ was considered to indicate statistical significance. Graphs present the mean value ± standard error of the mean (SEM).

## Results

*Effect of I/R on biliary, tissue and serum levels of ADMA, SDMA, Arg and Cit.*

Notably, both forms of methylarginines were detected in bile: a significant increase in ADMA and a decrease in SDMA were observed in bile at the end of reperfusion (fig. 1A).

The tissue levels of ADMA and SDMA are comparable with those reported by Bulua *et al.* [15]. In the present study, a decrease in liver ADMA levels and an increase in SDMA concentrations were detected after I/R (fig. 1C).

The ADMA and SDMA levels detected in plasma confirmed data previously reported in rats [16,17]. No serum changes in ADMA and SDMA were found after 30-min. ischaemia and 60-min. reperfusion (fig. 1E). A decrease in Arg and an increase in Cit were detected in bile, tissue and serum after I/R (fig. 1B,D,F).

Evaluation of the Arg/ADMA, Arg/SDMA and ADMA/SDMA ratio was performed; the results are reported in table 2. A biliary increase in Arg/SDMA and ADMA/SDMA and a decrease in Arg/ADMA was found in the I/R group. The hepatic Arg/SDMA and ADMA/SDMA ratios were found to be lower in tissue after 60-min. reperfusion as compared with sham-operated animals. Serum Arg/ADMA and Arg/SDMA ratios decrease after I/R injury (table 2). Arg/ADMA results

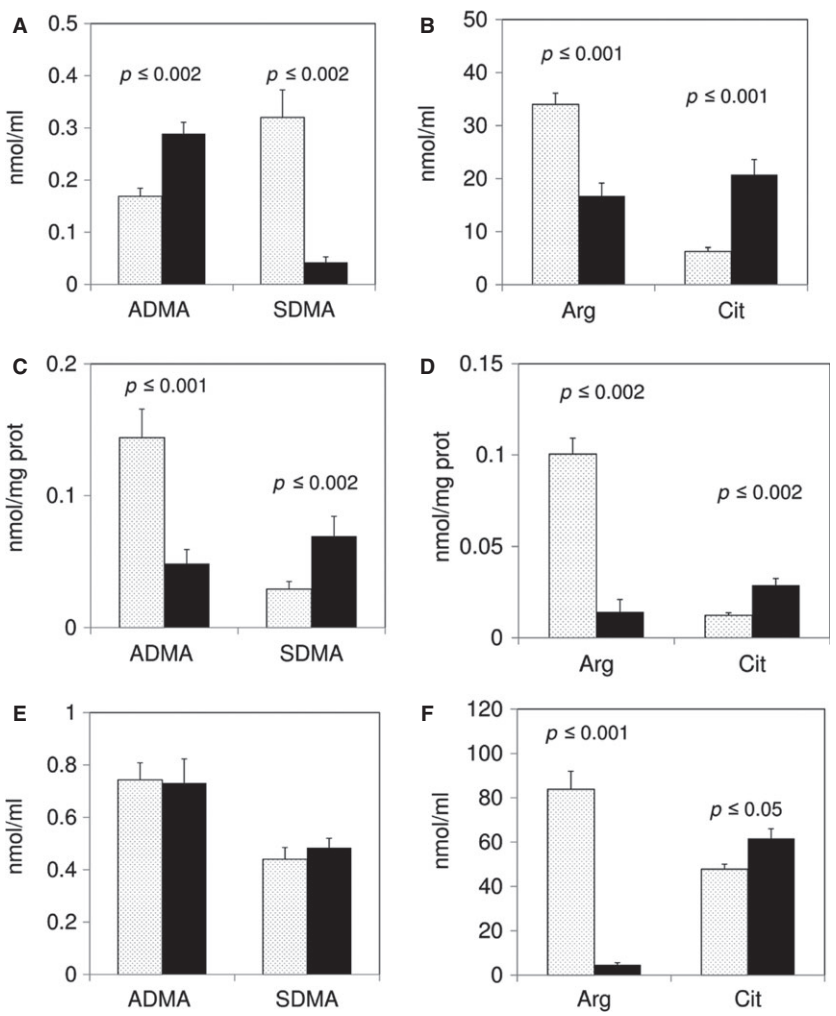


Fig. 1. Biliary (Panels A and B), hepatic (Panels C and D) and serum (Panels E and F) changes in ADMA, SDMA, Arg and Cit after I/R. Livers were submitted to 30-min. ischaemia followed by 60-min. reperfusion. Sham-operated control animals underwent similar manipulation without vascular occlusion. At the end of reperfusion, biliary and hepatic samples were collected. The results are reported as the mean ± S.E. of seven to eight different experiments. I/R: ■; sham: ▒.

*Table 2.*
Comparison of Arg/ADMA, Arg/SDMA and ADMA/SDMA ratios in rat bile, liver and serum after I/R.

| | Bile | | Tissue | | Serum | |
|---|---|---|---|---|---|---|
| | Sham | I/R | Sham | I/R | Sham | I/R |
| Arg/ADMA | 179 ± 13 | 63 ± 13** | 0.76 ± 0.1 | 0.40 ± 0.2 | 111 ± 17 | 6.3 ± 1.0** |
| Arg/SDMA | 136 ± 33 | 484 ± 73* | 3.85 ± 0.7 | 0.24 ± 0.07* | 191 ± 20 | 9.2 ± 1.4** |
| ADMA/SDMA | 0.7 ± 0.2 | 10 ± 2.1* | 5.9 ± 1.5 | 0.83 ± 0.2* | 1.9 ± 0.2 | 1.5 ± 0.1 |

*$p \leq 0.002$ and **$p \leq 0.001$ *versus* Sham.

*Table 3.*
Serum levels of AST, ALT and AP after 30 ischaemia followed by 60-min. reperfusion.

| | AST (mU/ml) | ALT (mU/ml) | AP (mU/ml) |
|---|---|---|---|
| Sham | 105 ± 14 | 50 ± 8 | 310 ± 30 |
| I/R | 1109 ± 222* | 933 ± 94* | 358 ± 45 |

*$p \leq 0.001$ *versus* Sham.

obtained in serum of sham animal are comparable with those previously reported [18] as is the marked Arg/ADMA decrease observed during reperfusion [17,19,20].

*Relationships between serum ALT and ADMA, SDMA or Arg/ADMA.*
As expected, serum AST, ALT and AP increased in animals submitted to ischaemia and reperfusion as compared with the sham-operated group (table 3). We examined ADMA and SDMA concentrations in bile to assess their association with liver necrosis. A particularly significant positive correlation was found between biliary ADMA and serum ALT (fig. 2, panel A), as well as a negative correlation was detected comparing biliary SDMA and serum ALT (fig. 2, panel B). No correlation was found comparing serum ADMA or SDMA versus serum ALT (fig. 2, panels C and D). A good correlation was also detected when biliary Arg/ADMA levels were compared with serum ALT (fig. 2, panel E) and were higher than those obtained when comparing serum Arg/ADMA with serum ALT (fig. 2, panel F). Other correlations between biliary and serum Arg, Arg/SDMA and ADMA/SDMA versus serum ALT are reported in table 4.

*DDAH activity and mRNA expression of DDAH-1 after I/R injury.*
Decreased DDAH activity was observed at the end of reperfusion as reported in fig. 3A. DDAH contains SH groups in the catalytic site. We evaluated the oxidative stress so as to provide an explanation for the reduction in its activity observed after I/R. No significant changes in the GSH/GSSG ratio were observed at the end of reperfusion between the control and I/R groups (9.3 ± 0.8 and 8.6 ± 0.6, respectively). The evaluation of TBARS and ROS formation showed the same trend and no significant difference in any of the experimental groups considered was found comparing control and I/R livers (TBARS, nmol/mg liver: 1.92 ± 0.4 *versus* 1.83 ± 0.6; ROS, Arbitrary Units: 1951 ± 44 *versus* 1978 ± 55, respectively).

Evaluation of mRNA expression of isoform DDAH-1 was performed: the amount of mRNA expression of DDAH-1 decreased at the end of reperfusion as reported in fig. 3B.

*Effects of I/R on mRNA expression of cationic transporters.*
mRNA of CAT-2A and CAT-2B were also quantified. A decrease in mRNA transporter CAT-2A but not in CAT-2B was detected at the end of reperfusion as compared with sham livers (fig. 3C and D).

*Effects of I/R on serum and hepatic NOx content.*
An increase, although not significant, in serum NOx was found in the I/R group (fig. 4A). Higher significant levels of NOx were detected at the end of reperfusion in tissue of liver submitted to I/R when compared with sham-operated animals as shown in fig. 4.

## Discussion

This work supports our previous data on the crucial role of the liver not just in the metabolism of ADMA, which has so far been taken into consideration, but also in the biliary elimination of ADMA and, as well as this, in the biliary excretion of SDMA which is documented for the first time in this study.

*Changes in ADMA, SDMA and Arg levels during I/R injury.*
ADMA and SDMA are endogenous molecules that can be detected in plasma, urine, cerebrospinal and bronchoalveolar fluids [2]. Our study suggests that they can also be excreted in the bile. The liver's newly documented ability to eliminate ADMA by bile confirms our recently published laboratory results and could provide an explanation for data previously reported by other authors: Mookerjee *et al.* (2007) showed that only cirrhosis by bile duct ligature (BDL) induces an increase in plasma ADMA levels and not thiocetamide (TAA)-induced cirrhosis. The BDL rats exhibited a decreased rate of ADMA removal of about 50% [21], suggesting that the impossibility of bile excretion induces an increase in circulating ADMA. Additionally, we reported that a short BDL period induced a significant increase in tissue ADMA [22]. Moreover, an increase in the plasma concentration of ADMA has also been found in human alcoholic cirrhosis only when high levels of plasma bilirubin have also been found, suggesting a correlation between compromised biliary excretion of bilirubin and increase in ADMA plasma [23].

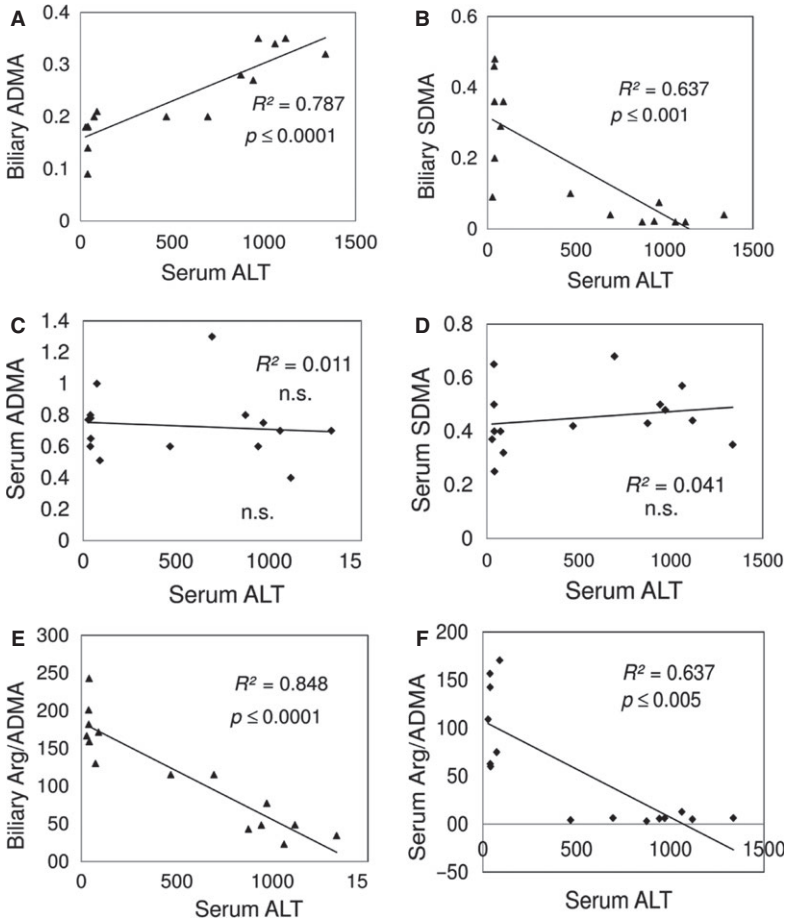


Fig. 2. Relationship between biliary ADMA (Panel A), SDMA (Panel B) and Arg/ADMA (Panel E) *versus* serum ALT. Relationship between serum ADMA (Panel C), SDMA (Panel D) and Arg/ADMA (Panel F) *versus* serum ALT. Livers were submitted to 30-min. ischaemia followed by 60-min. reperfusion. Sham-operated control animals had similar manipulation without vascular occlusion. n.s.: not significant.

*Table 4.*
Correlation between biliary and serum levels of Arg, ADMA, SDMA, Arg/ADMA, Arg/SDMA and ADMA/SDMA *versus* serum levels of ALT.

| | Biliary Arg, ADMA and SDMA versus serum ALT | | Serum Arg, ADMA and SDMA versus serum ALT | |
|---|---|---|---|---|
| | $R^2$ | *p* value | $R^2$ | *p* value |
| Arg | 0.743 | ≤0.0001 | 0.706 | 0.0010 |
| ADMA | 0.787 | ≤0.0001 | 0.011 | n.s. |
| SDMA | 0.637 | ≤0.0001 | 0.041 | n.s. |
| Arg/ADMA | 0.848 | ≤0.0001 | 0.637 | 0.0054 |
| Arg/SDMA | 0.524 | 0.0082 | 0.734 | 0.0047 |
| ADMA/SDMA | 0.689 | 0.0003 | 0.131 | n.s. |

n.s.: not significant.

Little attention has been paid to SDMA since this methylated form of Arg does not influence NOS activity but may be of clinical significance by reducing substrate availability of NO synthase through competition with Arg for CATs [24]. Siroen *et al.* demonstrated that the kidneys are not the sole organ responsible for the clearance of SDMA as the human liver also exhibits hepatic extraction of SDMA taking up substantial amounts of this methylarginine [25]. Although performed in an animal model, the biliary elimination of SDMA found in this study could provide an explanation for previously reported clinical results.

Significantly, this work also supports previous results regarding decreased Arg concentration in plasma after reperfusion [26]. A significant decrease in serum Arg associated with an increase in Cit after warm hepatic I/R has also been reported by Jeyabalan *et al.* [27]. In addition, a decrease in tissue Arg was observed in kidney after I/R in comparison with sham animals [28]. Our data also show that lower Arg levels associated with higher Cit levels were found in serum, tissue and bile after I/R as compared with sham rats. As reported by Silva *et al.*, low Arg in serum may reflect influx of the amino acid into hepatocytes, resulting in increased formation of NO [29]. This consumed Arg is thought to be converted to NO; this phenomenon has been confirmed by the increased plasma Cit detectable after hepatic I/R, possibly produced from Arg [30].

*I/R and changes in CAT-2A and CAT-2B.*

Free methylarginines and Arg can be released in extracellular space by cationic amino-acid transporters (CATs). CATs are also involved in the removal of circulating ADMA and SDMA

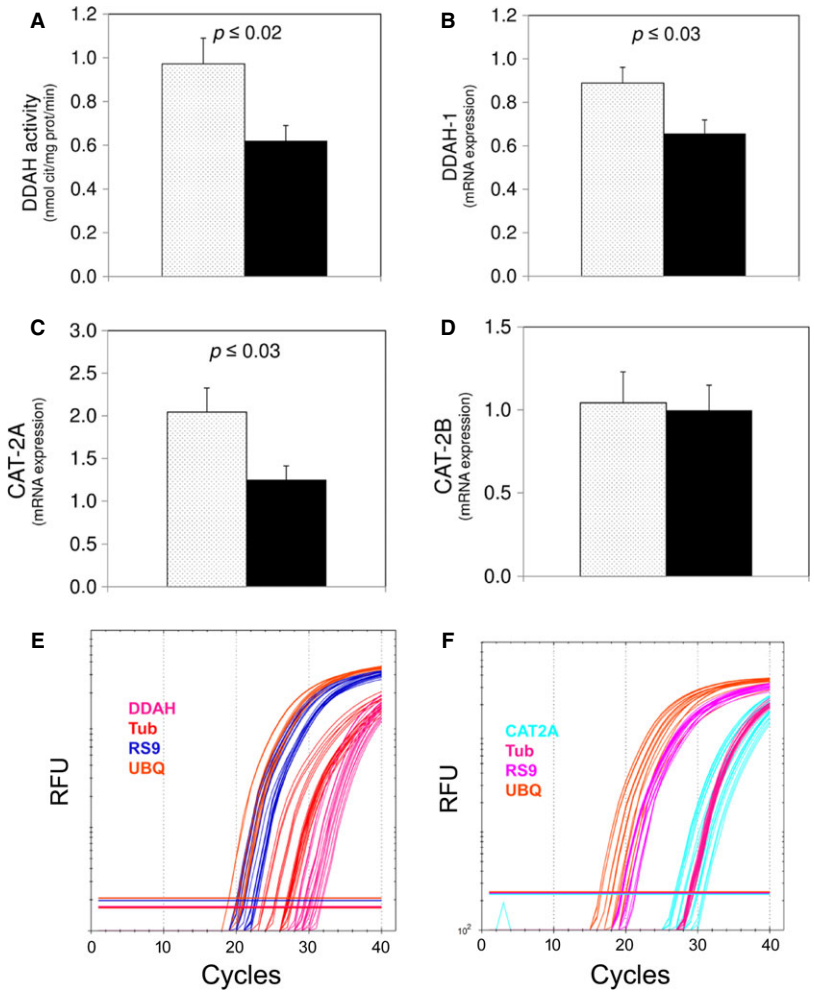


Fig. 3. DDAH activity (Panel A) and mRNA expression of DDAH-1 (Panel B), CAT-2A (Panel C) and CAT-2B (Panel D) in livers at the end of reperfusion. Panel E and F: examples of the qPCR for DDAH-1 and CAT-2A. Livers were submitted to 30-min. ischaemia followed by 60-min. reperfusion. Sham-operated control animals underwent similar manipulation without vascular occlusion. The results are reported as the mean ± S.E. of seven to eight different experiments. I/R: ■; sham: ▒.

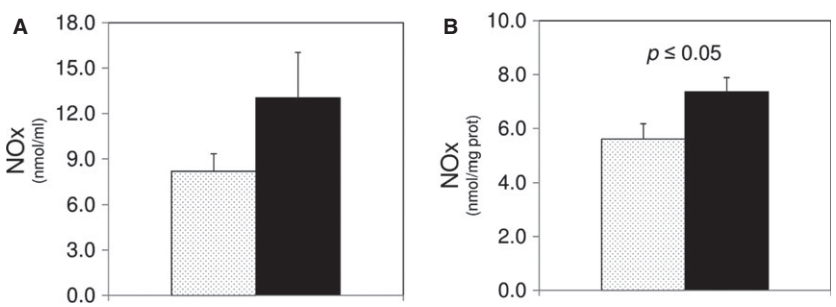


Fig. 4. NOx levels in serum (Panel A) and liver (Panel B) at the end of reperfusion. Livers were submitted to 30-min. ischaemia followed by 60-min. reperfusion. Sham-operated control animals underwent similar manipulation without vascular occlusion. The results are reported as the mean ± S.E. of seven to eight different experiments. I/R: ■; sham: ▒.

by the liver. In the portal area of the liver lobule, vessel cells and the bile duct show high CAT-2 mRNA expression [31]; furthermore, the abundant expression of CAT-2 mRNA in the liver confirms the crucial role of this organ in the elimination of ADMA from the circulation [5]. Based on the data reported, we now posit its involvement in the biliary excretion of Arg, ADMA and SDMA. We observed a decrease in CAT-2A mRNA; on the contrary, CAT-2B mRNA levels after I/R

remained unchanged, We previously reported that the protein expression of total CAT-2 did not change after I/R [9]. We have now evaluated the mRNA expression of both CAT-2A and CAT-2B demonstrating a difference in CAT-2B but not in CAT-2A. Thus, Betz *et al.* have also demonstrated that no difference in mRNA of CAT-2 occurs after kidney I/R; on the contrary, a decrease, although not significant, in mRNA CAT-2A was found [28]. The role of I/R injury in the regulation of hepatic transporters is not new in the literature, though not sufficiently explored: a previous study has demonstrated that hypoxia regulates the expression of hepatobiliary transporter genes [32]; further data in human hepatocytes have demonstrated that the organic solute transporters alpha and beta are induced by hypoxia [33].

The liver metabolizes ADMA by the enzyme DDAH, and both ADMA and SDMA might even be eliminated unchanged in the bile through CATs transporters. In this study, we suggest that CAT-2B could be directly involved in the biliary excretion of ADMA, SDMA and Arg, provoking a tissue decrease in ADMA and an increase in SDMA. A summary of events that occur after hepatic I/R is given in fig. 5. Significantly, similar events were seen in spontaneously hypertensive rats [16]: the trend in serum changes of Arg, ADMA, SDMA, Arg/ADMA and ADMA/SDMA described in that study reflected the changes observed in bile in the present study. The previous study suggested that the kidney in hypertensive rats might be protected against injury via a reduction in ADMA concentration in tissue, and this event is associated with an increase in plasma ADMA and a decrease in SDMA, Arg and CAT [16]. It has been suggested that the kidney of hypertensive rats self-protects against injury by decreasing ADMA uptake from the circulation; in the present study, we posit that liver not only reduces ADMA uptake but in addition increases its clearance by biliary excretion to reduce I/R damage. Significantly, previous data demonstrated that an ADMA-mediated increase in peroxinitrite generation leads to the translocation of e-NOS to the mitochondria resulting in mitochondrial dysfunction [34]. Furthermore, previous data documenting an involvement of CATs in the regulation of mean artery pressure in rats showed that a decrease in these transporters was associated with reduced Arg uptake in the renal medulla [35]. Our results support these events and suggest a possible involvement of CATs also in biliary hypertension that could occur after I/R injury. In addition, it has been shown that increased biliary hypertension represents a key proliferative trigger for the growth of bile ducts [36]. Other studies have also reported that high ADMA plasma levels in combination with low plasma Arg levels adversely affects systemic hemodynamics and reduces blood flow through the kidney, spleen and liver [37].

Several studies have demonstrated that plasma ADMA evaluation appears to be an early predictor for survival in patients with sepsis associated to acute liver failure [38]. Recently, SDMA levels have also been studied in association with liver dysfunction and mortality in chronic liver disease [39]. Furthermore, it has been shown that the Arg/ADMA ratio is a more powerful predictor of organ failure with respect to ADMA alone. Recent results have indicated that

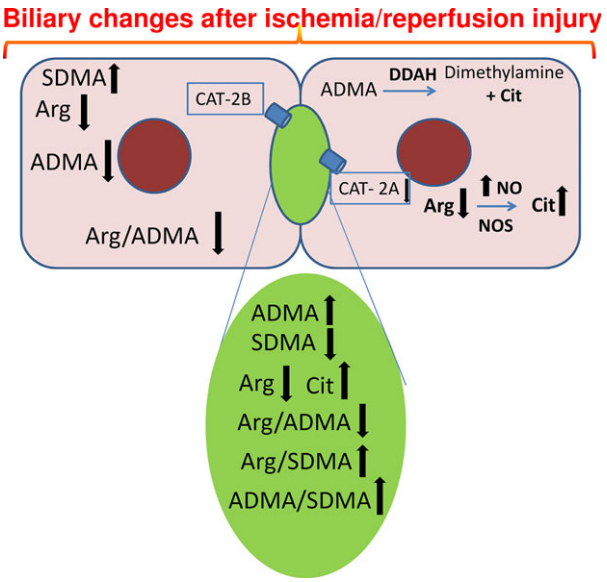


Fig. 5. Schematic representation of events that occur in bile after hepatic I/R. Liver is responsible for the biliary excretion of ADMA and SDMA, in unchanged form, from the body and I/R induced changes in the biliary levels of Arg and its methylated derivatives.

the Arg/ADMA ratio is associated with impaired organ functions and mortality [40]. The serum Arg/ADMA ratio in Intensive Care Unit patients is associated with circulatory failure, organ failure and mortality in septic patients [41,42]. In the present study, a marked decrease in serum Arg/ADMA was also detected in I/R livers in keeping with other published results [43,44]. Here, we have demonstrated, for the first time that the biliary Arg/ADMA ratio appears to be a better predictor of liver necrosis than the serum Arg/ADMA ratio. Because the Arg/ADMA ratio is a better predictor of morbidity and mortality than ADMA alone, it has been suggested that the restoration of this ratio, for example, by means of the administration of L-arginine, should be considered a suitable option when attempting to improve a patient's condition [45].

## Conclusions

In conclusion, this study supports our previous results on biliary ADMA clearance and has demonstrated, for the first time, that the liver is also responsible for the biliary excretion of SDMA: the kidney is not the sole organ responsible for the clearance of SDMA, as the liver also takes up SDMA from the portal and systemic circulation. This data also allows us to take a step forward in our understanding of the mechanisms involved in the control of Arg and its methylated derivatives during hepatic I/R; finally, changes in biliary Arg, ADMA and SDMA excretion could be considered as novel and early predictors of liver injury.

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## Conflict of Interest

The authors state that there is no conflict of interest.

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