Review Article

Biochemistry, pharmacology and *in vivo* function of arginases

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Abstract

Arginase catalyzes the hydrolysis of L-arginine into L-ornithine and urea. The two existing isoforms Arg1 and Arg2 show different cellular localizations and metabolic functions. Arginase activity is crucial for nitrogen detoxification in the urea cycle, synthesis of polyamines, and control of L-arginine bioavailability and nitric oxide (NO) production. Despite significant progress in the understanding of the biochemistry and function of arginases, several open questions remain. Recent studies have revealed that the regulation and function of Arg1 and Arg2 are cell-type-specific, species-specific, and profoundly different in mice and humans. The main differences were found in the distribution and function of Arg1 and Arg2 in immune and erythroid cells. Contrary to what was previously thought, Arg1 activity appears to be only partially related to vascular NO signaling under homeostatic conditions in the vascular wall, but its expression is increased under disease conditions and may be targeted by treatment with arginase inhibitors. Arg2 appears to be mainly a catabolic enzyme involved in the synthesis of L-ornithine, polyamine, and L-proline but may play a putative role in blood pressure control, at least in mice. The immunosuppressive role of arginase-mediated arginine depletion is a promising target for cancer treatment. This review critically revises and discusses the biochemistry, pharmacology, and *in vivo* function of arginases, focusing on the insights gained from the analysis of cell-specific Arg1 and Arg2 knockout mice and human studies using arginase inhibitors or pegylated recombinant arginase.

Significance statement. The review emphasizes the need for further research to deepen our understanding of the regulation of Arg1 and Arg 2 in different cell types under consideration of their localization, species-specificity, and multiple biochemical and physiological roles. This will lead to better pharmacological strategies to target arginase activity in liver, cardiovascular, hematological, immune/infection diseases and cancer.

Running title: Pharmacology of arginases

Keywords: Arginase inhibitors, enzymatic catalysis of arginase, endothelial dysfunction, liver disease, red blood cells

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Abbreviations

2-(S)-amino-5-boronohexanic acid, ABH; Arginase 1, Arg1; Arginase 2, Arg2; asymmetric dimethylarginine, ADMA; coronary artery disease, CAD; cardiovascular disease, CVD; endothelial cells, ECs; endothelial nitric oxide synthase, eNOS; endothelium-dependent vasodilation, EDV; global L-arginine bioavailability ratio, GABR; hepatic stellate cells, HSC; inducible nitric oxide synthase, iNOS; interleukin, IL; ischemia reperfusion, I/R; isonitrosopropiophenone ISPF;; maximum reaction rate, V_{max}; Michaelis constant, K_m; mitogen activated protein kinase, MAPK; mass spectrometry, MS; Myeloid-derived suppressor cells, MDSC; myocardial infarction, MI; nitric oxide synthase, NOS; nitric oxide, NO; Nω-hydroxy-nor-arginine, nor-NOHA; red blood cells, RBCs; polymorphonuclear neutrophils, PMN; sickle cell disease, SCD; signal transducer and activator of transcription, STAT; solute carrier family 25 member 29, SLC25A29; streptozotocin, STZ; type 2 diabetes mellitus,T2DM; T cell receptor

I. Introduction

The enzyme arginase (L-arginine-urea hydrolase; EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to L-ornithine and urea and thereby participates in the final step of the urea cycle (**Fig. 1**). In mammals there are two isoforms of arginase, defined as arginase 1 (Arg1) and arginase 2 (Arg2), which are codified by independent genes (Arg1 6q23; Arg2 14q24.1-24.3) (Sparkes et al., 1986, Gotoh et al., 1997). The two isoenzymes differ substantially in subcellular localization and function. Arg1 is mainly localized in the cytoplasm and is highly expressed in hepatocytes in the liver and participates in the urea cycle. Instead, Arg2 is a mitochondrial enzyme that was first identified in the kidney (Kaysen and Strecker, 1973), but its function in the kidney and elsewhere has been unclear for many years.

Arg1 was found to regulate L-arginine bioavailability and nitric oxide (NO) synthesis from nitric oxide synthases (NOSs) in the immune system and the cardiovascular system (Durante et al., 2007); therefore, Arg1 was proposed to modulate macrophage M1/M2 polarization, suppress T-cell responses and regulate vascular endothelial function and NO-mediated cardioprotection. However, There is accumulating evidence from cell-specific mice and multiomics studies demonstrating that some of these mechanisms are instead modulated by Arg2, depend on the cell type or health/disease conditions, are species-specific and, in many cases, profoundly different in mouse and man.

Moreover, by synthesizing L-ornithine, arginases are also crucial for the generation and intracellular availability of polyamines, such as putrescine, spermidine, and spermine (**Fig. 2**) Notably, polyamines have been shown to be involved in the promotion of stem cell self-renewal (James et al., 2018), the induction of autophagy (Hofer et al., 2022), the protection against neurological disorders (Ghosh et al., 2020), immune cell functions, and immune suppression in the tumor microenvironment (Hayes et al., 2014).

This review will summarize the current state of knowledge on the biochemistry, pharmacology, and *in vivo* function of arginases, focusing on *in vivo* data obtained from the

27   analysis of cell-specific Arg1 knockout (Arg1$^{-/-}$) and Arg2 knockout (Arg2$^{-/-}$) mice, and from

28   human studies. Specifically, we will summarize the biochemistry, genetics, and cellular

29   biology of arginases; their cell-specific and species-specific role in the liver, vasculature,

30   bone marrow, blood (immune cells, red blood cells (RBCs)), and kidney; and summarize the

31   current pharmacological strategies, and the results of human studies using recombinant

32   arginase and arginase inhibitors.

33   The careful characterization of the cell-specific role of Arg1 and Arg2 in mouse models,

34   human cells/organoids, and human cohorts with single-cell and multiomics approaches will

35   allow a deeper understanding of the role of arginase in specific cells and tissues, thus

36   proving better diagnostic, prognostic, and therapeutic strategies to address cardiovascular,

37   hematological, inflammatory, and genetic diseases related to L-arginine metabolism and

38   cancer.

II. Biochemistry and regulation of arginase expression and activity

A. Genetic characteristics and regulation

The enzyme arginase catalyzes the hydrolysis of L-arginine to L-ornithine and urea (**Fig. 1**). In mammals, there are two isoforms of arginase, defined as arginase 1 (Arg1) and arginase 2 (Arg2), which are codified by independent genes. In humans, Arg1 and Arg2 show 58% sequence homology (Morris et al., 1997, Perozich et al., 1998). Human ARG1 gene was cloned by independently by the groups of Cederbaum and Mori in the 1980s and mapped to chromosome 6q23 (Dizikes et al., 1986, Sparkes et al., 1986, Haraguchi et al., 1987). Human ARG2 was cloned by the same groups 10 years later and mapped on chromosome 14q24.1–24.3 (Gotoh et al., 1996, Vockley et al., 1996, Gotoh et al., 1997). The gene sequence presents highly conserved residues among species (Jenkinson et al., 1996) (**Tab. 1**). Structural studies identified high similarity (50%) and sequence homology in encoded genes of arginase of different species (Jenkinson et al., 1996, Perozich et al., 1998) (**Tab. 1**).

Evolutionary, it is most likely that the two paralogues ARG1 and ARG2 genes originated by gene duplication before amphibians and mammals diverged (Patterton and Shi, 1994, Morris et al., 1997). Among all species, arginase and other ureohydrolases share multiple conserved residues, which play crucial roles in protein folding, binding of manganese-ions, and substrate interaction (Kanyo et al., 1996, Perozich et al., 1998). The sequence around the catalytic site of both Arg1 and Arg2 is highly conserved, which makes it difficult to synthesize isoform-specific arginase inhibitors. Current arginase inhibitors resemble its substrate L-arginine, are mostly unselective, and display similar K_i or IC_{50} values (Adams et al., 2017). However, there is currently an effort for synthetizing isoform-specific drugs (Gzik et al., 2024) (see also Chapter VI B).

The human Arg1 protein exists in three isoforms obtained by alternative splicing (**Tab.1; Fig. 3**). The canonical isoform 1 (P05089-1) comprises 8 exons that encode 322 amino acids.

65 The isoform 2 (P05089-2) includes an 8 amino acids insertion and therefore consists of 330

66 amino acids (**Fig. 3**). The isoform 3 (P05089-3) lacks exons 4 and 5, which encode for the

67 amino acids 204-289 and therefore make a total of 236 amino acids. Only one sequence is

68 known for Arg2, which is built up of 8 exons as well and comprises 354 amino acids (Sayers

69 et al., 2022).

70 Apart from several similarities between Arg1 and Arg2, the localization, cellular function, and

71 tissue- or cell-specific expression are rather diverse. In humans and rodents, Arg1 is found

72 constitutively expressed at high levels in the liver, particularly in the cytosol of hepatocytes,

73 where it is responsible for catalyzing the last step of the urea cycle. This is also the reason

74 why it was called "liver arginase" (Morris et al., 1997). The functions of Arg1, besides its role

75 in the urea cycle, are less characterized and profoundly different in mice and man. In human,

76 Arg1 is also expressed to a lower extent in the bone marrow, the blood, and the skin (Morris

77 et al., 1997, Kim et al., 2002, Bruch-Gerharz et al., 2003, Munder et al., 2005). In rodents,

78 Arg1 is constitutively expressed in multiple tissues, mainly in the liver and gastrointestinal

79 tract, but also in the uterus and the skin (Yu et al., 2003, Choi et al., 2012).

80 At the cellular level, Arg1 is constitutively expressed in hepatocytes and inflammatory cells

81 (M2 macrophages) in all species; in other cell types including vascular endothelial cells,

82 smooth muscle cells, cardiomyocytes is expressed constitutively is species-specific way and

83 in general at very low level; but its expression is induced in disease conditions (Morris et al.,

84 1997, Teupser et al., 2006, Gonon et al., 2012, Caldwell et al., 2015), as described in detail

85 in the next chapters. In humans, polymorphonuclear neutrophils (PMN) constitutively

86 express Arg1, which is localized in granules and can be released under pro-inflammatory

87 conditions (Rotondo et al., 2011). Interestingly, human and primate erythrocytes

88 constitutively express higher levels of Arg1 and display increased arginase activity, while

89 rodent erythrocytes express very low levels (Spector et al., 1985).

10

90 The regulation of the expression either isozyme is cell type-specific and can be influenced by

91 health/disease conditions and by the presence of pro-inflammatory (Th1) or anti-

92 inflammatory (Th2) cytokines. In the mouse, Arg1 expression was shown to be induced upon

93 Th2 cytokines like interleukin (IL)-4 and -6 stimulation in M2 macrophages via signal

94 transducer and activator of transcription (STAT) 6 signaling, by mitogen-activated protein

95 kinase (MAPK) - activating transcription factor-2 (ATF-2) signaling pathway, by

96 CCAAT/enhancer binding protein (C/EBP) β, and by the transcription factor forkhead box O4

97 (FoxO4) (Gray et al., 2005, Sheldon et al., 2013, Shatanawi et al., 2015, Zhu et al., 2015,

98 Schmok et al., 2017, Caldwell et al., 2018)

99 In contrast, Arg2 is mainly localized in the mitochondria and is constitutively expressed at

100 higher levels in the kidney, the urinary bladder, the prostate but also in human skeletal

101 muscle (Vockley et al., 1996, Morris et al., 1997, Rath et al., 2014), but a lower constitutive

102 expression of Arg2 was found in almost all cells of the body. The expression of Arg2 can be

103 upregulated in inflammatory or disease conditions in different cell types and in a species-

104 specific way. For example it was shown to be up-regulated by interferon regulatory factor 3

105 in Jurkat cells (Grandvaux et al., 2005), by the hypoxia-inducible factor-2 in HUVECs

106 (Krotova et al., 2010), by activation of the ERK5 (extracellular signal-regulated kinase 5)-

107 CREB (cyclic AMP-responsive element-binding protein) pathway in human Jurkat cells and

108 mouse monocytes, (Barra et al., 2011) and by IL-10 stimulation likely via signal transducer

109 and activator of transcription 3 activation in the mouse. It is therefore generally different from

110 the regulation of Arg1 (Grandvaux et al., 2005, Krotova et al., 2010, Barra et al., 2011,

111 Dowling et al., 2021).

112 **B. Protein structure and catalysis**

113 Human Arg1 and Arg2 show a similar trimeric structure and catalytical mechanisms (Cama

114 et al., 2003, Di Costanzo et al., 2005). Each monomer of arginase exhibits a typical

115 Rossman-fold structure, where β-sheets are wrapped by α-helices (Li et al., 2022a). The

116 molecular weight of each monomer varies among isoforms and species ranging from 30 to

117 40 kDa (**Tab. 1**) (Li et al., 2022a). The crystal structure of human Arg1 was characterized by

118 Di Costanzo et al. (Di Costanzo et al., 2005). The catalytic center of each arginase monomer

119 contains a Mn^{2+} coordinated to one His and 3 Asp (**Fig. 4**). The spin-coupled Mn^{2+}-Mn^{2+}

120 structure is formed in the catalytic center and activates arginase (**Fig. 4**) (Li et al., 2022a).

121 Mn^{2+} is required as a cofactor for converting L-arginine to L-ornithine and urea. Interestingly,

122 if Mn^{2+} is replaced with Co^{2+} the catalytic efficiency (k_{cat}/k_M) of Arg1 is increased (Stone et al.,

123 2010).

124 Interestingly, the kinetics of the hydrolysis of L-arginine to L-ornithine and urea catalyzed by

125 isolated/recombinant arginase is dramatically different when compared to the enzyme

126 expressed in cellular compartments. Purified rat liver arginase is characterized by a

127 Michaelis constant (K_m) of 1 mM and a maximum rate (V_{max}) of 4380 μmol/min/mg in the

128 presence of 10 mM $MnCl_2$ at pH of 7.5 (Reczkowski and Ash, 1994). In contrast,

129 recombinant human Arg1 and Arg2 expressed in HEK293T cells are characterized by a K_m

130 of 3.3 mM and V_{max} of 34 nmol/min/mg and a K_m of 1.9 mM and V_{max} of 883 pmol/min/mg

131 respectively at physiological pH value of 7.4 (Tommasi et al., 2018). These differences need

132 to be taken into consideration for pharmaceutical preparations containing recombinant

133 arginase, like the pegylated-arginases, which was recently approved for treating

134 hyperarginemia in patients with genetic deficiency of arginase (see chapter VI).

135 Interestingly, it was proposed that arginase kinetics in cells is modified by the presence of

136 binding partners that regulates the catalytical activity of the enzyme. Examples of binding

137 partners regulating arginase activity are human flotillin, which was proposed to bind Arg1 in

138 human RBCs (Jiang et al., 2006) or the embryonic stem cell-expressed Ras, which was

139 found to interact with Arg1 in hepatic stellate cells (HSC) (Pudewell et al., 2022).

140 Also, the concentration and availability of L-arginine may contribute to regulate the kinetic

141 activity of arginase enzymes. For example, in cultured RAW 264.7 cells and primary murine

142 alveolar macrophages, Arg2 or Arg1, respectively, were described to compete for L-arginine

143   bioavailability with the inducible nitric oxide synthase (iNOS), which catalyzes the oxidation

144   of L-arginine to L-citrulline and leads to high-output nitric oxide (NO) synthesis (Wang et al.,

145   1995, Hey et al., 1997, Sonoki et al., 1997, Momma and Ottaviani, 2022). Therefore, Arg1

146   was proposed to control L-arginine bioavailability and NO production by iNOS in murine

147   alveolar macrophages and other cell types co-expressing iNOS during pro-inflammatory

148   conditions (Sonoki et al., 1997, Rath et al., 2014), which also include for example human

149   keratinocytes (Bruch-Gerharz et al., 2003). However, the intracellular L-arginine

150   concentrations (approx. 100 µM) and the K_m of iNOS (2.8 µM) are much lower than the K_m

151   for Arg1 (2 mM) (Garganta and Bond, 1986, Stuehr et al., 1991)

152   Arg1 was also proposed to compete with eNOS for the common substrate L-arginine in ECs.

153   The intracellular concentration of L-arginine in ECs is ca. 100 µM, which should saturate

154   eNOS; however, paradoxically, NO production by eNOS in ECs can be still increased by

155   increasing extracellular L-arginine concentration. It remains unclear how excess extracellular

156   L-arginine can increase eNOS activity. The ability of extracellular L-arginine to increase NO

157   production in the presence of arginase was defined as "the L-arginine paradox" and it is still

158   partially unresolved (Girerd et al., 1990, Lundberg and Weitzberg, 2022).

159   **C. Summary**

160   To summarize, the two isoenzymes Arg1 and Arg2 catalyze the same reaction and their

161   tertiary and quaternary structure are similar. The kinetics of the reaction are affected by the

162   cell type, sub-localization in the cells (cytoplasm, granula, mitochondria), the presence of a

163   binding partner (flotillin), and the availability of L-arginine for the reaction.

164 # III. Biochemical assays for the determination of arginase activity

165 # and L-arginine metabolomics

166 Biochemical assays for determination of arginase activity and the new metabolomics

167 approaches have been playing a crucial role in understanding arginase function and its

168 involvement in different cellular processes. This chapter will discuss the various biochemical

169 assays used to determine arginase activity and arginine bioavailability, and their applications

170 for studying its role in systemic L-arginine metabolism in humans.

171 **A. The urea assay: colorimetric determination of urea formation in cells and tissues**

172 The first method to determine arginase activity was developed in 1945. The arginase activity

173 in liver or RBCs was quantified colorimetrically as formation of urea by derivatization with α-

174 isonitrosopropiophenone (ISPF) (Archibald, 1945, Van Slyke and Archibald, 1946). Later on,

175 it was modified by others for fitting a 96-well plate format and applied for determination of

176 arginase activity in leucocytes with a detection limit of 0.02 μmol urea (Corraliza et al.,

177 1994). Then the method was further improved and adapted by other groups for other sample

178 types (Romero et al., 2008, Yang et al., 2013, Bhatta et al., 2017, Heuser et al., 2022,

179 Pudewell et al., 2022).

180 In this assay, the samples (cells or tissue lysates) are pre-incubated at 58-60 °C with 7-10

181 mM $MnCl_2$ as cofactor for the activation of arginase. Afterwards, a millimolar concentration of

182 L-arginine (500 mM) is added into the mixture incubated for 1 min - 1 hour or more at 37 °C

183 according to specific arginase activity (i.e. activity/mg protein) expected in the specific

184 cell/tissue of interest. The enzymatic reaction is terminated by adding an acidic mix

185 composed of H_2SO_4, H_3PO_4, and H_2O, in v/v/v, 1:3:7 to denature the proteins in the sample

186 and to allow the derivatization reaction, which requires acidic conditions. Then a solution of

187 ISPF (9% in ethanol) is added to the samples and incubated at least 30 - 45 min at 100 °C.

188 Urea concentration in the samples is then determined colorimetrically by measuring the

189 absorbance of the pink ISPF-adduct with urea at 540 nm and quantified by comparing the

14

190 absorbance of a standard curve prepared by using standard concentrations of urea. Based

191 on this method, the arginase-dependent urea production in different cells and tissues

192 including kidney, liver, and brain was determined and compared among health and disease

193 conditions as well as different species (Iwata et al., 2002, Romero et al., 2008, Bagnost et

194 al., 2009, Bhatta et al., 2017, Heuser et al., 2022, Pudewell et al., 2022).

195 The main disadvantage of the urea assay for analysis of arginase activity in cells and tissues

196 is the lower sensitivity and signal-to-noise ratio due to the presence of urea and other

197 colored contaminants (like heme) in biological samples. For example, in plasma urea

198 concentration is very high and can be removed by size exclusion centrifugation filters. This

199 procedure is however not always applicable to tissues or cell lysates, thus limiting the

200 accuracy of the assay for samples with very low specific activity. An example of cells with

201 low specific activity of arginase are rodent RBCs or platelets (see below paragraph II.C.).

202 **B. Colorimetric determination of L-ornithine**

203 In 1952 Chinard et al. described a single cuvette assay based on the colorimetric reaction of

204 ninhydrin reagent with L-ornithine at very low pH (Chinard, 1952). Later on, the method was

205 optimized for microplates for the detection of arginase activity by analyzing the production of

206 L-ornithine in human hemolysates and cultured erythroleukemic cells (K562 cells) (Iyamu et

207 al., 2008). If applied for determination of arginase activity in cells and tissues, this method

208 has some major disadvantages including the interference of endogenous L-ornithine found in

209 sample at the steady state, as well as its complex trafficking and metabolism, which also

210 includes the formation of downstream products like polyamine.

211 **C. The radioactive assay: Conversion of ^{14}C-L-arginine into L-ornithine and ^{14}C-urea**

212 Rüegg and Russell first proposed the determination of arginase activity in bovine liver, calf

213 serum, and murine macrophage extract by applying L-[guanido-^{14}C]-arginine (Ruegg and

214 Russell, 1980). Before application into the assay the substrate, L-[^{14}C]-arginine was purified

215 by using an ion exchange column prepared by using a Dowex 50W X8 (hydrogen form)

15

216 resin, which removes potential contaminants (including spontaneously hydrolyzed arginine)

217 and leads to a very low background. According to their method, 1 volume of glycine buffer

218 containing 75 mM glycine, 2 mM $MnCl_2$, and 0.02% thymol blue at pH 9.7 was premixed with

219 7 volumes of 1 M L-arginine solution containing L-[^{14}C]-arginine and water (v/v, 1:6) and 10

220 mM $MnCl_2$, and then incubated at 56 °C, pH 7.5. The reaction was terminated by adding

221 acetic acid with 7 M urea, 10 mM L-arginine, and 0.001% methyl red at pH 4.5 in each

222 sample. The time-dependent formation of ^{14}C-urea was measured by scintillation counting at

223 different time points from 2 to 120 min (Ruegg and Russell, 1980).

224 By using L-[guanido-^{14}C]-arginine Spector et al. established a similar assay for the detection

225 of arginase activity in erythrocyte lysate (Spector et al., 1980). The produced ^{14}C-urea was

226 converted into ammonia and $^{14}CO_2$ by Jackbean urease, which was trapped by filter paper

227 soaked with NaOH as $Na_2{}^{14}CO_3$ followed by scintillation counting. By using this method, the

228 authors compared arginase activity in RBCs of different species, including humans and

229 primates, rats, rabbits, cats, and dogs; the arginase activity was normalized as μmol L-

230 arginine hydrolyzed per gram hemoglobin per hour (Spector et al., 1985). They found less

231 than 1 μmol urea/g hemoglobin/h of RBCs from Blab/c mouse, rat, rabbit, cat, and dog; in

232 contrast to more than 900 μmol urea/g hemoglobin/h of that in humans.

233 A further optimization of this method was carried out by Morris Jr. et al. later on (Morris et al.,

234 1998). This assay was applied by many authors to analyze arginase activity in cells, tissues,

235 and blood, and it is still one of the most sensitive and accurate assays. By applying this

236 assay, they found that the arginase activity in the plasma and RBCs of individuals with sickle

237 cell disease (SCD) were significantly higher than those in healthy controls (humans with

238 SCD vs healthy controls, 37.7 ± 2.9 vs 23.5 ± 1.7 nmol/mg/min in plasma and 2.1 ± 2.1 vs

239 0.4 ± 0.2 μmol/mL/h in RBCs) (Morris et al., 2005a).

240 **D. Systemic analysis of L-arginine bioavailability and L-arginine metabolism.**

241 With the discovery that upregulation of arginase activity may play an important role in

242 disease conditions, there has been a growing interest in the analysis of arginase activity and

243 L-arginine bioavailability in human cohorts and in pre-clinical studies in experimental

244 animals. As mentioned above, L-arginine is the common substrate for arginase and the NOS

245 enzymes; therefore, systemic L-arginine bioavailability is thought to be dependent on the

246 relative activity of both enzyme classes, their expression, and the compartmentalization of L-

247 arginine (Elms et al., 2013). For the analysis of L-arginine bioavailability, the "global L-

248 arginine bioavailability ratio" (GABR) was proposed. GABR is calculated as the ratio

249 between L-arginine level and the levels of L-ornithine and L-citrulline (GABR = L-arginine/(L-

250 ornithine + L-citrulline), which are metabolic products respectively of arginase and NOS

251 activity (Tang et al., 2009). Further studies have investigated the levels of L-arginine, L-

252 ornithine, and L-citrulline in plasma of human cohorts (Kovamees et al., 2016b, De Santo et

253 al., 2018, Burrage et al., 2019, Fan et al., 2021).

254 Alternatively, for determination of serum levels of L-arginine, L-ornithine, L-citrulline,

255 asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA), some

256 authors applied an HPLC method based on fluorescent derivatization with 6-aminoquinolyl-

257 N-hydroxysuccinimidyl carbamate (AccQ-Fluor™) (Heresztyn et al., 2004, Miyazaki et al.,

258 2018).

259 L-arginine, L-ornithine and L-citrulline, and the derived GABR were also measured directly in

260 plasma of subjects with coronary artery disease (CAD) by electrospray ionization tandem

261 mass spectrometry (LC/ESI/MS/MS) online with an API 365 triple quadruple mass

262 spectrometer using $^{13}C_6$-arginine as the internal standard for the quantitation (Tang et al.,

263 2009).

264 Metabolomics approaches were also established to investigate the L-arginine metabolism

265 and its corresponding regulation *in vivo* by using LC-qTOF-MS. One example is a method for

17

266 the determination of 16 amino acids, amino acid derivatives, and related compounds in

267 plasma to identify potential biomarkers in pediatric chronic kidney disease (Benito et al.,

268 2016). The metabolites comprised L-homoarginine, L-homocysteine, L-arginine, symmetric

269 dimethylarginine (SDMA), ADMA, dimethylglycine, S-adenosylhomocysteine, S-

270 adenosylmethionine, L-citrulline, betaine, creatine, creatinine, glutathione, methionine,

271 glycine, and cysteine (Benito et al., 2016). Another example is the quantitative analysis of L-

272 arginine metabolites, polyamines, and acetylated polyamines in various biological matrices

273 such as liver, muscle, adrenal glands, and brain in mice (Langner et al., 2022). Further

274 examples of applications of the analysis of arginase metabolites in human cohorts are

275 discussed in Chapter IV and summarized in the related **Tab. 4**.

276 **E. Summary**

277 To summarize, the arginase activity in cells and tissues was analyzed by measuring the

278 production of urea by derivatization and colorimetric assay, which has limitations due to

279 interference with endogenous urea (e.g. in plasma) or heme-containing proteins (hemoglobin

280 in RBCs). A more accurate method is the detection of the conversion of isotope-labeled (^{13}C

281 or ^{14}C) L-arginine into their enzymatic products ^{13}C or ^{14}C -L-ornithine/urea from arginases or

282 ^{13}C or ^{14}C-L-citrulline from NOS by scintillation or for nonradioactive isotopes by MS. MS has

283 the advantage that can be applied for metabolic analysis of L-arginine metabolites on a

284 larger scale and does not require the use of radioactive compounds.

IV. The biological role of arginase in cells and tissues

In this section, we aim to review and discuss the cell-specific role of arginase in the liver, immune cells, vasculature, RBCs, and the kidney and to highlight their significance in health and disease. Specifically, we will explore the diverse function of Arg1 and Arg2 in the tissues and point on the species-specific differences, their function, and regulation in rodents and humans. These differences are particularly important when pharmacological therapies are tested in pre-clinical models. The available cell-specific mice models and their phenotypes are summarized in **Tab. 2**.

A. Role of arginase in the liver

The liver is built up out of four major cell types: hepatocytes (~70%), HSCs (~13%), sinusoidal endothelial cells (~10%), and liver resident macrophages named Kupffer cells (~7%), (Racanelli and Rehermann, 2006, Chen and Tian, 2021). Interestingly, all of these cells express Arg1 and are of major importance to maintain liver homeostasis (MacParland et al., 2018).

Arg1, the so-called liver-type arginase, is predominantly and constitutively expressed in the liver, especially in hepatocytes (MacParland et al., 2018). The main role of arginase in the liver is the detoxification of the body from ammonia, which is produced as a result of the catabolism of amino acids and is potentially neurotoxic. Ammonia is detoxified in the urea cycle by forming urea, which can be then excreted by the kidney (Krebs and Henseleit, 1932, Strong et al., 2021) (**Fig. 5**).

The urea cycle can be divided into five enzymatic steps. The first two steps are located in the mitochondria, the last three in the cytoplasm of hepatocytes: (1) Ammonium, carbon dioxide, and two ATP are converted into carbamoylphosphate by the carbamoylphosphate synthetase I; (2) carbamoylphosphate is combined with L-ornithine to L-citrulline; (3) L-citrulline and aspartate are condensed to argininosuccinate by the argininosuccinate synthetase using one ATP; (4) argininosuccinate is cleaved into L-arginine and fumarate by

19

311   the argininosuccinase; and finally (5) Arg1 hydrolyzes L-arginine into L-ornithine and urea

312   (Pelley, 2012, Barmore et al., 2024). Urea is exported from the hepatocytes towards the

313   kidney where it is excreted in the urine.

314   The transport of L-ornithine and L-citrulline in and outside of the mitochondria is carried out

315   by L-ornithine carrier 1 and 2 and the solute carrier family 25 member A29 (SLC25A29)

316   (Fiermonte et al., 2003, Porcelli et al., 2014). However, the expression of SLC25A29 is lower

317   as compared to the other transporters. Therefore, the role of SLC25A29 in the urea cycle is

318   likely limited (Camacho and Rioseco-Camacho, 2009).

319   Besides the urea cycle, L-arginine can also be consumed by eNOS or iNOS to produce L-

320   citrulline and NO in liver ECs (Poisson et al., 2017), but also in hepatocytes, HSC, and

321   Kupfer cells macrophages (Cunningham and Porat-Shliom, 2021, Pudewell et al., 2022).

322   Furthermore, in the liver L-arginine can be converted into agmatine or creatine, whereas L-

323   ornithine can be further catabolized into polyamines, L-proline, or glutamate. The urea cycle

324   and its intermediates are tightly regulated (Wu and Morris, 1998). In individuals carrying a

325   genetic mutation of liver Arg1 the levels of L-arginine in plasma are elevated (Diez-

326   Fernandez et al., 2018), indicating that the higher arginase activity in the liver limits the

327   export of L-arginine in plasma. In line with those findings, global constitutive Arg1^{-/-} mice

328   display hyperarginemia and die between day 10 and 14 after birth because of

329   hyperammonemia (Iyer et al., 2002).

330   Conditional mouse models of Arg1 deficiency (Arg1[flox/flox] mice) were generated by targeting

331   exons 7-8 (El Kasmi et al., 2008) or exon 4 (Van den Bossche et al., 2012). Late-onset

332   global Arg1^{-/-} mice or hepatocyte-specific Arg1^{-/-} mice showed a similar phenotype

333   characterized by hyperarginemia, hyperammonemia, and dysregulation of amino acid

334   metabolism, but without any increase in L-ornithine (Kasten et al., 2013, Sin et al., 2013).

335   Interestingly, the same phenotype was found in another study in hepatocyte-specific Arg1^{-/-}

336   mice (Ballantyne et al., 2016), demonstrating that Arg1 in hepatocytes plays a major role in

20

337   regulating the systemic levels of ammonia and L-arginine. Other pathways that metabolize L-

338   arginine, like NO production by eNOS or iNOS, are mainly influenced by the extracellular L-

339   arginine concentration (MacKenzie and Wadsworth, 2003, Shin et al., 2011).

340   Polyamines play an important role in liver regeneration and homeostasis and are regulated

341   by arginase levels, import and export of polyamines and amino acids, as well as the

342   expression of the rate-limiting enzyme L-ornithine decarboxylase that converts L-ornithine to

343   putrescine (Luk, 1986, Dayoub et al., 2006, Uemura and Gerner, 2011, Okumura et al.,

344   2016, Sagar et al., 2021). Recently, it was shown that in HSC, Arg1 plays a major role in the

345   maintenance of quiescence, suggesting an effect of downstream polyamine synthesis

346   (Pudewell et al., 2022). The detailed role of arginase in sinusoidal liver endothelial cells is

347   less known. The consequence of EC-Arg1 knockout in mice was not specifically studied for

348   the liver in detail. However, mice lacking EC Arg1 did not show specific liver phenotype (at

349   least in our hands) (Heuser et al., 2022) and other authors did not mention any liver

350   phenotype in similar models (**Tab 2**) (Bhatta et al., 2017). Arg1 is also expressed in resident

351   liver macrophages (or Kupfer cells) in mouse liver. Although the canonical role of arginase in

352   mouse bone marrow and alveolar macrophages has been well studied (see paragraph IV.

353   B), cell-specific analysis of the effects of arginase in Kupfer cells on liver pathophysiology is

354   still lacking.

355   **B. Role of arginase in the immune system**

356   Arginases are crucially involved in various aspects of inflammation and immunomodulation

357   both in health and disease conditions. Increased arginase activity has been involved in

358   inflammation-triggered immune dysfunction, tumor immune escape, fibrosis,

359   immunosuppression, and immunopathology of infectious diseases (Bronte and Zanovello,

360   2005, Munder, 2009, Murray, 2016, Martí and Reith, 2021). The regulatory role of Arg1 and

361   Arg2 in the immune response profoundly differs between mice and humans.

362 According to a classical view, mouse macrophages can be classified as belonging to two

363 subtypes named M1 and M2 based on their role in inflammatory response and their

364 expression of iNOS (M1) or Arg1 (M2) (**Fig. 6**) (Thomas and Mattila, 2014, Murray, 2017). In

365 this model, the balance between arginase and iNOS activity in macrophages dictates the

366 outcome of immune responses. M1 macrophages preferentially metabolize L-arginine via

367 iNOS into NO and L-citrulline and orchestrate the first pro-inflammatory phase of the immune

368 response; instead, M2 macrophages metabolize L-arginine via Arg1 into L-ornithine and

369 urea and are mainly involved in anti-inflammatory responses (Thomas and Mattila, 2014,

370 Murray, 2017). The underlying mechanism involves the activation of iNOS expression in M1

371 macrophages by T helper 1 -derived cytokines (IL-1β, TNFα) and IFN-γ induces iNOS

372 expression via activation of transcription factors like NFκB, AP1 and drives the classical M1

373 activation pathway. T helper 2 cytokines like IL-4, IL-10, and IL-13 suppress iNOS activity

374 and promote Arg1 expression (Bronte and Zanovello, 2005, Thomas and Mattila, 2014, Martí

375 and Reith, 2021). Recently it was shown that Arg2 is present in the mitochondria of pro-

376 inflammatory M1 macrophages and is essential for IL-10 metabolic downregulations to

377 resolve the cell inflammatory status (Dowling et al., 2021). According to this view of M1

378 macrophages expressing iNOS and M2 macrophages expressing Arg1, it is tempting to

379 speculate that control of L-arginine bioavailability via these enzymes may also involve L-

380 arginine transporters like CAT1 and CAT2 and act via inter-cellular communication

381 In humans, the M1/M2 dichotomy is not well defined and controversially discussed in the

382 literature (Munder, 2009, Thomas and Mattila, 2014). In human blood circulating monocytes

383 do not express Arg1; instead, human Arg1 is constitutively expressed in PMN granules, is

384 released in response to pro-inflammatory stimuli and regulates immune T-cell responses

385 (Munder et al., 2005, Munder et al., 2006, Oberlies et al., 2009). Less is known about the

386 role of Arg1 in human tissue macrophages, and in general about the role of Arg2, which is

387 constitutively expressed in mitochondria and contributes to L-arginine metabolism (Martí and

388 Reith, 2021).

389　T lymphocytes play a central role in the adaptive immune response. It is well known that L-

390　arginine starvation impairs T cell functions by multiple mechanisms (Geiger et al., 2016,

391　Martí and Reith, 2021). T cell proliferation is dose-dependent on L-arginine, with maximal

392　proliferation occurring at plasma concentrations (~100 μmol/L) (Ochoa et al., 2001). CD8^{+} T

393　cells show a more pronounced dependency on L-arginine availability than CD4^{+} T cells.

394　Moreover, dietary L-arginine supplementation improves thymic weight and T-cell reactivity in

395　both rats and humans (Martí and Reith, 2021).

396　Mechanistically, L-arginine starvation impairs T cell function through the downregulation of

397　the CD3ζ subunit of the T cell receptor (TCR) complex, crucial for TCR assembly and

398　activation signaling (Munder et al., 2006, Munder, 2009). Additionally, L-arginine deprivation

399　disrupts TCR signaling, reduces IL-2 production, and affects cell cycle regulators, causing T

400　cell arrest in the G0-G1 phase (Martí and Reith, 2021). L-arginine starvation also inhibits

401　glycolysis in T cells without affecting mitochondrial function, although high L-arginine levels

402　can enhance CD8^{+} T cell anti-tumor activity *in vivo* (Grzywa et al., 2020).

403　Human T cells constitutively express mitochondrial Arg2(Lowe et al., 2019, Martí i Líndez et

404　al., 2019), while the constitutive expression of Arg1 is under debate(Martí and Reith, 2021)

405　(Murray, 2016). Therefore Arg2 regulates T-cell intracellular L-arginine metabolism and plays

406　a critical role in T cell function (Martí and Reith, 2021). Inhibition or deletion of Arg2

407　enhances T-cell activation and anti-tumor responses, independent of extracellular L-arginine

408　levels (Grzywa et al., 2020, Martí and Reith, 2021). Arg2 also supports regulatory T cell

409　(Treg) function and survival, indicating its potential as a therapeutic target in autoimmune

410　and neoplastic diseases (Grzywa et al., 2020). Based on the mechanism of arginase

411　inhibiting T cell function in the tumor microenvironment to promote cancer growth, novel

412　immunotherapy vaccines targeting Arg1 or Arg2 are developed and are now under clinical

413　trials (Martinenaite et al., 2019, Weis-Banke et al., 2020, Lorentzen et al., 2022, Niu et al.,

414　2022).

23

415 Human granulocytes subpopulations and especially polymorphonucleated neutrophiles

416 express both isoforms of arginase. In these cells, Arg1 is not found in the cytoplasm, but

417 rather in the cytoplasmic granules and, as briefly mentioned before, Arg1 release in the

418 extracellulare space exerts immunosuppressive functions by depletion of L-arginine and

419 inhibition of T effector cell responses (Munder et al., 2005, Munder et al., 2006, Oberlies et

420 al., 2009). Surprisingly the expression of Arg1 is not regulated by Th2-cytokines and other

421 stimuli in these cells (Munder, 2009, Murray, 2016). Similar T-cell immunosuppressive

422 activity of arginases is found in myeloid-derived suppressor cells (MDSCs). MDSCs are a

423 heterogeneous population of immature myeloid cells at different stages of myelopoiesis

424 exerting immunosuppressive function through their ability to metabolize and deplete L-

425 arginine, which is needed for T-cells-mediated responses (Bronte et al., 2016, Ostrand-

426 Rosenberg and Fenselau, 2018). Indeed MDSCs express high levels of both Arg1 and iNOS

427 (Gabrilovich and Nagaraj, 2009), and were described to inhibit T cells by high-output NO

428 production (Jia et al., 2010), and by inducing L-arginine starvation of T cells (Raber et al.,

429 2014). Arg1 is also crucial for the inhibition of allo-stimulated T cell inhibition by MDSCs

430 (Bronte et al., 2003). Hence, arginases and in general L-arginine metabolic enzymes in

431 MDSCs are considered as excellent molecular targets of immunoregulatory compounds in

432 infectious diseases and cancer (**Tab. 3**).

433 In the tumor microenvironment, abundant arginase activity is mainly related to the presence

434 of MDSCs and L-arginine metabolism is one of the metabolic pathways responsible for

435 tumor progression (Kim et al., 2018). Moreover, upregulation of either Arg1 or Arg2

436 expression/activity has been reported in several cancer types (Graboń et al., 2009, de

437 Boniface et al., 2012, Bedoya et al., 2014). Accumulating research indicates that inhibiting

438 the potent immunosuppressive mechanisms of MDSCs can be a therapeutic target to restore

439 T-cell activity and immunotherapy success in antifungal therapy (Law et al., 2020). Indeed,

440 pharmacological inhibition of MDSC-derived Arg1 expression by either SB202190, which is a

441 specific inhibitor of p38, or vandetanib, an orally available receptor tyrosine kinase inhibitor,

442   significantly enhanced T-cell-mediated antifungal responses against C. neoformans infection

443   (Li et al., 2022b). It has also been reported recently that by using the arginase inhibitor OAT-

444   1746, the negative effects of Arg1 in ovarian carcinoma can be mitigated (Czystowska-

445   Kuzmicz et al., 2019). Please refer also to Chapter IV and Table 3 and 4.

446   In conclusion, arginases significantly influence immune responses and inflammation, with

447   elevated activity linked to various pathological conditions such as immune dysfunction, tumor

448   progression, and immunosuppression. The roles of Arg1 and Arg2 differ between mice and

449   humans, in particular regarding their modulation of macrophage and T cell functions. The

450   use of pharmacological inhibitors to improve immunotherapy outcomes or recombinant

451   arginase to induce cancer cell L-arginine starvation is showing a great potential in cancer

452   therapy.

453   **C. Role of arginase in red blood cells**

454   It has long been known that Arg1 is present in circulating RBCs and that its protein level

455   differs considerably among species (Azizi et al., 1970, Spector et al., 1983). Humans and

456   primates have a high level/activity of arginase, while rodents, cats, and dogs have a rather

457   low arginase activity (which results often under the detection limits of common methods)

458   (Azizi et al., 1970, Spector et al., 1983).

459   In general, the proteome of circulating RBCs is made of proteins, which were synthesized

460   during their maturation from proerythroblasts to reticulocytes in the bone marrow. Human

461   erythroid cells express both Arg1 and Arg2 (Kim et al., 2002, Grzywa et al., 2021a). In

462   human erythroid cells, the expression of arginases starts during the late phase of

463   erythropoiesis, when the hematopoietic stem cell differentiates into proerythroblasts, while

464   the highest protein level is found in circulating human RBCs (Grzywa et al., 2021a, Grzywa

465   et al., 2021b). Importantly, Arg2 was found to be upregulated by 12-fold during erythroid

466   differentiation and remained elevated in late-stage erythroblasts, whereas Arg1 was

467   upregulated at very late-stage terminal differentiation (Grzywa et al., 2021a, Grzywa et al.,

468 2021b). Likely, Arg2 is lost when the mitochondria and the nucleus are extruded (Grzywa et

469 al., 2021a, Grzywa et al., 2021b). In human erythroid cells, there is also an alternatively

470 spliced variant of Arg1 with preserved activity (Kim et al., 2002).

471 The strong induction of Arg1 and Arg2 in human proerythroblasts was associated with a

472 continuous requirement for extracellular L-arginine throughout the erythroid differentiation

473 process. Notably, L-arginine in this context was not required for the synthesis of creatine or

474 NO, but rather for polyamine biosynthesis and the hypusination of the eIF5A transcription

475 factor (Shima et al., 2006, Gonzalez-Menendez et al., 2023). Interestingly, mouse

476 proerythroblasts express Arg1 at lower levels as compared to humans (Grzywa et al., 2021b,

477 Shahbaz et al., 2021).

478 There is compelling evidence that Arg1 levels in human RBCs are increased in people with

479 SCD (Iyamu et al., 2005, Morris et al., 2005a). SCD is caused by different mutations in the

480 β-globin chain of hemoglobin. Hypoxia induces sickling of RBCs due to polymerization of

481 abnormal hemoglobin (Pauling et al., 1949). Sickle cells are stiffer, more fragile, more prone

482 to rupture, and show an increased arginase activity (Iyamu et al., 2005). Specifically, it was

483 proposed that the release of RBC protein content due to cell rupture/damage induces an

484 increase of free hemoglobin and Arg1 in plasma. The increase in free hemoglobin in plasma

485 leads to systemic oxidative stress and NO scavenging, while the increased arginase activity

486 in plasma leads to reduced L-arginine bioavailability. Therefore, both free hemoglobin and

487 arginase in plasma may contribute to the pathophysiology of SCD by promoting endothelial

488 dysfunction and pulmonary hypertension. (Morris et al., 2005a). A similar pathophysiology

489 was also observed in the hemolytic uremic syndrome (Friberg et al., 2024).

490 Interestingly, other authors proposed that liberation of arginase from human RBCs into the

491 plasma may also exert immunosuppressive effects by L-arginine depletion (Bernard et al.,

492 2008, Munder, 2009). This immunosuppressive effect of arginase released from RBCs could

493 also be a possible explanation for the increased risk of invasive bacterial infection in humans

494 with SCD.

495 Another role attributed to Arg1 in RBCs is the control of systemic NO bioavailability and NO

496 release from RBCs (Yang et al., 2013). We and others have shown that eNOS is present in

497 RBCs (Kleinbongard et al., 2006, Cortese-Krott et al., 2012). In line with this finding, Yang et

498 al. showed that the inhibition of Arg1 in human RBCs regulates eNOS-dependent export of

499 NO-metabolites and contributes to cardioprotection in a Langendorff bioassay (Yang et al.,

500 2013). By comparing the cardiovascular hemodynamics and the outcome of acute

501 myocardial infarction in RBC- and EC-specific eNOS$^{-/-}$ mice, we recently demonstrated that

502 eNOS present in RBCs regulates blood pressure, the levels of circulating NO metabolites

503 and is cardioprotective (Leo et al., 2021, Cortese-Krott et al., 2022). Recently, an erythroid

504 cell targeted Arg1$^{-/-}$ mouse was generated by using mice expressing a Cre-recombinase

505 under the control of the promoter for erythropoietin receptor, and crossed on an apoE$^{-/-}$

506 background. These mice showed increased vascular calcification on high-fat diet and

507 increased S-nitrosoglutathione reductase activity in the vessels (Gogiraju et al., 2022).

508 It is important to point out that mice and rats are unlikely to be good models for studying the

509 role of Arg1 in RBCs *in vivo*. According to all studies investigating arginase activity

510 monitoring the conversion of isotopically labeled L-arginine into L-ornithine and urea, the

511 arginase activity in mouse and rat RBCs is very low or even undetectable under some

512 conditions (Azizi et al., 1970, Spector et al., 1983). Moreover, the expression and activity of

513 arginase in mouse monocytes is very high (Munder et al., 2005), which may contaminate

514 RBC samples. In addition, when considering the results of mouse studies obtained with the

515 loxP/Cre system, the specificity of the promoter and its regulation often also determine the

516 quality of the results. As pointed out before the Tie2 promoter drives gene expression in

517 both, ECs and cells of the myeloid cell lineage (leukocytes) (Payne et al., 2018), and the

518 phenotype may derive from vascular or immune cell dysfunction.

519   There is no doubt however that an increase in arginase activity in human RBCs has an

520   important pathophysiological role. Independent human studies found that the levels of L-

521   arginine in human RBCs correlated to the levels of Arg1 expression and activity in RBCs

522   (Spector et al., 1985, Morris et al., 2000, Morris et al., 2005a). As pointed out before, RBC

523   arginase activity was found to be elevated in hematological diseases (especially in mutations

524   of hemoglobin like SCD) and also in cardiovascular disease (CVD) (Azizi et al., 1970, Iyamu

525   et al., 2005, Morris et al., 2005b).

526   In the late '80s Cederbaum et al. proposed the absence of arginase in RBCs from lower

527   animals and its presence in RBCs from primates may be the results of an evolutionary

528   adaptation, rather than the "vestigial presence of an arcane function" (Spector et al., 1985).

529   It is unclear whether the presence of arginase expression in the RBCs confers any obvious

530   advantage or disadvantage on the animal who carries it. This interesting perspective was

531   sadly not followed further.

532   To summarize, while human RBCs carry high levels of Arg1, mice, and rats express Arg1 at

533   a very low level. An increase in RBC arginase activity plays a major role in SCD

534   pathophysiology and was proposed to be also immunomodulatory. More studies with human

535   erythroid precursor cells and human cohorts are required to understand the

536   pathophysiological role of arginase in RBCs and how its levels and activity may be

537   modulated under disease conditions.

538   **D. Role of arginase in the vasculature**

539   In the vasculature, L-arginine is mainly converted into NO by the eNOS (EC:1.14.13.39)

540   expressed in the ECs. NO is involved in the modulation of endothelial function, vascular

541   tone, organ perfusion, and blood pressure (Moncada et al., 1991, Farah et al., 2018,

542   Ostrand-Rosenberg and Fenselau, 2018, Lundberg and Weitzberg, 2022). Reduced

543   bioavailability of NO results in endothelial dysfunction and promote hypertension,

544   atherosclerosis, and myocardial infarction.

28

545 In the vascular wall ECs and vascular smooth muscle cells may express both isoforms of

546 arginase (although there are some species-specific patterns for Arg1 or Agr2, as carotid

547 porcine EC for example express Arg2 and not Arg1 (Thacher et al., 2010)) (**Fig. 7**).

548 Nevertheless, multiple papers describe arginase as a counterpart of eNOS in vascular ECs

549 for modulating endothelial function (Kim et al., 2009, Chung et al., 2014, Krause et al.,

550 2015). Thus, increased arginase activity in the vessel wall was proposed to limit the

551 bioavailability of L-arginine for eNOS and therefore decrease NO production resulting in

552 endothelial dysfunction and hypertension (Zhang et al., 2001, Toque et al., 2013, Caldwell et

553 al., 2018, Mahdi et al., 2020a, Li et al., 2022c).

554 Multiple pre-clinical studies in rodents showed that the oral administration of arginase

555 inhibitors, such as 2(S)-Amino-6-BoronoHexanoic acid (ABH) and N-hydroxy-nor-arginine

556 (nor-NOHA) improved eNOS dependent vasorelaxation, endothelial function, and decreased

557 blood pressure in spontaneously hypertensive rat, old rats or rats fed with high-fat diet (Kim

558 et al., 2009, Bagnost et al., 2010, Chung et al., 2014).

559 A similar phenotype was observed in EC-specific Arg1$^{-/-}$ mice generated by using Cadherin-5

560 (Cdh5)-promoter Cre-recombinase mice and put on a high-fat and high-sucrose diet. In

561 these mice, the deletion of Arg1 in ECs protected mice from vascular dysfunction (Bhatta et

562 al., 2017, Yao et al., 2017). Interestingly, EC/myeloid cell-specific Arg1$^{-/-}$ mice generated by

563 using Tie2-Cre-recombinase mice did not improve vasomotor function in diabetic mice

564 (Chennupati et al., 2018). It is important to point out, the Tie2-promoter drives Cre-

565 recombinase expression in all subtypes of ECs but Cre-expression was also found in the

566 hematopoietic cell lineage or, depending on the gene construct, also in the heart valves

567 (Payne et al., 2018). The Cdh5-promoter drives the expression of Cre-recombinase

568 specifically in ECs and it is generally considered as the most specific promoter, in particular

569 when the activity of the Cre-recombinase is also inducible by tamoxifen (Cdh5ET2-Cre

570 mice); however, in some models where Chd5-Cre recombinase expression is constitutive,

571 the expression of Cre-recombinase was also observed in hematopoietic cells and the

572 cardiac valve.

573 Work from our own laboratory demonstrated that under homeostatic conditions EC Arg1^{-/-}

574 mice (tamoxifen-inducible Cdh5ET2-Cre) show a downregulation of eNOS in the aorta and a

575 fully preserved vascular function and NO-metabolites under basal conditions (Heuser et al.,

576 2022). We also observed a compensatory upregulation of Arg1 in the aorta, which points to

577 an upregulation of Arg1 in vascular smooth muscle cells (Heuser et al., 2022). Therefore, the

578 relationship between eNOS and Arg1 in ECs *in vivo* is far more complex than a competition

579 for their common substrate. Accordingly, a recent study provided quantitative evidence that

580 in murine macrophages and human umbilical artery ECs, there was no direct competition

581 between Arg1 and the NOS enzymes if a constant flux of L-arginine is provided (Momma

582 and Ottaviani, 2022).

583 The role of Arg2 in vessels is less known. Arg2 expression in the mitochondria of ECs and

584 smooth muscle cellss is lower as compared to Arg1, and their functions are difficult to

585 discern without genetic manipulation. There are few studies investigating the role of

586 mitochondrial Arg2 in the endothelium in mice. One animal study showed that Arg2 in aged

587 mice is the key isoform responsible for the total arginase activity in the aorta of aging mice

588 leading to eNOS uncoupling and endothelial dysfunction (Shin et al., 2012). This finding is

589 supported by another study showing that mice overexpressing Arg2 in the endothelium

590 showed endothelial dysfunction, hypertension, and enhanced atherosclerosis (Vaisman et

591 al., 2012). In addition, Arg2 is reported to promote a pro-inflammatory effect, contributing to

592 insulin resistance and atherogenesis (Ming et al., 2012, Yang and Ming, 2014). (Ming et al.,

593 2012, Yang and Ming, 2014). In addition, Arg2 is reported to promote inflammation,

594 contributing to insulin resistance and atherogenesis (Ming et al., 2012, Yang and Ming,

595 2014). Interestingly, in porcine carotid endothelial cell Arg2 was upregulated by oscillatory

596 shear stress; as a result, porcine carotid arteries subjected to oscillatory shear stress

597 showed a decreased bradykinin-induced vasorelaxation, which could be recovered by

598   treatment with the arginase inhibitor NorNOHA (Thacher et al., 2010). Interesting porcine

599   carotid arteries and their cellular components (ECs and SMCs) express Arg2, but not Arg1,

600   showing a further species-specificity feature of arginases in the vasculature.

601   Overall, these results indicate that Arg1 does not appear to be involved in the regulation of

602   NO-dependent vasorelaxation in homeostatic conditions and that increase in arginase

603   activity is correlated to regulation of vascular remodeling and stiffens probably via L-arginine

604   depletion and synthesis of polyamines.

605   Further studies are needed to understand whether and how arginase expression or activity

606   in the vascular endothelium is regulated by pathophysiological stimuli like shear stress or

607   turbulent flow and how this regulation is coordinated with eNOS activity.

608   **E. Role of arginase in the heart**

609   In the heart, Arg1 is expressed in coronary ECs and cardiomyocytes in a species-specific

610   way. The expression of Arg1 has been found to be upregulated in coronary arterioles in

611   humans with type 2 diabetes mellitus (T2DM) and in homogenate of the right atrial

612   appendage samples collected during cardiac surgery (Chen et al., 2006, Beleznai et al.,

613   2011). Arg1 is constitutively expressed in cardiomyocytes of felines and affects

614   cardiomyocyte NO signaling, whereas Arg2 is not constitutively expressed in feline

615   cardiomyocytes (Jung et al., 2006). Rat heart lysate shows expression of both Arg1 and

616   Arg2 but cardiomyocytes from rats express only Arg2 (Steppan et al., 2006).

617   Nor-NOHA-mediated arginase inhibition during ischemia-reperfusion (I/R)-injury in rats

618   resulted in reduced infarct size and elevated plasma nitrite levels *in vivo* (Jung et al., 2010,

619   Tratsiakovich et al., 2013). Furthermore, Arg1 expression was significantly increased in the

620   ischemic myocardium of rats (Jung et al., 2010). Whether these effects are due to the

621   expression of Arg1 in the myocardial cells (cardiomyocytes, vascular cells) or from infiltrated

622   neutrophiles or other blood cells was not further investigated. Indeed infiltrated neutrophiles

623   are known to contribute to the infarct size at least in rat (Williams et al., 1994). It has also

624 been shown that in pigs, Arg1 expressed in coronary arterioles modulates NO-mediated

625 vasorelaxation (Zhang et al., 2001). Furthermore, it has been proposed that coronary

626 endothelial cell dysfunction plays a role in the microvascular injury occurring after I/R injury.

627 This hypothesis is supported by the finding that mice overexpressing TNF-α show an

628 increase in arginase activity and the expression of Arg1 in ECs at basal conditions and after

629 I/R-injury as well (Gao et al., 2007). In addition, these mice show a significant reduction in

630 maximal vasodilation after I/R injury as well as a decrease in eNOS expression in coronary

631 arterioles.

632 To summarize, also in the heart, arginases show a species-specific and cell-specific

633 expression. It appears that Arg1 plays a role in the pathophysiology of myocardial infarction

634 whereas Arg2 plays an immunosuppressive and protective role, at least in rodents. The data

635 on the expression/activity and function of arginases in human heart tissue is still too sparse

636 to be able to make a clear conclusion about its role in the heart. More research is needed in

637 this direction, perhaps by using novel single cell sequencing and mapping in heart biopsies.

638 **F. L-Arginine metabolism and role of arginase in the kidney**

639 The kidney plays an essential role in the endogenous synthesis of L-arginine. L-Arginine is

640 synthesized by arginosuccinate synthetase and arginosuccinate lyase out of L-citrulline

641 (Szepesi et al., 1970, Morris et al., 1989). As mentioned in paragraph IV.A, there is a high

642 turnover of L-arginine through the urea cycle in the liver; however, the urea cycle is tightly

643 regulated in a way that L-arginine is promptly metabolized further, and thereby does not

644 contribute to the circulating levels of L-arginine under homeostatic conditions. In contrast, in

645 the kidney only a small part of the synthesized L-arginine is used for the production of

646 polyamines and creatine from L-ornithine, while most of it is released into the circulation,

647 making it available for other tissues (Rogers et al., 1972) (**Fig 8**).

648 L-Arginine is synthesized throughout the whole length of the proximal tubule by ASS and

649 ASL, but in the proximal convoluted tubule synthesis is the highest, which is consistent to the

32

650   highest expression of ASS and ASL in the nephron (Levillain et al., 1990, Levillain, 2012). L-

651   Arginine synthesis gradually decreases in the terminal part in the outer medulla, i.e. in the

652   proximal convoluted tubule, where a lower but significant synthesis takes place.

653   Notably, approximately 83% of L-citrulline released from the small intestine undergoes renal

654   metabolism. Thus, circulating L-citrulline availability is the limiting factor in renal L-arginine

655   production (Windmueller and Spaeth, 1981, Dhanakoti et al., 1990). At least in rats,

656   endogenous production of L-arginine from L-citrulline is necessary for normal growth and it

657   cannot be completely restored by the diet, demonstrating the importance of renal production

658   of L-arginine for the optimal growth in young animals (Hoogenraad et al., 1985). Surprisingly,

659   in rats, the level of L-arginine in plasma is normally stable even after chronic renal failure

660   due to the increased plasma concentration of L-citrulline and the rise of urea that may inhibit

661   the arginase activity (Moradi et al., 2006).

662   The total arginase activity is low in the kidney, and its function is still not fully understood.

663   The predominant arginase isoform expressed in the kidney is the mitochondrial isoenzyme

664   Arg2, which is also known as "the kidney arginase". It is constitutively expressed in the

665   kidneys of humans, rodents, and likely in all mammals, whereas Arg1 is not expressed in the

666   kidney under homeostatic conditions (Spector et al., 1983, Morris et al., 1997, Miyanaka et

667   al., 1998, Morris et al., 2011). In rats, Arg2 is expressed mostly in the proximal straight

668   tubule (Miyanaka et al., 1998). Studies carried out on male and female mice showed that

669   female mice have a 3-fold higher Arg2 expression and activity as compared to male mice

670   (Levillain et al., 2005a). In rats, Arg2 is expressed at higher levels in the inner medullary

671   collecting ducts as compared to the thin descending and ascending limbs of Henle's loop.

672   Accordingly, enzymatic activity of Arg2 is not homogenous in the whole kidney, but it occurs

673   mainly in the outer stripe of the outer medulla and inner medulla (Levillain et al., 1989).

674   Interestingly, there is evidence that all three NOS isoforms are expressed in the inner

675   medullary collecting duct (as summarized previously (LoBue et al., 2023)), which may

676 indicate a mutual regulation between these two enzyme classes in the metabolism of L-

677 arginine in the kidney (Wu et al., 1999, Levillain et al., 2005b, Hyndman et al., 2013). It has

678 been hypothesized that the renal arginase activity may be important for L-ornithine

679 production and subsequently polyamine metabolism for the maintenance of normal tissue

680 homeostasis and, only in small part, for producing urea, which may contribute to concentrate

681 the urine in the medulla (Levillain et al., 1989, Waddington et al., 1998, Brosnan and

682 Brosnan, 2004).

683 Interestingly, diabetic Arg2$^{-/-}$ mice chronically treated with streptozotocin (STZ) were

684 protected against diabetic nephropathy. The lack of Arg2 in these mice protected them

685 against STZ-induced albuminuria, macrophage recruitment, and histopathological changes,

686 leading to renal tissue protection and suggesting a role of Arg2 in diabetic nephropathy.

687 Moreover, the lack of Arg2 protected for a decrease in renal modular blood flow, which is

688 consistent with preserved renal NO production (Morris et al., 2011). Accordingly, it was

689 shown that a specific lack of Arg2 in ECs reduced renal fibrosis in mice by restoring NO

690 levels and mitochondrial function in the kidney (Wetzel et al., 2020). Another interesting

691 study indicates that Arg2 plays a role in the circadian clock. In this study, it was shown that

692 the lack of Bmal1 in the nephron led to increased urea levels in the plasma which correlated

693 with tubular dysfunction (Nikolaeva et al., 2016). The increase in urea could be explained by

694 an increase in the activity of Arg2 in the kidney.

695 Differently from Arg2, Arg1 expression in the kidney occurs only in pathological conditions,

696 mainly as a consequence of inflammation or tissue damage. For example, in nephritic

697 glomeruli in rats, arginase activity was found to be six-fold higher as compared to control

698 glomeruli due to the induction of Arg1 expression, while Arg2 was not upregulated

699 (Waddington et al., 1998). The expression of Arg1 is likely due to the presence of infiltrating

700 macrophages in the tissue. In fact, high expression of Arg1 was also found in the

701 macrophages located in the outer medulla after I/R injury in mice (Shin et al., 2022). In this

702   study, the authors proposed that Arg1 activity may contribute to stimulate the reparative

703   proliferative response to replace the cells in the medullary tubule.

704   To summarize, the kidney is a key organ involved in the synthesis of L-arginine and the

705   maintenance of L-arginine levels in plasma. Although expression of Arg2 is constitutive in

706   kidney cells, its function is not fully understood. The main role of Arg2 is likely to keep

707   normal tissue structure/homeostasis through the production of L-ornithine and the

708   subsequently metabolism of polyamines; however, this needs to be further investigated. In

709   contrast, the expression of Arg1 is mainly induced during tissue damage and inflammation

710   and contribute to immunomodulation and tissue repair.

711   **G. Summary and outlook**

712   In summary, Arg1 and Arg2 play multiple, often species-specific roles across cells and

713   tissues in the body. In this context, recent studies carried out with cell-specific transgenic

714   mice models are providing new and somehow unexpected information on the biological roles

715   of arginase in specific cells and compartments. Accumulating evidence also indicate that

716   there are important species-specific differences, in particular between rodents and humans,

717   regarding the role of arginases in immune cells and RBCs. These differences need to be

718   taken into account in pharmacological and translational studies, as well as in pre-clinical

719   testing.

720   In the liver, the cytosolic isoenzyme Arg1 is crucial for detoxifying ammonia via the urea

721   cycle, while the role of mitochondrial Arg2 appears to be mainly the synthesis of L-ornithine

722   as a precursor of L-proline and polyamines.

723   In the immune system, Arg1 is well known to modulate immune cell function and promote

724   immunosuppression, host protection, and resolution of inflammation, mainly by limiting L-

725   arginine bioavailability for downstream metabolic pathways and iNOS-mediated high-output

726   NO synthesis. In mouse, arginase expression in M2 macrophages drives the anti-

727   inflammatory responses, as well as participates in tissue repair via polyamine and proline

728 synthesis. In humans, Arg1 is constitutively expressed in granules of PMN cells and MDSCs,

729 and its L-arginine-depleting activity drives immunosuppression of T-cell responses. The

730 expression and function of Arg1 in monocytes/macrophages in humans is still debated. The

731 immunosuppressive role of arginases is part of the pathophysiology of chronic inflammatory

732 conditions, infections, and cancer; for example, some parasites and tumor cells express their

733 own arginase or induce arginase expression in the cells of the host. Also, Arg2 is emerging

734 as a further important regulator of the immune response and a possible pharmacological

735 target.

736 In the blood, Arg1 is also present at higher levels in human and primate RBCs, but its

737 presence in the RBCs of other mammals including mice and rats is very low. In humans,

738 arginase activity is increased in RBCs of people with SCD, thalassemia, and other

739 hemoglobinopathies, and it was shown to promote endothelial dysfunction and pulmonary

740 hypertension by hemolysis-induced arginase release into the plasma and systemic L-

741 arginine depletion. High levels of RBC arginase are also found in people with CVD and

742 diabetes and are proposed to contribute to endothelial dysfunction and cardiovascular

743 events. Interestingly, an immunosuppressive function of arginase release from RBCs has

744 been also proposed.

745 In the vasculature, arginase activity is increased under pathological conditions like diabetes

746 and atherosclerosis and promotes endothelial dysfunction mainly by competing with eNOS

747 for L-arginine and affecting endothelial NO production. The specific role of the cytoplasmic

748 Arg1 or the mitochondrial Arg2 in the vessel wall is not fully understood. Mice lacking EC

749 Arg1 show no changes in vascular endothelial function *ex vivo* or *in vivo* under homeostatic

750 conditions.

751 In the kidney, Arg2 is likely involved in L-arginine metabolism into L-ornithine and

752 polyamines, it is upregulated and exerts a protective function of the kidney tissue in diabetic

753 nephropathies, at least in the mouse.

36

754   These findings underscore the need for further research to reveal the complex roles of Arg1

755   and Arg2, by considering their cellular and subcellular localization and regulation, their

756   species specificity, and their significance in cellular processes. These differences need to be

757   taken into consideration when pharmacological therapies are tested in pre-clinical models.

37

V. L-Arginine metabolism and arginase activity in human disease

There are multiple human studies investigating L-arginine metabolism in human cohorts. L-Arginine levels and metabolism in humans have been extensively studied to investigate the role of arginase in the urea cycle and the consequences of genetic defects (like hyperarginemia) in liver homeostasis, as well as in the immune system and cancer cells in relationship to iNOS activity. Moreover, L-arginine bioavailability was studied in the context of arginase as a counterpart of eNOS for endothelial dysfunction in CAD and diabetes. Arginase has shown potential as a therapeutic target for various diseases and conditions, and its inhibition has been explored in clinical trials to evaluate its efficacy and safety. Details about the human studies discussed in the text are summarized in **Tab.5**.

A. Measurements of arginase expression and activity in human disease

The main pathophysiological consequence of autosomal ARG1 mutations in humans is hyperargininemia, which leads to an autosomal inborn error in the urea cycle (Diez-Fernandez et al., 2018). Other symptoms are progressive intellectual impairment and neurological impairment, persistent growth retardation, and spastic paraparesis (Diez-Fernandez et al., 2018). In a study, 66 mutations of the ARG1 gene have been identified in 112 humans with hyperargininemia, 30 of those were missense mutations, 15 deletions, 10 splicing, seven nonsense, one small insertion, and one translation initiation codon mutation. The estimated incidence of this disease is around 1:726,000 (Catsburg et al., 2022). At the beginning of this year, pegzilarginase, a recombinant, cobalt-substituted, and pegylated human ARG1 enzyme therapy, received approval as an orphan drug in the EU for the treatment of Arg1 deficiency (Russo et al., 2024). Interestingly, mutations of the ARG1 gene could also play a role in other diseases. In subjects with erectile dysfunction (n=110), two different polymorphisms in the ARG1 gene were associated with the severity of erectile dysfunction, but there was no correlation with plasma Arg1 levels. (Lacchini et al., 2015).

783   It was proposed that an upregulation of Arg1 in the lungs leads to an imbalance in L-
784   arginine/NO availability resulting in pulmonary hypertension and/or smooth muscle
785   contraction as well as lung tissue remodeling of lung. In fact, an upregulation of Arg1 has
786   been found in pulmonary diseases, including chronic obstructive pulmonary disease,
787   pulmonary hypertension, pulmonary fibrosis, tuberculosis, and asthma. (North et al., 2009,
788   Henno et al., 2015, Monin et al., 2015, Lucca et al., 2018, Wu et al., 2019).

789   Increased levels of Arg1 in human RBCs and plasma were proposed to contribute to the
790   pathophysiology of hemoglobinopathies like SCD or thalassemia. It has been shown that
791   arginase activity in plasma is higher and L-arginine plasma level is lower in humans with
792   thalassemia (n=14) or SCD (n=140) (Morris et al., 2005a, Morris et al., 2005b). The authors
793   of these elegant studies proposed that intravascular hemolysis with release of hemoglobin
794   and arginase in plasma causes on one hand a reduction in L-arginine concentration in
795   plasma, and on the other hand scavenging of NO, leading to endothelial dysfunction and
796   pulmonary hypertension. Interestingly, the treatment of humans with SCD with hydroxyurea
797   (n=23) reduced arginase activity in the plasma (Iyamu et al., 2005).

798   In addition, there are first evidences that Arg1 present in RBCs can modulate endothelial
799   dysfunction and the outcome of I/R injury as tested in bioassays (Yang et al., 2018, Zhou et
800   al., 2018, Mahdi et al., 2020b). RBCs from humans with T2DM (n=20) showed an increased
801   arginase activity and arginase level in RBCs as compared to healthy individuals (n=15) (Zhou
802   et al., 2018). Furthermore, these authors showed in a bioassay that the co-incubation of
803   RBCs from humans with T2DM with rat aortas induces endothelial dysfunction, which can be
804   prevented by the *ex vivo* inhibition of arginase. In addition, RBCs from people with T2DM
805   also induced an increase in arginase activity in co-incubated human carotid arterial ECs. The
806   authors of the study proposed that this upregulation is induced by peroxynitrite (Mahdi et al.,
807   2020b). In another study, the same authors showed that RBCs from people with T2DM
808   (n=13) aggravate myocardial I/R injury (Yang et al., 2018). These studies indicate that Arg1

39

809 present in human RBCs play a role in the complex vascular and cardiac pathophysiological

810 consequences of T2DM in humans.

811 There are multiple studies showing an increased Arg1 activity/protein levels in serum of

812 humans with myocardial infarction (Porembska and Kedra, 1975, Bekpinar et al., 2011), and

813 was linked to endothelial dysfunction via decrease of L-arginine bioavailability.

814 An interesting hypothesis that should be also taken into consideration is the release of

815 arginase and decrease of endogenous levels of L-arginine may also exert an

816 immunosuppressive effect as hypothesized by Munder et al. (Munder, 2009). The

817 immunosuppression may become pathophysiological in various infections with parasites and

818 viral infections. For example, in HIV-infected humans, a high expression of Arg1 in lymph

819 nodes correlated with an increase in HIV viral load whereas iNOS expression negatively

820 correlates with the HIV viral load (n=52) (Zhang et al., 2016). Such upregulation may

821 contribute to the suppression of antiviral immunity in HIV-infected humans, thus, Arg1

822 expression can be used as a parameter to predict disease progression.

823 There are no known genetic defects of Arg2 that cause human disease. However, it was

824 shown that Arg2 expression and activity were increased in pulmonary artery ECs of humans

825 with pulmonary arterial hypertension (PAH) (n=41 ± 3) (Xu et al., 2004). Similar to Arg1, Arg2

826 expression is increased in humans with asthma caused by chronic airway inflammation (Xu

827 et al., 2017).

828 In summary, both arginase isoforms play a crucial role in various disease conditions.

829 Mutations in the ARG1 gene lead to a defect of the urea cycle causing hyperargininemia and

830 leading to early death. Since the beginning of 2024, the first therapy using human

831 recombinant pegylated cobalt-substituted arginase enzyme is available. On the other hand,

832 the upregulation of arginase expression and activity seems to be involved in various

833 diseases like T2DM and SCD. In addition, arginase shows immunosuppressive properties

834 which is involved in infections with parasites and viral infection.

40

835 **B. Measurement of L-arginine bioavailability in human cohorts with cardiometabolic**

836   **disease**

837 The majority of the human studies having L-arginine bioavailability or metabolism as a

838 primary outcome measure is based on the hypothesis that arginase activity in the

839 vasculature contributes to the consumption of L-arginine, which contributes to endothelial

840 dysfunction. Consumption of L-arginine and the production of L-ornithine should affect GABR

841 in plasma, as an index of L-arginine bioavailability. Therefore, GABR was measured in the

842 plasma of humans with CAD (humans with no significantly obstructive CAD n=402 vs.

843 significantly obstructive CAD n=608) by monitoring the levels of free L-arginine, L-ornithine,

844 L-citrulline, and ADMA. GABR was lower in humans with obstructive CAD (>50% stenosis),

845 as compared to the humans without obstructive CAD, which implied that decreased GABR is

846 associated with obstructive CAD and subsequent increased incidence of major adverse

847 cardiovascular events (Tang et al., 2009). Similarly, the levels of L-arginine, L-ornithine, and

848 L-citrulline were determined in the serum of humans (n=2236) collected before coronary

849 angiography (Sourij et al., 2011). In this study, they found that GABR was significantly

850 decreased in humans with T2DM and also was inversely correlated with some known

851 biochemical markers of endothelial dysfunction such as the expression of intracellular

852 adhesion molecule-1, vascular adhesion molecule-1, and von Willebrand factor.

853 Furthermore, they also observed that decreased GABR and the arginine-to-ornithine ratio

854 were associated with increased cardiovascular mortality. A further study investigated the

855 changes of GABR in people with T2DM (n=41) after intensifying the therapy according to the

856 guideline for 3 months to reach therapy targets like, HbA$_{1c}$, LDL cholesterol 2.6, or blood

857 pressure (Tripolt et al., 2012). They showed that targeting those risk factors improves GABA

858 and arginine-to-ornithine ratio. It is important to note that this improvement was only found in

859 people with T2DM for less than 5 years.

860 L-arginine levels and metabolism were also determined in plasma samples from subjects

861 during the acute phase of myocardial infarction collected before percutaneous coronary

862 intervention and again 6 months after myocardial infarction (n=70) (Molek et al., 2021). L-

863 arginine, L-ornithine, and ADMA levels were determined and calculated into indexes of L-

864 citrulline/L-arginine, L-citrulline/L-ornithine, and L-arginine/ADMA. They observed that the

865 index of L-citrulline/L-arginine significantly decreased in the acute phase of MI but the

866 indexes of L-citrulline/L-ornithine and L-arginine/ADMA were unchanged. The authors

867 proposed that this may indicate a shift of L-arginine utilization from NOS towards arginase

868 and/or an increase in the activity of arginase.

869 The L-arginine and L-ornithine concentrations were also measured in the plasma from lean

870 and obese humans with asthma, obese humans without asthma, and also corresponding

871 healthy controls by using LC-MS/MS (Winnica et al., 2019). In this study they demonstrated

872 that the L-arginine levels in the plasma were decreased in both lean and obese humans with

873 asthma, and also obese humans without asthma as compared to healthy individuals.

874 Moreover, the L-arginine levels in obese humans with asthma were significantly lower than in

875 healthy lean controls.

876 Recently, L-arginine level, L-arginine-to-L-ornithine ratio, and GABR were analyzed in adult

877 humans diagnosed with COVID-19 (n=32) and pediatric humans with COVID-19/multisystem

878 inflammatory syndrome (n=20). They found that all these parameters were significantly lower

879 in COVID-19-positive adult and COVID-19/MIS-C pediatric groups as compared to the

880 COVID-19-negative controls. The authors proposed that low arginine-to-ornithine ratio in

881 these people might be due to an elevated arginase activity, and that low GABR may

882 contribute to immune dysregulation and endothelial dysfunction in COVID-19 (Rees et al.,

883 2021).

884 **C. Arginase as a pharmacological target**

885 Depending on the pathophysiological role of arginase and the specific type of disease

886 targeting arginase involves two possible strategies including arginase-mediated L-arginine

42

887   depletion or inhibition of arginase to restore L-arginine bioavailability. Established and

888   potential therapeutic applications are listed in table 3.

889   Administration of pegylated recombinant arginase has been recently approved by the EMEA

890   to treat humans with genetic hyperarginemia. Moreover, pegylated arginase has been

891   proposed as an anti-tumor agent (similar to the antileukemia agent L-asparaginase) for

892   tumors susceptible to L-arginine deprivation, like tumors lacking ASS enzymes (Cheng et al.,

893   2021). If properly targeted to the site of inflammation, another possible application of

894   recombinant Arg1 may be immunosuppression in autoimmunity and unwanted inflammatory

895   reactions (Munder, 2009).

896   Applications of arginase inhibitors were proposed against diseases with pathological

897   upregulation of arginase like specific infection diseases, cancer, or against endothelial

898   dysfunction in T2DM and CAD, and to treat pulmonary hypertension in SCD and

899   hemoglobinopathy. (see chapter IV paragraph B)

900   There are three generations of arginase inhibitors which are summarized in **Tab. 4**.

901   These inhibitors do not show isoform specificity, need to be administrated systemically via

902   i.p. or i.v injection, have a short half-life, and are rapidly eliminated by the kidney. A well-

903   known and often-used arginase inhibitor in cell culture, animal studies but also in human

904   studies is nor-NOHA. Nor-NOHA is a derivate of NOHA, a stable intermediate of NO

905   synthesis by NOS. It is a competitive non-specific inhibitor of arginase and is considered as

906   one of the most potent arginase inhibitors (Colleluori and Ash, 2001, Pudlo et al., 2017).

907   There are already numerous studies investigating the effect of arginase inhibitors in animals

908   (Kim et al., 2009, Jung et al., 2010, El-Bassossy et al., 2013, You et al., 2013, Pera et al.,

909   2014), and lately, in humans (**Tab. 4**).

910   There are very promising studies showing that arginase inhibition by nor-NOHA improves

911   endothelium-dependent vasodilation (EDV) (Shemyakin et al., 2012, Kövamees et al., 2014,

912   Kovamees et al., 2016a, Mahdi et al., 2018). In human studies, NorNOHA (0.1 mg/min for 2

43

913   hours) was administrated by infusion (Shemyakin et al., 2012, Kövamees et al., 2014,

914   Kovamees et al., 2016a, Mahdi et al., 2019). Administration of nor-NOHA improved

915   endothelial function in healthy elderly humans (n=21) as determined by forearm venous-

916   occlusion plethysmography (Mahdi et al., 2019).

917   Recently, a third generation of arginase inhibitors was developed and tested (**Tab.4**). One

918   notable compound from this generation is INCB001158 (formerly CB1158), an oral arginase

919   inhibitor that exhibits higher specificity for Arg1 (IC_{50}=86 nm) compared to Arg2 (IC_{50}=296

920   nm). INCB001158 has been evaluated in phase 1/2 clinical trials for the treatment of solid

921   tumors with increased arginase activity in combination with chemotherapy (Steggerda et al.,

922   2017, Kuboki et al., 2024, Naing et al., 2024). The most notable result of these studies is that

923   arginase inhibitors may be effective for therapies of specific tumors with up-regulation of

924   arginase activity as a mechanism for L-arginine depletion and immunosuppression.

925   In conclusion, pegylated arginase is an approved drug for hyperarginemia and genetic-

926   defect. Potentially for L-arginine auxotrophic tumors suspectable to L-arginine deprivation

927   and for immunosuppression.

928   Instead, the clinical applications of arginase inhibitors have shown potential to improve

929   endothelial function and vasodilation in individuals with T2DM, enhancing the effects of L-

930   arginine supplementation in individuals with heart failure and coronary artery diseases, and

931   serving as a promising therapeutic target for therapy of specific cancers. Further

932   pharmacological research focused on developing and testing arginase inhibitors will

933   contribute to the development of novel treatments and therapies in the future.

934   **D. L-arginine/L-arginine metabolites supplementation**

935   Oral supplementation of L-arginine was proposed as a way to increase L-arginine

936   bioavailability and to boost production of NOS-derived NO in humans (Girerd et al., 1990).

937   For example, L-arginine supplementation was proposed for alleviate endothelial dysfunction

44

938 (Lerman et al., 1998, Bai et al., 2009). However, the results of these studies are

939 controversial.

940 Oral L-arginine intake was shown to positively affect humans with heart failure and in

941 peripheral artery occlusive disease by increasing the distance in a 6-minute walk test,

942 increasing the forearm blood flow during forearm exercise, or reducing the symptom score

943 significantly (Rector et al., 1996, Lerman et al., 1998). On the other hand, oral administration

944 of L-arginine in healthy human (n=26) did not improve systemic hemodynamics *in vivo* or

945 vascular function of gluteal subcutaneous arteries assessed *in vitro* (Chin-Dusting et al.,

946 1996).

947 **E. Summary and outlook**

948 The involvement of arginases in the urea cycle, liver function, immune response, and cancer

949 cell dynamics, particularly in relation to iNOS activity, has been extensively examined. In

950 addition, arginase activity in the vessel wall has been studied in the context of endothelial

951 dysfunction in CAD and diabetes, revealing its potential as a therapeutic target. The GABR is

952 a measure of L-arginine bioavailability, which has been found to be lower in humans with

953 obstructive CAD and T2DM, correlating with major adverse cardiovascular events and

954 endothelial dysfunction markers. Pharmacological interventions that improve risk factors for

955 T2DM have been shown to improve GABR, particularly in humans recently diagnosed with

956 diabetes. Mutations in the ARG1 gene cause hyperargininemia, leading to various health

957 complications. Arginase activity is also increased in several pulmonary diseases and

958 hemolytic anemias, affecting NO availability and contributing to disease pathology. The

959 arginase inhibitor nor-NOHA has demonstrated efficacy in improving endothelial function in

960 people with T2DM and healthy elderly individuals. Supplementation with L-arginine or L-

961 citrulline has been explored to enhance L-arginine bioavailability and NO production, with

962 varying results depending on the health status of individuals.

VI. Conclusion and outlook

Arginases exhibit diverse functions across various tissues. In the liver, Arg1 is indispensable for ammonia detoxification, while the specific role of mitochondrial Arg2 need further investigation. It is important to point out that there are significant differences in Arg1 and Arg2 expression, cellular localization, and activity in humans and primates as compared to rodents (mice, rats) and other mammals, in particular in the blood and bone marrow, and vascular endothelial cells.

In the immune system, the distribution and function of Arg1 are very different in mouse and man. While in mice blood Arg1 is mainly expressed in myeloid cells and it is up-regulated by Th2-cytokines, in humans Arg1 is constitutively expressed in PMN cells and is not regulated by Th2 cytokines. In both species, arginase activity has an anti-inflammatory and immunosuppressive effect on T cells.

In human and primate RBCs and proerythroblasts Arg1 expression and activity are high, while in rats, mice, and other mammals it is very low and/or barely detectable. In humans, elevated arginase activity in RBCs from individuals with SCD or hemoglobinopathies suggests a contributory role in disease pathogenesis, with similar observations in CVD and diabetes, underlining its fundamental importance in human disease.

In CVD conditions like diabetes and atherosclerosis, increased arginase activity can lead to endothelial dysfunction by competing with eNOS for the bioavailability L-arginine, thus impairing NO production and leading to endothelial dysfunction. Accordingly, administration of arginase inhibitors in people with T2DM improve vascular function. Surprisingly, EC Arg1$^{-/-}$ revealed no changes in endothelial function or CV hemodynamics, and instead global Arg2$^{-/-}$ mice show hypertension. Indicating that at least *in vivo,* the role of vascular Arg1 in maintaining endothelial function under homeostatic conditions in mice is limited.

Although the kidney plays a major role in the control of L-arginine synthesis, systemic levels and bioavailability, the role of arginases is the kidney is not fully understood. The main

989 isoform expressed is Arg2 and appears to be crucial for tissue integrity and repair (at least in

990 rodents).

991 Clinical and translational studies have highlighted the therapeutic potential of targeting

992 arginase/L-arginine metabolism in various diseases. Clearly, the described multiple,

993 complex, species, cell-, and isoform-specific roles of arginase make pharmacological

994 targeting difficult. The only approved drug is pegylated recombinant arginase for the

995 treatment of humans with genetic hyperarginemia. However pegylated arginase was

996 proposed as a possible anti-tumor therapy for tumors sensitive to L-arginine deprivation. The

997 control of L-arginine bioavailability by arginases, as a key factor in endothelial dysfunction in

998 CVD, has been extensively studied and arginase is still considered as a promising

999 therapeutic target. The use of arginase inhibitors (e.g., nor-NOHA) was tested in small

1000 cohorts of humans with endothelial dysfunction due to CAD or T2DM. Moreover,

1001 administration of arginase inhibitors was proposed for blocking circulating arginase in

1002 hemoglobinopathies (SCD, thalassemia) and specific infection disease. Arginase inhibition

1003 has shown efficacy in improving endothelial function in subjects with T2DM and healthy

1004 elderly individuals, while L-arginine and L-citrulline supplementation have demonstrated

1005 potential benefits in heart failure and peripheral arterial occlusive disease. A strong limitation

1006 of the currently available inhibitors is that they do not show isoform specificity, need to be

1007 administrated systemically via i.p. or i.v injection, and have a short half-life, and are rapidly

1008 eliminated by the kidney. The third generation of arginase inhibitors can be administrated

1009 orally and have better pharmacodynamics and have been tested already in cancer therapy.

1010 More studies are needed to understand the complex biological and species-specific roles of

1011 Arg1 and Arg2, their importance in cellular processes, and their potential as therapeutic

1012 targets. The new pharmacological cell-specific targeting and the applicability of single-cell

1013 analysis for personalized medicine will allow better pharmacological strategies to target Arg1

1014 and Arg2 in humans.

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VIII. Tables

Table 1: Characteristics of arginases in humans, rats, and mice. Please note that according to NCBI, human proteins are abbreviated with ARG1 and ARG2 (with all capital letters), for all other species abbreviation is Arg1 and Arg2.In the text, we use Arg1 and Arg2 to indicated the Arg1 and Arg2 enzymes from all species.

	Gene Name	Protein Names	Chromo-some	Size	Uni-prot ID	Co-factors	Localization
Human	ARG1	Arginase 1 (ARG1) Arginase I (AI) Liver-Type Arginase Type I Arginase	6q23.2	322 aa (35 kDa)	P05089		Cytosol
		Three identified isoforms by alternative splicing (Isoform 1: 322 aa / Isoform 2: 330 aa / Isoform 3: 236 aa)					
Rat	Arg1	Arg1	1p12	323 aa (35 kDa)	P07824		
Mouse	Arg1	Arg1	10 A4	323 aa (35 kDa)	Q61176		
Zebrafish	SI:zC146F4.4	Arg	12	341 aa (37 kDa)	E7F8R4	Two Mn^{2+} ions each Oligomerization (Homotrimer)	
Human	ARG2	Arginase 2 (ARG2 Arginase II (AII) Kidney-type Arginase Non-hepatic Arginase Type II Arginase	14q.24.1	354 aa (39 kDa)	P78540		Mitochondria
Rat	Arg2	Arg2	6q24	354 aa (39 kDa)	O08701		
Mouse	Arg2	Arg2	12 C3	354 aa (39 kDa)	O08691		
Zebrafish	ARG2	Arg2	13	347 aa (38 kDa)	Q6PH54		

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Table 2: Summary of arginase specific knock out models

Mice Model	Targeted isoforms	Gene targeting strategy	Phenotype	Reference
Effect of genetic modification on home strain				
Global Arg1^{-/-}	Arg1	The replacement vector consisted of genomic sequences from exon 2 to exon 8, in which exon 4 was replaced by the neomycin resistance cassette (Neo[R])	• Hyperammonaemia • Early lethal (between day 10 and 14)	(Iyer et al., 2002)
Global Arg2^{-/-}	Arg2	Partly deletion of exons 4 and 5 of the Arg2 gene (by Sin et al.)	• Homozygous Arg2^{-/-} mice were viable • Plasma L-arginine level ↑ • Plasma norepinephrine turnover ↑ • Hypertension	(Shi et al., 2001, Huynh et al., 2009)
Global Arg2^{-/-}	Arg2	Arg2[flox/flox] (exon 2+3) FVB/NTgN(ACTB-Cre)2Mrt mice	• Arg2^{-/-} mice are unable to produce intestinal mucin • Arg2^{-/-} mice became highly susceptible to experimentally induced colitis	(Park et al., 2009)
Inducible global Arg1^{-/-}	Arg1	Arg1[flox/flox] (exons 7 and 8) CreER[T2]	• Lethal 2 weeks after Tamoxifen treatment • L-citrulline and guanidinoacetic acid ↑ • L-ornithine levels = • other amino acids ↓	(Sin et al., 2013)
Arg1^{-/-}Arg2^{-/-} mice	Arg1 Arg2	Arg1^{-/-} Arg2^{-/-} mice were generated by crossing Arg1^{+/-} mice (by Lyer et al.) with Arg2^{-/-} mice (by Sin et al.)	• L-arginine level ↑ • L-ornithine levels ↓ • Liver L-ornithine levels reduced to 2% with L-arginine very highly elevated.	(Deignan et al., 2006)
Cell-specific arginase knockout models				
Liver-specific Arg1^{-/-}	Arg1	Deletion of exons 7 and 8 of the Arg1 gene after i.p. injection of Arg1[flox/flox] (exons 7 and 8) mice with AAV-TBG-Cre-Promoter-Cre recombinase vector	• Lethal phenotype similar to the inducible Arg1^{-/-} mice phenotype • Delivery of Arg1-eGFP AAV vector prolongs lifespan	(Ballantyne et al., 2016)

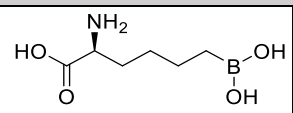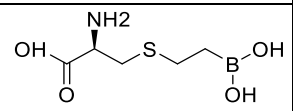|				
EC/HC Arg1^{-/-}	Arg1	Arg1[flox/flox] (exon 4) Tie2-Cre deleter	• IL-4-induced polyamine production ↑	(Van den Bossche et al., 2012)
EC Arg1^{-/-}	Arg1	Arg1[flox/flox] (exons 7 and 8) Cdh5-Cre/ERT2^{pos}	• Expression of eNOS in the aorta ↓ • L-arginine & NO bioavailability = • Vascular endothelial function in conductance and resistance arteries = • Preserved systemic hemodynamic and cardiac performance • Increased contractile response to phenylephrine in aorta rings	(Heuser et al., 2022)
Microglial-specific Ar1^{-/-}	Arg1	Arg1[flox/flox] (exons 7 and 8) Cx3cr1[CreER]	• No notable morphological differences • Impaired cholinergic innervation and dendritic spine maturation in the hippocampus • Deficits in long-term memory acquisition in females	
**Effect of disease conditions (selected)**				
*Infectious disease*				
Macrophage Arg1^{-/-} Toxoplasmid gonid/tuberculosis	Arg1	Arg[flox/flox] : (exon 7 and 8) Tie2Cre[tg/–] Promoter LysMCre[tg/-] Promoter	• Knockout[Tie2] showed complete Arg1 ablation in all macrophage types, knockout[LysM] showed less deletion • Host survival in Toxoplasma gondii infection ↑ • Lung bacterial load in tuberculosis infection ↓	(El Kasmi et al., 2008)
Asthmatic Arg1 deficient BM chimeric mice	Arg1	Transfer Arg1 deficient BM into irradiated recipient mice Asthma model: OVA-induced or *Aspergillus fumigatus*-induced	• M-derived Arg1 is not required for baseline immune cell development and allergen-induced inflammation • BM-derived Arg1 is the main source of allergen-induced lung arginase	(Niese et al., 2009)
Global Arg2^{−/−} mice Infected with *H. pylori*	Arg2	Partly deletion of exons 4 and 5 of the Arg2 gene (by Sin et al.)	• Macrophages of Arg2^{-/-} mice iNOS-protein levels & NO levels ↑ • Inhibition of arginase in Arg2^{-/-} mice did not have additional effects on iNOS or NO levels	(Lewis et al., 2010)
Global Arg2^{-/-} mice infected with *H.pylori*	Arg2	Partly deletion of exons 4 and 5 of the Arg2 gene (by Sin et al.)	• Arg2^{-/-} macrophages undergo less apoptosis • Arg2^{-/-} macrophages more abondend • Arg2^{-/-} macrophages iNOS ↑ • Arg2^{-/-} macrophages nitrotyrosine staining ↑	(Lewis et al., 2011)
EC & HC Arg1^{-/-} in endotoxemia	Arg1	Arg1[flox/flox] : Exon 4 Tie2Cre[tg/–] Promoter	• Inflammatory response ↑ • NO production by iNOS ↑ • Depressed microcirculatory flow in the jejunal	(Wijnands et al., 2014)
Double Arg2^{-/-} Nos2^{-/-} mice infected with *H. pylori*	Arg2	Double Arg2^{-/-} Nos2^{-/-} obtained bv crossing Arg2^{-/-} mice (obtained by deletion of exons 4 and 5 of the Arg2 gene) and Nos2^{-/-}	• In Arg2^{−/−}, gastric polyamine synthesis and catabolism ↑ • Arg2^{−/−} and Arg2^{−/−},Nos2^{−/−}, gastritis ↑ colonization ↑ • In Arg2^{−/−}, M1 macrophage activation ↑, NOS2^{−/−} and Arg2^{−/−};Nos2^{−/−}, M1 macrophage activation =	(Hardbower et al., 2016)

		mice obtained by deletion exons 12 and 13 of the NOS2 gene		

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Inflammatory disease/asthma

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Asthmatic EC/HC Arg^{-/-}	Arg1	Arg1[flox/flox] : exon 4 Tie2Cre[tg/−] Promoter or LysMCre[tg/-] Promoter Asthma model: OVA-induced allergic asthma in female mice	• Arg1 allele was virtually completely deleted in the lungs of knockout[Tie2] mice, but incompletely in knockout[LysM] mice • Improved peripheral lung function in OVA-treated knockout[Tie2] mice • Knockout[Tie2] mice did not alter air hyperreactivity and lung inflammation	(Cloots et al., 2013)
Asthmatic Myeloid cell Arg1^{-/-} in female mice	Arg 1	Arg1[flox/flox] : exon 4 Tie2Cre[tg/−] Promoter LysMCre[tg/-] Promoter Asthma model: OVA-induced allergic asthma in female mice	• Arg1 positive cells completely absent from the lungs of OVA-treated knockout[Tie2] mice, but only reduced in knockout[LysM] mice • Compared to male mice, females show more decline of arginine-metabolizing and -transporting genes, OVA-specific IgE ↓ • methacholine responsiveness and accumulation of inflammatory cells =	(Cloots et al., 2017)

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Diabetes/CVD

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Diabetic Arg2^{-/-}	Arg2	Partly deletion of exons 4 and 5 of the Arg2 gene (by Sin et al.)	• Albuminuria ↓ • Macrophages recruitment ↓ • Renal blood flow ↑	(Morris et al., 2011)
Diabetic Arg1^{+/-} Arg 2[-/-]	Arg1 Arg2	Arg 1 -/- Arg 2 -/- mice were generated by crossing Arg 1 +/- mice (by Lyer et al.) with Arg 2 -/- mice (by Sin et al.) + STZ treatment	• Impairment of EC-dependent vasodilation ↓ • Tissue oxidation, vascular stiffness, and coronary fibrosis ↓	(Romero et al., 2012)
EC Arg1^{-/-} mice fed a high-fat/high-sucrose diet	Arg1	Arg[flox/flox] (exon 7&8) Cdh5-Cre[pos/neg]	• Prevention of endothelial dysfunctions	(Bhatta et al., 2017)
Diabetic EC/HC Arg1^{-/-}	Arg1	Arg1[flox/flox] (exon 4) Tie2Cre[tg/−] mice Diabetic model, induced by STZ treatment	• L- arginine concentration in plasma ↑ Diabetes-induced alterations in arterial smooth muscle reactivity and endothelium-dependent relaxations =	(Chennupati et al., 2018)

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Renal disease

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EC Arg2^{-/-} & proximal tubular cell Arg2^{-/-} with unilateral ureteral obstruction	Arg2	Arg2[flox/flox] (exon 3) Tie2-Cre[pos/neg] Ggt1-Cre[pos/neg]	• EC Arg2 knockout, level of renal fibrosis ↓ • Proximal tubular epithelial cell Arg2 knockout, level of fibrosis =	(Wetzel et al., 2020)
Renal tubular cells Arg2^{-/-}	Arg2	Arg2[flox/flox] (exons 3, 4, 5 and 6) Pax8-rtTA/LC1 mice	• Urea concentration and os-molality gradients along the corticomedullary axis ↓ • Tissue damage after unilateral I/R-injury • Albuminuria and aminoaciduria	(Ansermet et al., 2020)
Atherosclerosis				
Erythroid Arg1^{-/-} on apoE^{-/-} background + HFD	Arg1	Arg[flox/flox] (exon 7&8) apoE^{-/-} EpoR-Cre[pos/neg] mice + Western diet (high cholesterol)	• Atherosclerotic lesion size at the aortic root = • Vascular NO bioactivity, smooth muscle osteoblastic differentiation, and atherosclerotic lesion calcification ↑ • L-Ornithine, proline in vascular smooth muscle cells expression ↑	(Gogiraju et al., 2022)

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Table 3 Established and potential therapeutic applications

Therapeutic intervention	Indication	Status	Reference
Pegylated arginase1	1. Arg1 deficiency 2. Arginase auxotrophic tumors 3. Immunosuppression	1. Approved as orphan drug by the EMEA 2. open-label Phase II trial 3. Not tested	1.(Russo et al., 2024) 2.(Cheng et al., 2021) 3. (Munder, 2009)
L-Arginine/L-Citrulline supplementation	1. Chest Pain 2. T2DM 3. Endothelia dysfunction	1. Clinical trial 2. Clinical trial 3. Clinical trial	1.(Lerman et al., 1998) 2.(Shatanawi et al., 2020) 3.(Chin-Dusting et al., 1996)
Arginase inhibitor	1. T2DM (+CVD) 2. Advanced/metastatic solid tumors with upregulation of Arg1 3. Infection with parasites 4. Pulmonary hypertension in SCD	1. clinical tests/ Phase I clinical trial 2. phase I clinical trial 3. Mice studies 4. Mice studies	1. (Shemyakin et al., 2012, Kövamees et al., 2016) 2. (Steggerda et al., 2017, Kuboki et al., 2024, Naing et al., 2024) 3. (Li et al., 2022b) 4. (Morris et al., 2005a, Steppan et al., 2016)

Table 4 arginase inhibitors used in human-based *in vitro/ex vivo* studies

Name	Structure	Test	U.S. National Clinical Trial number	Administration	application	Reference
First generation						
ABH (2-(S)-amino-6-boronohexanoic acid)		In vitro	n.a.	n.a.	n.a.	(Van Zandt et al., 2019)
BEC (S-(2-boronoethyl)-L-cysteine)		In vitro/ex vivo/clinical trial	n.a.	Intradermal microdialysis in combination with nor-NOHA	CVD	(Busnel et al., 2005, Holowatz et al., 2006)

nor-NOHA (Nω-hydroxy-nor-arginine)		In vitro/ex vivo/clinical trial	NCT 02009527 NCT 05536934 NCT 02687152 NCT 05806502	Intrabrachial infusion/ sublingual perfusion/ intradermal microdialysis/ intra-arterial infusion	CVD, D2TM, Obese	(Kövamees et al., 2014, Van Zandt et al., 2019)
Second generation						
ABH analogues (synthesized based on Ugi reaction)		In vitro/in vivo	n.a.	n.a.	n.a	(Golebiowski et al., 2013)
Third generation						
NED 3238		In vitro	n.a.	n.a.	n.a	(Van Zandt et al., 2019)
INCB001158 (formerly named as CB-1158)		Clinical trial phase 1/phase 2/in vitro	NCT 03314935 NCT 02903914 NCT 03910530 NCT 03361228 NCT 03837509	Oral application	Solid tumours	(Steggerda et al., 2017, Kuboki et al., 2024, Naing et al., 2024)

956 **Table 5. Summary of human studies on arginases and its substrates and products**

Study subjects Cohort (number of subjects)	Parameters	intervention	Main findings	Reference
Measurement of L-arginine bioavailability in human cohorts				
Subjects without significantly obstructive CAD (402) vs subjects with significantly obstructive CAD (608)	GABR in plasma	N.A.	• GABR ↓ and L-citrulline level ↑ • associated with the development of significantly obstructive atherosclerotic CAD and raise the risk of MACE	(Tang et al., 2009)
CAD (2236)	GABR, L-arginine-to-L-ornithine ratio in serum	N.A.	• GABR inversely correlated with endothelial markers such as ICAM-1 and VCAM-1 • GABR ↓ and arginine-to-ornithine ratio ↓ are associated with a significant increase in cardiovascular mortality • GABR ↓ in subjects with T2DM than subjects without diabetes	(Sourij et al., 2011)
T2DM (41)	GABR In plasma	Intensified risk factor intervention	• GABR and L-arginine-to-L-ornithine ratio ↑ after 3 months with intensified risk factor invention such as glucose-lowering treatment, anti-hypertensive treatment, and lipid-lowering treatment	(Tripolt et al., 2012)
adults with COVID-19 (32) and children with COVID-19/MIS-C (20) vs adult controls (28)	GABR, L-arginine-to-L-ornithine ratio in plasma	N.A.	• L-arginine ↓, L-arginine-to-L-ornithine ratio ↓, and GABR ↓ in the COVID-19−positive adult and COVID-19/MIS-C pediatric group • Low GABR might contribute to immune dysregulation and endothelial dysfunction in COVID-19 • Low L-arginine-to-L-ornithine ratio might be due to the elevated arginase activity	(Rees et al., 2021)
subjects with STEMI (70)	L-arginine metabolite levels in plasma	NOS inhibitor, L-NAME	• Median concentration of L-arginine in acute phase of myocardial infarction > 6-month follow-up measurements correlated with ischemia risk area and infarct size • Median L-citrulline/L-arginine ↓, L-citrulline/L-ornithine and arginine/ADMA = indicating a shift of L-arginine metabolism from NOS towards arginase • Low L-arginine concentration associated with worse long-term outcomes	(Molek et al., 2021)

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Arginase expression and activity in human disease				
Early phase of myocardial infarction (100)	Arginase activity in serum after myocardial infarction (measured from a few hours after the first attack of coronary pain until 5 days)		• Arginase activity ↑ in the10-30 hours after the attack • Normal values after 3-5 days • No changes in individuals with angina pectoris, acute coronary insufficiency, left cardiac failure, right cardiac failure, or cardiac insufficiency	(Porembska and Kedra, 1975)
PAH (41) vs controls (37)	L-arginine metabolites, arginase activity in pulmonary artery ECs	none	• Arginase activity ↓ and Arg2 expression ↓ in the pulmonary artery ECs from the lung of individuals with PAH	(Xu et al., 2004)
Sickle cell disease (228) vs controls (36)	Amino acid levels (Arg, Orn, Cit, and Pro) and arginase activity in plasma, pulmonary hypertension, Mortality	N.A.	• Plasma arginase activity ↑ • Correlation between arginase activity and L-arginine-to-L-ornithine ratio • Correlation between arginase activity and increased intravascular hemolytic rate • Low L-arginine-to-L-ornithine ratio associated with greater severity of pulmonary hypertension and mortality	(Morris et al., 2005a)
Thalassemia (14) vs controls (36)	Amino acid levels (Arg, Orn, Cit, and Pro) and arginase activity in plasma	N.A.	• L-arginine levels ↓ , L-ornithine levels ↑ L-citrulline ↑ • L-arginine-to-L-ornithine ratio ↓ • arginase activity in plasma ↑	(Morris et al., 2005b)
Sickle cell disease (35) vs controls (10)	Arginase and NOS activity in plasma and RBCs, Fetal hemoglobin levels blood count	23 humans with SCD with hydroxyurea-therapy, 12 humans with SCD without HU-therapy	• Arginase activity ↓ in individuals with hydroxyurea-therapy, a treatment with ribonucleotide reductase inhibitor • Fetal hemoglobin levels ↑ NOS activity ↑in subjects with hydroxyurea-Therapy	(Iyamu et al., 2005) (Iyamu et al., 2005)
Asthma (6) vs controls (7)	Arg1 expression in lung tissues	N.A.	• Arg1 expression ↑ in subjects with asthma	(North et al., 2009)
MI (43) vs controls (33)	Arg1 activity and expression in serum, L-arginine, and ADMA concentrations in plasma	N.A.	• Arginase activity ↓ and arginase expression ↑ in blood serum from people with MI • Arginase expression negatively associated with left ventricular ejection fraction • Low L-arginine/ADMA ratio in plasma MI people	(Bekpinar et al., 2011)

Endothelial dysfunction (110) vs controls (106)	Arg1 and Arg2 in plasma (levels and activities)	N.A.	• Arg1 genetic variations affect ED severity • Arg2 concentrations ↑ in plasma of people with ED	(Lacchini et al., 2015)
HIV-asymptomatic (19), AIDS (33) vs controls lymph nodes (13), controls peripheral blood (20)	Arg1 expression in lymph nodes and peripheral blood	N.A.	• Arg1 expression ↑ in the lymph nodes from HIV-infected people	(Zhang et al., 2016)
Asthmatics (52) vs controls (51)	Arginase activity in serum, expression of Arg2 in airway epithelium	none	• Arg2 expression ↑ in the airway of asthmatic human	(Xu et al., 2017)
T2DM (46) vs controls (34)	Arginase activity in RBCs forearm blood flow, RBC (human)-aorta (rat) co-incubation	Incubation of RBC with ABH	• Arginase activity in RBCs from subjects with T2DM ↑ • RBC from subjects with T2DM-induced endothelial dysfunction • Inhibition of ROS and arginase prevented endothelial dysfunction in *ex vivo* bioassay	(Zhou et al., 2018)
T2DM (27) vs controls (23)	Arginase expression and activity in RBCs, effect of glucose on RBC arginase *ex vivo*, I/R on Langendorff heart		• RBC arginase activity ↑ and production of ROS ↑ in RBCs • RBCs from people with T2DM aggravate myocardial I/R injury in Langendorff heart • Inhibition of arginase in RBCs improves post-ischemic myocardial recovery	(Yang et al., 2018)
T2DM (18) vs controls (20)	RBC (individuals)-aorta (rat) co-incubation, arginase activity in aortic rings		• Peroxynitrite scavenging with FeTTPS in RBCs reversed endothelial dysfunction in bioassay *ex vivo* • Upregulation of arginase in T2D-RBCs and vasculature is peroxynitrite-dependent	(Mahdi et al., 2020b)
Administration of arginase inhibitors				
CAD (16), CAD and T2DM (16) vs controls (16)	Arginase expression in the arteries, EDV	nor-NOHA	• Inhibition of arginase significantly improves endothelial function in humans with CAD and T2DM • Upregulation of arginase activity is a critical factor in endothelial dysfunction	(Shemyakin et al., 2012)
CAD (12) vs CAD and T2DM (12)	EDV	nor-NOHA	• Inhibition arginase provides protection against I/R-induced endothelial dysfunction in humans with CAD	(Kövamees et al., 2014)
CAD (16), CAD and T2DM (16) vs controls (16)	EDV	arginase inhibitor nor-NOHA	• Inhibition of arginase improves microvascular endothelial function in humans with T2DM and microvascular dysfunction • Inhibition of arginase protects against I/R-induced endothelial dysfunction in humans with CAD	(Kovamees et al., 2016a, Mahdi et al., 2018)

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Controls (21)	EDV	Arginase inhibitor nor-NOHA	• Baseline EDV inversely associated with the age of the participants • Inhibition of arginase improves EDV, associated with the age of the participants • Inhibition of arginase improves endothelial function in elderly healthy subjects, age-dependent.	(Mahdi et al., 2019)
Supplementation of L-arginine/L-citrulline				
Subjects referred to tertiary treatment for heart failure	Forearm blood flow, 6-minute walk test, symptom scores	supplementation of L-arginine	• Supplementation of L-arginine significantly increased the forearm blood flow during forearm exercises, the distance in 6-minute walk test, and lowered symptom scores	(Rector et al., 1996)
Healthy individuals (26)	Forearm resistance arteries, major amino acids in plasma	28-day supplementation of L-arginine	• L-arginine led to no effect on endothelial function in healthy adults and changes in the total amino acid profile but not L-arginine concentration in plasma.	(Chin-Dusting et al., 1996)
Subjects with chest pain and coronary endothelial dysfunction	Coronary blood flow	6-month supplementation of L-arginine	• Long-term supplementation of L-arginine increased the coronary blood flow, associated with improved symptom scores and a decrease in plasma endothelin concentrations	(Lerman et al., 1998)
T2DM (25)	Arginase activity in plasma; levels of nitrite and nitrate in plasma	supplementation of L-citrulline	• Supplementation of L-citrulline reduced arginase activity and plasma NO levels in individuals of T2DM.	(Shatanawi et al., 2020)
Treatment with PEG-Arginase				
Arginase auxotrophic tumor (23)	L-arginine level in plasma, PEG-BCT-100 level in plasma, change in tumor size	Intravenous PEG-BCT-100	• Median L-arginine maintained at 2.5 µM after the second PEG-BCT-100 injection • Therapeutic L-arginine depletion found in 1.7 and 2.7 mg/kg/week cohorts with anti-tumor activities	(Cheng et al., 2021)
Arg1 deficiency (32)	L-arginine level in plasma, functional mobility (Gross Motor Function Measure part E and 2-min walk test)	Intravenously/subcutaneously, one-weekly pegzilarginase treatment	• Pegzilarginase treatment lowered mean L-arginine in plasma from 354.0 µM to 86.4 µM as compared to patients treated with placebo from 464.7 µM to 426.6 µM • Patients treated with pegzilarginase observed clinically relevant functional mobility improvements	(Russo et al., 2024)

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958 **IX. Footnotes**

959 **Authorship contribution**

960 All authors contributed with manuscript writing, drafting of figures and editing the manuscript.

966 **Data Availability Statement**

967 This review article contains no datasets generated or analyzed during the current study

968 **Conflict-of-interest statement**

969 No author has an actual or perceived conflict of interest with the contents of this article.

X. Legend of figures

Graphical Abstract: *Biochemistry, pharmacology, and in vivo function of arginases revisited: lessons from cell-specific Arg1 and Arg2-/- mice and human studies. Arginase converts L-arginine to L-ornithine and urea and plays important roles in various biological processes, including the urea cycle in the liver, the synthesis of polyamine and L-proline, and the control of L-arginine local bioavailability for NO production in the vessel wall and inflammatory cells. Recent studies performed in cell-specific transgenic mice and in human specimens indicate that the regulation and function of Arg1 and Arg2 appear to be cell type-specific, species-specific and profoundly different in mouse and human. Specificity and availability of arginase inhibitors in human are still limited by their lack of isoform specificity and for their poor pharmacodynamic properties, which means that they have to be administrated i.v., or i.p. The only approved drug is so far a pegylated-arginase for the treatment of hyperarginemia related to genetic defects of the ARG1 gene. Understanding the regulation of Arg1 and Arg2 in different cell types under consideration of their localization, species-specificity, and multiple biochemical and physiological roles could lead to better pharmacological strategies to target arginase in liver, cardiovascular, hematological, immune/infection diseases and cancer*.

Figure 1 Hydrolysis of L-arginine to L-ornithine and urea catalyzed by arginase

Figure 2 Synthesis of polyamines from L-ornithine

Figure 3 Domain organization of Arg1 and Arg2 in homo sapiens – *Arg1 comprises 322 aa and exists as the canonical protein (isoform 1), a longer variant that includes the sequence VTQNFLIL following Q43 (isoform 2) and an aa 204-289 depletion variant (isoform 3). Two substrate-binding regions (dark blue) were identified as well as Mn2+ interacting aa that are not marked here. Arg2 sequence starts with a mitochondrial transit peptide (Marselli et al., 2021) followed by the main body of the enzyme (blue) and closes of with a disordered region (yellow). The substrate binding regions are marked in dark blue*

Figure 4 Active site of a monomer of Arg1 showing a binuclear manganese center coordinated with 2 His and 4

Figure 5 Urea cycle in the liver. *The urea cycle is divided into five steps. The first two steps consist in the generation of carbamoylphosphate from ammonia and synthesis of L-citrulline which takes place in the mitochondria. L-Citrulline is transported to the cytosol and undergoes conversion into argininosuccinate by arginosuccinate synthetase. Arginosuccinate is then cleaved into L-arginine and fumarate by the arginosuccinase, and then Arg1 in the cytosol hydrolyzes L-arginine to L-ornithine and urea in a final step. L-Ornithine is transported back into the mitochondria, where is transformed again into L-citrulline. Instead, urea is exported into the blood and is excreted in the urine via the kidney.*

Figure 6 Arginase/iNOS pathway in mouse macrophages *(figure created with biorender.com)*

Figure 7 L-arginine metabolism in endothelial cells. *In endothelial cells, L-arginine serves as a key substrate for both nitric oxide (NO) synthesis by endothelial nitric oxide synthase (eNOS) and catabolism by arginases 1 (Arg1) and arginase 2 (Arg2). eNOS is well-known to produce NO, which diffuses to vascular smooth muscle cells where it activates soluble guanylate cyclase (sGC), leading to vasorelaxation. Given the co-expression of both arginase isoforms within the endothelium, it is proposed that they act as functional counterparts to eNOS, indirectly regulating its activity. Data from cell-specific mice models reveal that this competition becomes only relevant in disease conditions leading to an increase in arginase activity, and instead is less relevant under homeostatic conditions. Abbreviations: cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; (figure created with biorender.com)*

Figure 8 Synthesis of L-arginine in the kidney. *L-Arginine is endogenously synthesized in the kidney in a reaction catalyzed by argininosuccinate synthetase and argininosuccinate lyase by using circulating L-citrulline produced in the intestine. Synthesis occurs throughout the whole length of the proximal tubule, but is particularly high in the early part closest to the glomerulus, the proximal convoluted tubule (PCT), and gradually decreases in the terminal part in the outer medulla, the proximal straight tubule (PST). Only a small part of the L-arginine synthesized in the kidney is used for the production of polyamines and creatine from L-ornithine, while most of it is released into the circulation, making it available for other tissues (figure created with biorender.com).*
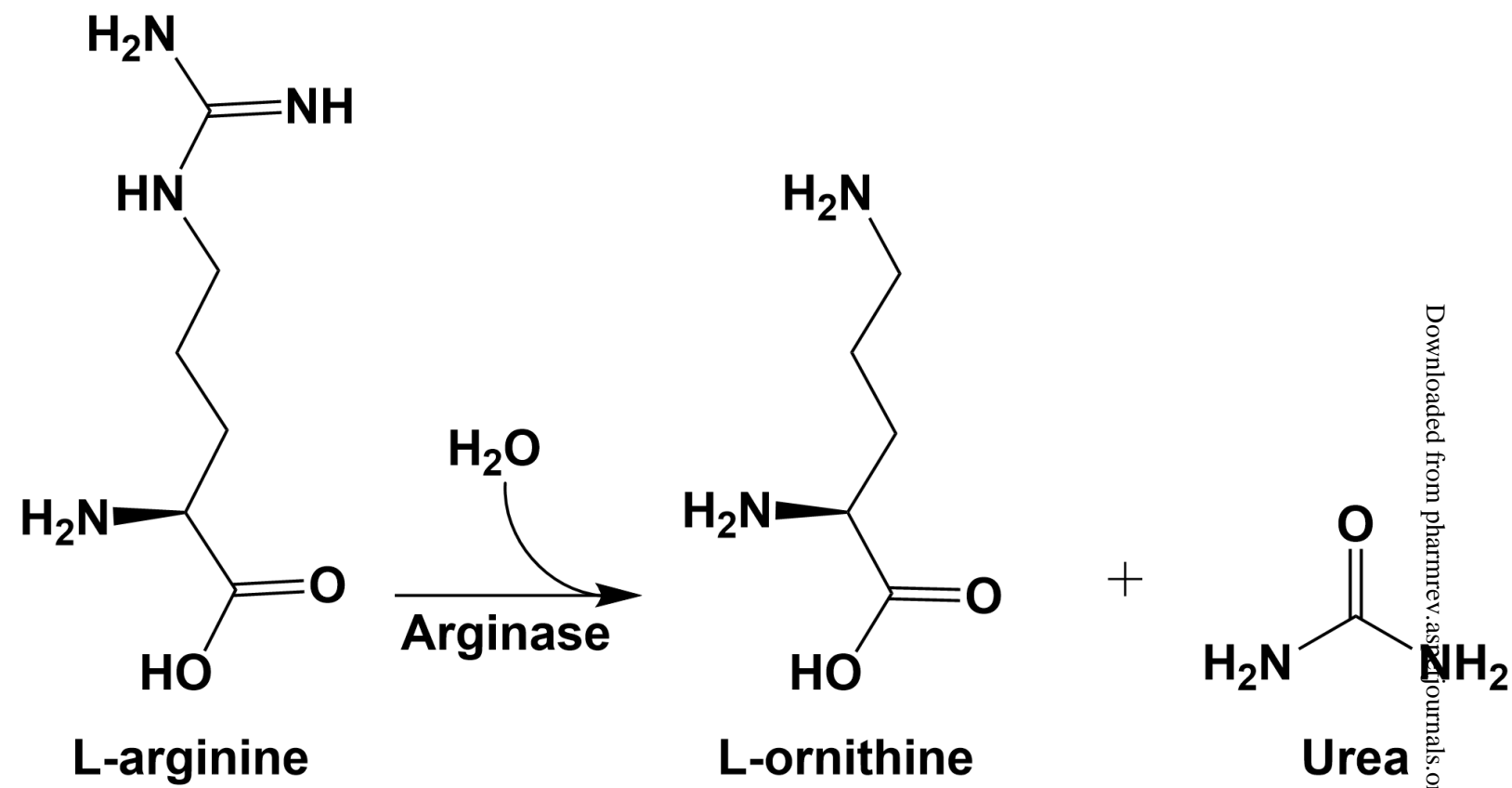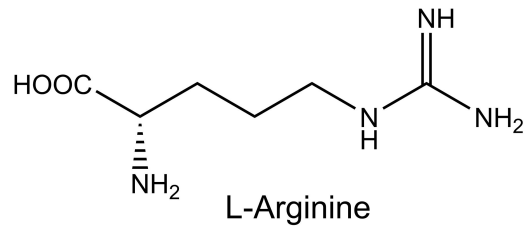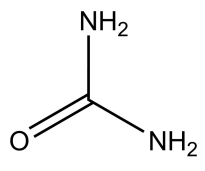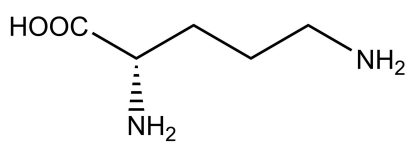
Fig. 1

L-arginine → (Arginase, H_2O) → L-ornithine + Urea

Fig. 2

Fig.3

Arginase 1

Isoform 2:
Insertion VTQNFLIL
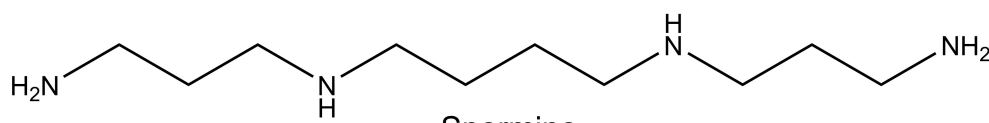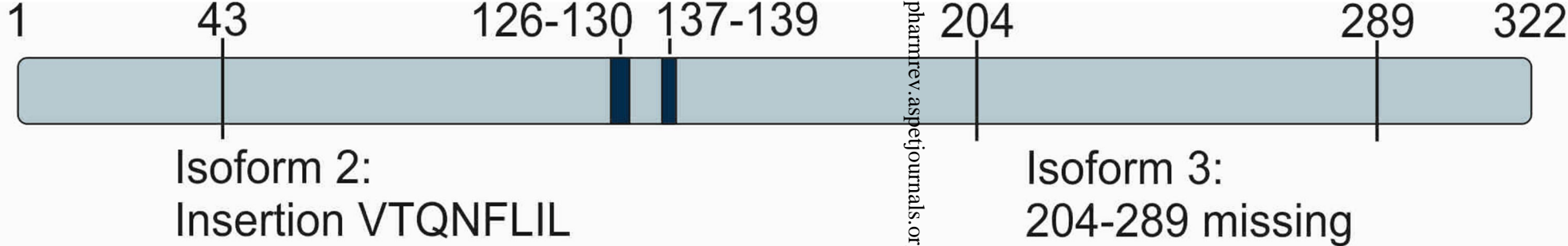
Isoform 3:
204-289 missing
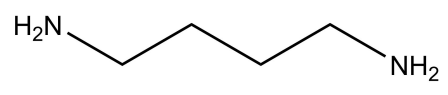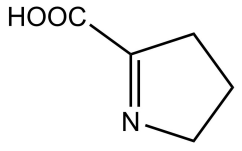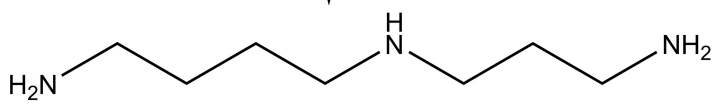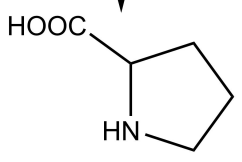
Arginase 2

■ Substrate binding

■ Mitochondrial transit peptide

■ Disordered region

Fig. 4

His-101

Asp-232

Asp-234

Mn^{2+}

Mn^{2+}

O

O^{-}

O

O

Asp-128

Asp-124

N

NH

His-126

Mitochondria   Cytosol

$2\ ATP + HCO_3^- + NH_4^+$

Carbamoylphosphate
synthetase I

$2\ ADP + Pi$

Carbamoylphosphate

Citrulline

Ornithine
transcarbamoylase

Pi

Ornithine   Citrulline

                                             ATP   Aspartate

                                                         Argininosuccinate-
                                                         synthetase

                                    AMP + PPi

                                             Arginino-
                                             succinate

Ornithine

                                                         Arginino-
                                                         succinase

Urea

Arginine   Fumarate

Arginase 1 H_2O

Fig.5

Fig. 6

Fig.7

Fig. 8

Circulating
L-Citrulline
(from intestine)

Circulating
L-Arginine

Bloodstream

L-Ornithine
Urea

Arginase 2

L-Citrulline

L-Arginine

Aspartate
ATP

Argininosuccinate
synthase

Argininosuccinate
lyase

AMP
PPi

Argininosuccinate

Fumarate

PCT