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**Presence of tumour inhibits the normal post-operative response in arginine and NO production in non-cachectic mice**

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## Abstract

We recently described that cancer patients have low plasma arginine concentrations, even without weight loss being present, suggesting that decreased arginine availability may be a specific feature of the presence of tumour. Since arginine is important in post-operative repair, we hypothesized that abnormalities in arginine metabolism in cancer lead to an aberrant post-operative response in arginine and NO metabolism. To investigate this, we studied post-operative alterations in arginine and NO production and the acute phase response in MCA sarcoma-bearing mice. Controls, mice with small MCA tumours (<15% of carcass weight) and large MCA tumours (>15% of carcass weight) were studied, either with or without undergoing laparotomy. The stable isotopes L-[guanidino-<sup>15</sup>N<sub>2</sub>-<sup>2</sup>H<sub>2</sub>]arginine and L-[ureido-<sup>15</sup>N]citrulline were used to study whole-body arginine and NO production rates. Serum amyloid P component (SAP) concentrations were measured to assess the acute-phase response. Significance was tested using Mann-Whitney U test. In healthy FVB mice, laparotomy significantly increased whole-body arginine production (from 42±3 to 54±3 nmol/10g/min), NO production (from 1.1±0.1 to 1.4±0.2 nmol/10g/min) and levels of serum amyloid P component (from 4±1 to 115±23 ng/ml), while in all MCA tumour-bearing mice baseline values of arginine metabolism and [SAP] were already elevated and the response to laparotomy was absent. In conclusion, MCA tumour-bearing mice had a disturbed post-operative metabolic response as evidenced by attenuated post-operative arginine and NO production, concomitant with an attenuated acute-phase response. This indicates that altered arginine metabolism may be an important characteristic of the metabolic changes in cancer.

## Introduction

We recently described that patients with malignant tumours inducing various degrees of metabolic derangements have decreased plasma arginine concentrations, even without weight loss being present [1], suggesting that decreased arginine availability may be a specific feature of host metabolism in the presence of cancer. Moreover, up to one third of cancer patients die from cachexia rather than from cancer itself [2], even after surgical removal of the tumour [3], indicating that cancer-related alterations in the metabolism of the host are a very important factor in determining mortality. In addition, cancer patients undergoing surgical treatment have more post-operative complications [4] and higher mortality [5] than patients undergoing surgery for non-malignant disease. Apparently, the host's response to surgery in cancer patients is different from the response of healthy subjects undergoing surgery. Since arginine plays a central role in the host response to surgical trauma, both via nitric oxide (NO) (produced from arginine in the nitric oxide synthase pathway) and ornithine (produced from arginine in the arginase pathway), arginine deficiency could compromise post-operative recovery of cancer patients.

In the last two decades, many studies investigating the peri-operative metabolic changes in cachectic cancer patients have been conducted. Still, the mechanism of cancer-related post-operative disturbances remains to be unravelled, which may be due to the complexity of the metabolic abnormalities that are present in the advanced stage where symptoms such as weight loss or anorexia have already become obvious. Therefore, we chose to investigate early derangements in the post-operative host response in a non-cachectic tumour mouse model.

The primary aim of the study was to investigate whether the post-operative response in arginine and NO production was different in the presence of a malignant tumour. The secondary aim was to investigate whether tumour-bearing mice undergoing surgery had an impaired post-operative acute-phase response. Thus, we studied basal and post-operative response in arginine/NO production in MCA sarcoma-bearing mice and healthy controls. To monitor the magnitude of the immune response after surgery, the acute – phase response was measured as plasma concentration of the acute-phase reactant serum amyloid P component (SAP) [6], the mouse homologue of C-reactive protein (CRP) in humans.

## Methods

### *Animals*

FVB mice were bred at the Central Animal Facilities of Maastricht University. Mice were fed standard lab chow (Hope Pharms, Woerden, The Netherlands) and subjected to a standard 12 hour light-dark cycle. Room temperature was maintained at 22°C. Water was provided ad libitum throughout the experiment. Male mice of 20-23 grams were randomized into six groups: 1) control; 2) control undergoing laparotomy; 3) small tumour of 5-15% of carcass weight; 4) small tumour undergoing laparotomy; 5) large tumour of more than 15% of carcass weight; 6) large tumour undergoing laparotomy.

Based on a power calculation using whole-body arginine production as primary outcome parameter, we calculated the group size to be n=8. To compensate for mortality during the course of the experiment (related to bleeding during catheter placement and cardiac arrest due to anesthesia), 12 animals were initially included per group. Food intake was measured daily by weighing the amount of chow in the cage and comparing it with the chow weight of the day before. Experiments were approved by the Ethical Committee of Animal Research of Maastricht University.

### *Tumour model*

MCA tumours were initially induced by subcutaneous injection of 1 mg methylcholantrene (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in mice in our laboratory. These tumours were maintained in vivo by serially transplanting tumour tissue through a 15G needle. The MCA tumour has been widely used as a model for cancer in metabolic studies in mice and rats and has the histological characteristics of malignant sarcoma, with locally aggressive growth [7, 8]. In our hands, the tumour does not induce anorexia or weight loss. Control mice were sham implanted.

### *Surgical trauma*

When the tumour reached 5-15% of carcass weight (small tumour groups) or more than 15% (large tumour groups) [9], a midline laparotomy was performed as a model for surgical trauma. In short, ketamine/medetomidine anaesthesia, fluid and temperature control were performed as described previously [10]. Under aseptic conditions, a midline incision from the level of the superior iliac spines until the xyphoid process was made. Intestines were put aside and wrapped in wet gauze. Five minutes later, intestines were

put back and the abdomen was closed with wound clips (Autoclip, Clay Adams, Becton Dickinson and Company, Parsippany, NJ, USA). After surgical trauma, food was taken away from all mice to prevent differences in food intake between groups. Water was provided ad libitum.

#### *Metabolic measurements*

Twenty-four hours after laparotomy, ketamine/medetomidine anaesthesia and fluid and temperature maintenance were performed as described by Hallemeesch et al. [10]. A primed-constant infusion of the stable isotopes L-arginine [guanidino- $^{15}\text{N}_2\text{-}^2\text{H}_2$ , 99% atom percent excess] ( $^{15}\text{N}_2\text{-}^2\text{H}_2\text{Arg}$ ) and L-citrulline [ureido- $^{15}\text{N}$ , 99% atom percent excess] ( $^{15}\text{N}\text{Cit}$ ) (Mass Trace, Woburn, MA, USA) was given in the jugular vein (prime: 146 nmol/10g  $^{15}\text{N}_2\text{-}^2\text{H}_2\text{Arg}$  and 44 nmol/10g  $^{15}\text{N}\text{Cit}$ ; infusion: 960 nmol/10g/h  $^{15}\text{N}_2\text{-}^2\text{H}_2\text{Arg}$  and 90 nmol/10g/h  $^{15}\text{N}\text{Cit}$ ). This stable isotope protocol yields tracer-tracee steady state in plasma within 30 minutes. Blood was collected from the carotid artery and processed as described before [10]. Amino acid concentrations and tracer/tracee ratios (TTR) were determined in plasma using HPLC and LC-MS as described by Van Eijk [11, 12].

#### *Serum amyloid P component ELISA*

Serum amyloid P component (SAP) concentrations in plasma were measured using a sandwich ELISA as described before [13].

#### *Calculations*

Plasma arginine and citrulline production rates were calculated from the arterial isotope TTR values of respectively  $^{15}\text{N}_2\text{-}^2\text{H}_2\text{Arg}$  and  $^{15}\text{N}\text{Cit}$  as described recently [14]. Whole-body NO production was calculated as plasma arginine to citrulline flux [14], based on the fact that conversion of arginine to NO yields stoichiometric amounts of citrulline. Data are expressed per 10g carcass weight, which was calculated by subtracting tumour weight from body weight including tumour.

#### *Statistics*

Results are presented as mean  $\pm$  SEM. Data were analyzed with SPSS [15]. To reduce the chance of false-positive significances, we performed as few tests as possible. Therefore, we only tested the effect of i) laparotomy in control mice, ii) the presence of tumour per se, iii) laparotomy in mice bearing small tumours, iv) laparotomy in mice bearing large

tumours. Since data were not normally distributed, non-parametric Mann-Whitney U test was used. Significance was defined as  $p < 0.05$ .

## Results

### *Drop out*

As a consequence of drop out due to intra-operative mortality, the final group sizes were: 1) control (n=12); 2) control undergoing laparotomy (n=12); 3) small tumour of 5-15% of carcass weight (n=9); 4) small tumour undergoing laparotomy (n=9); 5) large tumour of more than 15% of carcass weight (n=7); 6) large tumour undergoing laparotomy (n=7).

### *Normal post-operative response*

In healthy mice, arginine and NO production increased post-operatively by respectively 29 and 27% (figure 1), concomitant with a 30-fold increase in SAP concentrations (table 1). Laparotomy did not affect total amino acid concentrations, including arginine (table 1).

### *Effects of tumour*

There were no significant differences between tumour-bearing and control mice in carcass weight (control 25.4±0.4; small tumour 25.5±0.7; large tumour 23.9±0.8g) or food intake (control 4.2±0.1; small tumour 4.1±0.2; large tumour 4.3±0.2g/day).

Compared with controls, plasma arginine concentrations were specifically decreased in mice with small tumours without changes in total amino acid concentrations including glutamine and citrulline (table 1). Concomitantly, plasma ornithine concentrations were increased (table 1). In mice with small tumours, whole-body arginine and NO production were unaffected (figures 1 and 2). However, in mice bearing large tumours, whole-body arginine and NO production were 88% and 54% higher than in controls ( $p < 0.05$ , figures 1 and 2), without changes in plasma arginine concentration (table 1). The presence of a small tumour increased SAP concentrations by 80-fold, while in the presence of a large tumour SAP concentrations rose even 280-fold higher than in controls (table 1).

### *Attenuated post-operative response in tumour-bearing mice*

In contrast to controls, laparotomy did not increase whole-body arginine and NO production in the presence of a small or a large tumour (figures 1 and 2). In mice with large tumours, whole-body NO production decreased by 24% after laparotomy (figure 2). In contrast to healthy mice undergoing surgery, the post-operative SAP concentration did not increase in the presence of a small tumour or a large tumour (table 1). Similar to controls, laparotomy did not affect total amino acid concentrations including arginine in



the presence of small tumour. Unlike in healthy animals, laparotomy decreased total amino acid concentrations by 25% in mice with large tumours (table 1).

## Discussion

This study shows that the post-operative increase in arginine and NO production and the acute-phase response that were observed in healthy control mice, were absent in MCA tumour-bearing mice. This indicates that the presence of tumour compromised post-operative arginine metabolism and attenuated the host inflammatory response to surgical trauma.

Arginine is a non-essential amino acid that can be metabolized by NO synthase (NOS) or arginase. Conversion by NOS produces NO and citrulline, whereas conversion by arginase yields ornithine. Various malignant human tumours including lung, colon, prostate and breast carcinoma contain large quantities of arginase [16-18]. Recently, arginase activity in cancer was shown to be a mechanism for tumour-induced immune-suppression via depletion of arginine in the microenvironment of murine lung tumours [19], allowing the tumour to escape the immune response of the host. Subsequently, this mechanism was confirmed in patients with renal cell carcinoma's [20]. This may imply that maintenance amounts of arginine in the tumour environment could play a role in host defence against the tumour. The present study demonstrates that the presence of tumour also induces changes in arginine metabolism at whole body level, which appeared different in mice bearing small and large tumours. In the first group, plasma arginine concentrations were decreased without significantly affecting whole-body arginine production. In mice bearing large tumours, whole-body arginine production was increased with slightly (but not significantly) raised plasma arginine concentrations. It can be calculated that arginine clearance (the amount of plasma that is completely cleared from arginine each minute [21]) was increased both in the presence of small and large tumours.

It is important at this stage to address the relevance of these observations for human cancers. MCA rodent tumour models have the histological appearance of sarcoma's and induce several metabolic characteristics of human malignancies, with wasting of fat tissue [8] and altered protein turnover [22]. In analogy to common human malignancies such as colonic carcinoma and breast carcinoma, MCA tumours do not induce weight loss. MCA tumour-bearing mice may therefore represent cancer patients in whom no weight loss is present. If so, this may imply that cancer at a phase where no cachexia is

present already induces disturbances in host amino acid metabolism. Because human tumours are usually small compared to the patient's body weight, the experimental model with the small tumour might be the most clinically relevant of the two.

A common treatment for cancer is surgical removal of the tumour. In the post-operative period, several immune processes rely on arginine as substrate. NO is required for the cytotoxic effect of macrophages, both in animals [23] and humans [24]. Furthermore, arginine is necessary for a functional T cell response after trauma in mice [25] and humans [26]. Besides, both arginine and its products NO and ornithine are indispensable for wound healing in mice [27]. Recently, NO was shown to enhance collagen synthesis in human tendon cells [28]. Therefore, the disturbances in arginine metabolism induced by a tumour could have consequences for the host repair response of cancer patients to surgical trauma. We thus investigated the post-operative response in arginine and NO metabolism in relation to the acute-phase response in tumour-bearing mice.

In the current study we demonstrate that the presence of MCA tumour prohibited a post-operative increase in arginine and NO production. If we assume the response of control mice to be normal, this indicates that tumour-bearing mice had a defective post-operative metabolic response. Thus, cancer appears to induce disturbances in arginine metabolism that affect host arginine metabolism, which become even more pronounced when metabolism is challenged during metabolic stress. Concomitantly, SAP concentrations did not increase further. In a previous study, an attenuated CRP response was also reported in undernourished cancer patients that underwent surgery [29]. However, at that time, no metabolic information was present to explain this phenomenon. Some years later, we reported an impaired metabolic response to surgery in tumour-bearing rats in muscle [9] and intestine [30]. This suggested that the attenuated response to surgery as observed in cancer was not related to malnutrition, but rather due to the presence of tumour per se. The current study characterizes the metabolic inability in cancer further, describing an impaired capacity to mobilize arginine and NO after surgical stress with concomitant suppression of the acute-phase response. Unfortunately, in the MCA-tumor model, tumor removal is not possible without mortality, at least not in our hands, probably due to the relatively large size (ca. 10x20mm) of the tumor. Strictly, the

currently used model thus compares with patients in whom radical resection of tumor was not possible.

Strikingly, there was a “chronic” acute-phase response ongoing in the presence of tumour. This is probably different from the “acute” acute-phase response that usually occurs after surgical trauma. We speculate that the ongoing acute-phase response as observed in tumour-bearing mice attenuates the initiation of the inflammatory response upon acute metabolic stress, such as surgical trauma. Hypothetically, this may be due to exhaustion of endogenous sources (possibly amino acids from muscle protein) for substrate for the acute-phase response (possibly acute-phase protein). Because absolute SAP concentrations in tumour-bearing mice were higher than post-operative values in healthy animals, point for discussion remains whether absolute values or relative increases are important for post-operative recovery. Further studies are needed to solve this issue.

In conclusion, our data suggest that the acute-phase response normally observed after surgery does not occur in the presence of cancer. This non-responsiveness was concomitant to absence of the increase in whole-body arginine and NO production after surgery, indicating a role for altered arginine metabolism in cancer. Hypothetically, such changes may contribute to post-operative morbidity and mortality, and if similar changes would occur in humans with cancer undergoing surgery this could have consequences for clinical practice.

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## Figures and Tables

### *Legend figure 1: Arginine production*

Values are means  $\pm$  SEM. White bars: without laparotomy; black bars: with laparotomy.

Whole-body arginine production was measured using [ $^{15}\text{N}_2$ - $^2\text{H}_2$ ]Arg. Statistics with Mann-Whitney U test, #p<0.05 tumour effect, \*p<0.05 laparotomy effect.

### *Legend figure 2: NO production*

Values are means  $\pm$  SEM. White bars: without laparotomy; black bars: with laparotomy.

Whole-body NO production was measured by calculating [ $^{15}\text{N}_2$ - $^2\text{H}_2$ ]Arg to [ $^{15}\text{N}$ - $^2\text{H}_2$ ]Cit flux. Statistics with Mann-Whitney U test, \*p<0.05 laparotomy effect.

Table 1: Arterial plasma amino acid and SAP concentrations

	CO	LA	ST	ST+LA	LT	LT+LA
ARG	117± 8	114± 5	90± 10 <sup>#</sup>	77± 9	147± 35	93± 30
GLN	408± 10	385± 16	444± 26	459± 22	435± 19	319± 38
CIT	88± 4	78± 3	77± 6	70± 5	86± 5	70± 5 *
ORN	83± 8	85± 5	139± 12 <sup>#</sup>	120± 13	134± 11 <sup>#</sup>	105± 10
SUMAA	3128±143	2861±90	3735±237 <sup>#</sup>	3271±221	4295±244 <sup>#</sup>	3206±140 *
SAP	<u>4± 1</u>	<u>115± 23 *</u>	<u>330± 87 <sup>#</sup></u>	<u>518± 124</u>	<u>1116± 136 <sup>#</sup></u>	<u>996± 116</u>

Values of amino acids are means ± SEM in µmol/L, values of SAP are means ± SEM in ng/ml. CO: control, LA: laparotomy, ST: small tumour of 5-15% of carcass weight, LT: large tumour of more than 15% of carcass weight. SUMAA included Glu, Asn, Ser, Gln, Gly, Thr, His, Cit, Ala, Arg, Tau, Tyr, Val, Met, Ile, Phe, Trp, Leu, Orn, and Lys. Statistics with Mann-Whitney U test: \*p<0.05 laparotomy effect, <sup>#</sup>p<0.05 tumour effect.

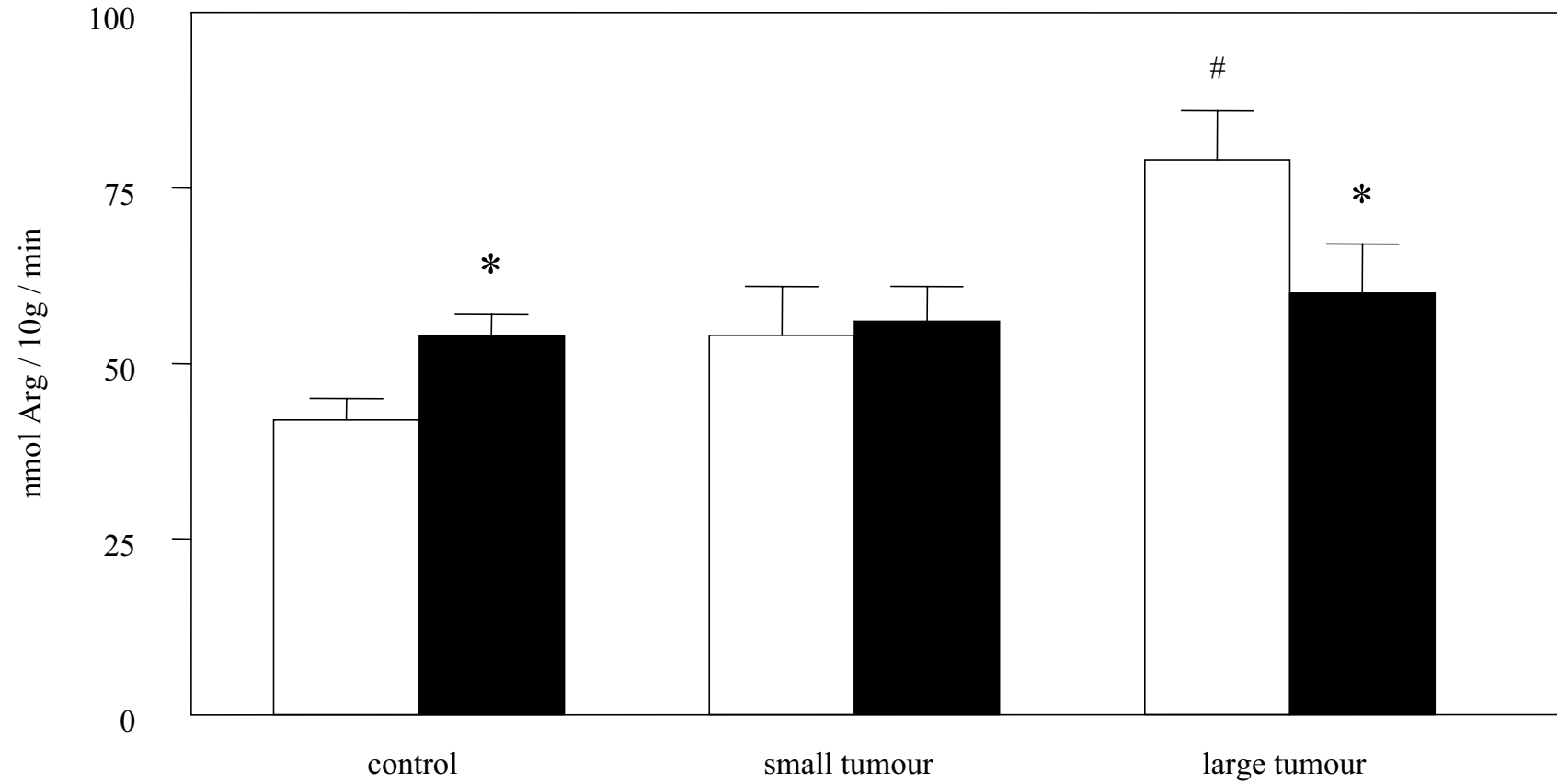


Figure 1

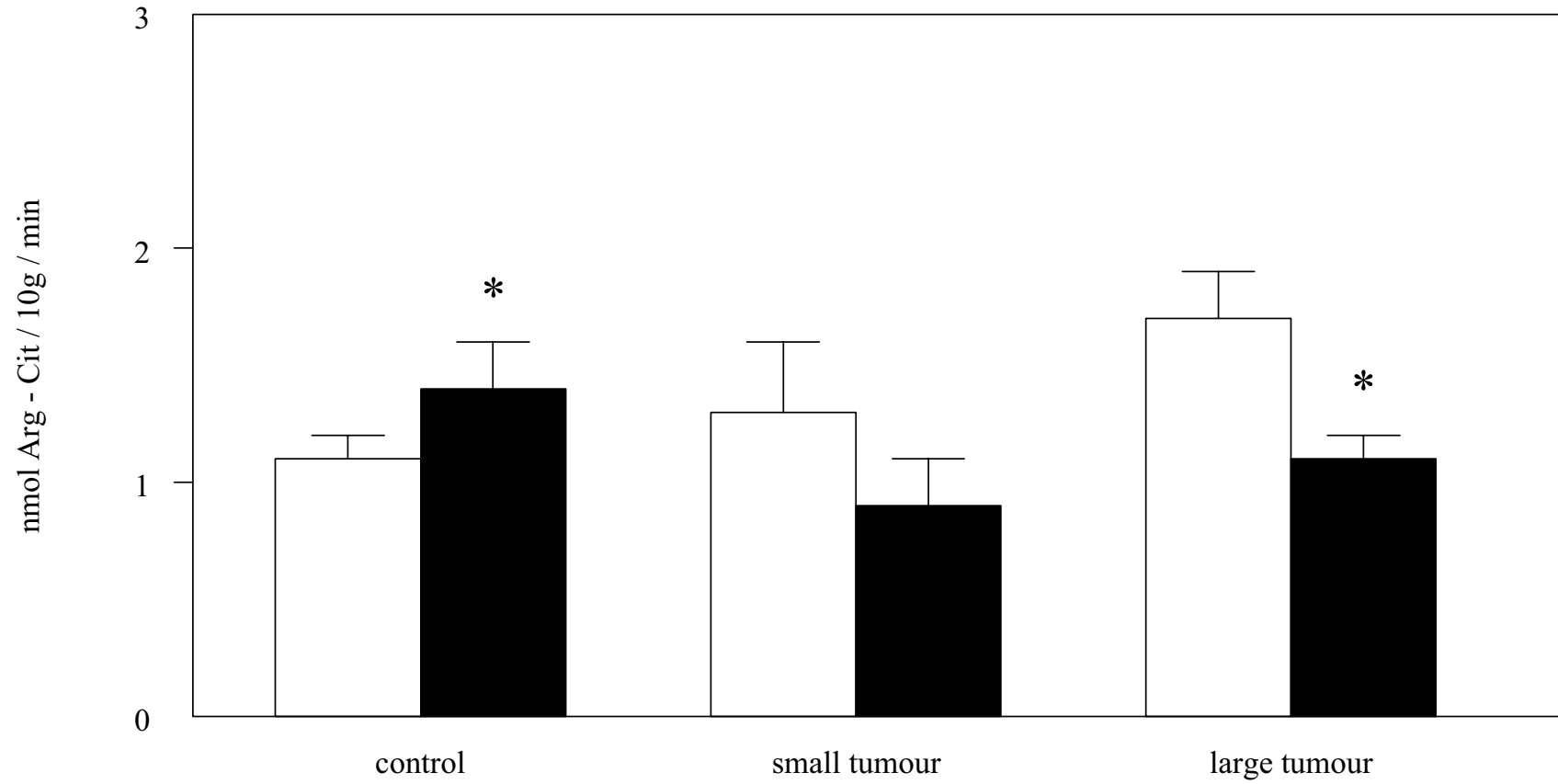


Figure 2