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REVIEW ARTICLE

Mitochondrial transporters for ornithine and related amino acids: a review

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Abstract Among the members of the mitochondrial carrier family, there are transporters that catalyze the translocation of ornithine and related substrates, such as arginine, homoarginine, lysine, histidine, and citrulline, across the inner mitochondrial membrane. The mitochondrial carriers ORC1, ORC2, and SLC25A29 from *Homo sapiens*, BAC1 and BAC2 from *Arabidopsis thaliana*, and Ort1p from *Saccharomyces cerevisiae* have been biochemically characterized by transport assays in liposomes. All of them transport ornithine and amino acids with side chains terminating at least with one amine. There are, however, marked differences in their substrate specificities including their affinity for ornithine (K_M values in the mM to μM range). These differences are most likely reflected by minor differences in the substrate binding sites of these carriers. The physiological role of the above-mentioned mitochondrial carriers is to link several metabolic pathways that take place partly in the cytosol and partly in the mitochondrial matrix and to provide basic amino acids for mitochondrial translation. In the liver, human ORC1 catalyzes the citrulline/ornithine exchange across the mitochondrial inner membrane, which is required for the urea cycle. Human ORC1, ORC2, and SLC25A29 are likely to be involved in the biosynthesis and transport of arginine, which can be used as a precursor for the synthesis of NO, agmatine, polyamines, creatine, glutamine, glutamate, and proline, as well as in the degradation of basic amino acids. BAC1 and BAC2 are implicated in some processes similar to those of their human counterparts and in nitrogen and amino acid metabolism linked to stress conditions and the development of plants. Ort1p is involved in the biosynthesis of arginine and polyamines in yeast.

Keywords Mitochondrial carrier · Mitochondrial transporter · Ornithine · Arginine · Lysine · Histidine · Homoarginine · Urea cycle · Mitochondria · Membrane transport

Abbreviations

BAC1	Basic amino acid carrier 1
BAC2	Basic amino acid carrier 2
HHH	Hyperornithinemia-hyperammonemia-homocitrullinuria
MC	Mitochondrial carrier
NO	Nitric oxide
NOS	NO synthase
ORC1	Ornithine carrier 1
ORC2	Ornithine carrier 2
SLC25A29	Member 29 of the SLC25 protein family

Introduction

The mitochondrial carriers (MCs) constitute a family of eukaryotic intracellular transport proteins that, apart from a few exceptions (Palmieri et al. 2001b; Fukao et al. 2001; Bedhomme et al. 2005; Leroch et al. 2008; Bouvier et al. 2006; Thuswaldner et al. 2007; Kirchberger et al. 2008; Arai et al. 2008; Eubel et al. 2008; Linka et al. 2008; Palmieri et al. 2009; Agrimi et al. 2012), are localized to the inner membranes of mitochondria (Palmieri 2004,

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2013). Each family member has three repeated domains of about 100 residues (Saraste and Walker 1982) enclosing two hydrophobic transmembrane segments and the conserved signature sequence motif PX[D/E]XX[K/R]X[K/R] (20–30 residues) [D/E]GXXXX[W/Y/F][K/R]G (PROSITE PS50920, PFAM PF00153 and IPR00193) (Palmieri 1994). The signature motif sequence has been used to identify MCs in genomic sequences: 53 MCs are found in man, 35 in yeast, and 58 in *Arabidopsis thaliana* (Palmieri et al. 2006a, 2011; Palmieri and Pierri 2010a). Until now, about half of these have been biochemically characterized, and their different transport functions are involved in many metabolic pathways by connecting reactions in the matrix and the cytoplasmic compartments. MCs are responsible for the transport of nucleotides, amino acids, carboxylic acids, inorganic ions, and cofactors that are important for oxidative phosphorylation, transfer of reducing equivalents of NADH, gluconeogenesis, and fatty acid metabolism as well as for mitochondrial replication, transcription, and translation. The fact that mutations in some mitochondrial carriers cause human diseases underlines their physiological importance (Palmieri 2014).

The structure and transport mechanism of MCs are thought to be similar. The only reported atomic-resolution structures of the integral membrane part of MCs are those of the carboxyatractyloside-inhibited ADP/ATP carrier (Pebay-Peyroula et al. 2003; Nury et al. 2005; Ruprecht et al. 2014). In these basket-like structures, the membrane embedded part consists of a six-transmembrane α-helical bundle (H1-H6) with the signature motif prolines kinking the α-helices H1, H3, and H5 causing closure of the basket structure toward the matrix side. This closure is further stabilized by a salt-bridge network engaging the two charged residues following the proline of the signature motif. The signature motif glycines are found at the beginning of the transmembrane α-helices H2, H4, and H6, and other important glycines are found in α-helices H1, H3, and H5 nine residues before the signature motif prolines (Palmieri and Pierri 2010b). Due to the high conservation of the signature motifs, the highly variable amino acid sequences of MCs may be aligned well, and 3D homology models based on the alignments are fairly reliably. Based on sequence and structure analyses residues in certain positions on the even-numbered transmembrane α-helices have been suggested to constitute "contact points" participating in substrate binding and explaining substrate specificity (Robinson and Kunji 2006). This hypothesis is supported by the fact that many single mutations of contact point residues in various MCs are inactive (Palmieri 2008; Monné et al. 2013b). The determinants for substrate specificity have been further deduced by the identification of specific and conserved residues in each subfamily transporting the same substrates (Palmieri et al. 2011). Based on these analyses and the set of MCs biochemically characterized (Palmieri 2004, 2013; Porcelli et al. 2014; Todisco et al. 2014; Di Noia et al. 2014), the MC family can be divided into three major classes with carriers that transport nucleotides, carboxylic acids, and amino acids. Most MCs work as antiporters exchanging an intermembrane space/cytoplasmic substrate for a matrix substrate, but some of them can also work as uniporters or symporters, and they may be dependent on or independent of the electrical and pH gradients across the inner mitochondrial membrane (Monné and Palmieri 2014).

Among the human MCs that transport amino acids, isoforms of three subfamilies have been characterized biochemically: the aspartate/glutamate carrier (Palmieri et al. 2001a), the glutamate carrier (Fiermonte et al. 2002), and the ornithine carrier (Fiermonte et al. 2003). In addition, some MCs transport substrates related to amino acids such as the *S*-adenosylmethionine carrier (Marobbio et al. 2003; Agrimi et al. 2004) and the aspartate-transporting UCP2 (Vozza et al. 2014) that belongs to the carboxylic acid class of MCs. The aspartate/glutamate carrier is involved in the malate/aspartate shuttle together with the 2-oxoglutarate/malate carrier (Indiveri et al. 1987; Palmieri 2004; Monné et al. 2013a) to transfer reducing equivalents of NADH across the mitochondrial inner membrane. The glutamate carrier catalyzes symport of glutamate and proton into the mitochondrial matrix, a transport step that is important for nitrogen metabolism and amino acid degradation. The exchange of ornithine for citrulline catalyzed by the ornithine carriers is important in connecting the matrix and cytoplasmic reactions of the urea cycle.

Several MCs that transport ornithine and related substrates, such as arginine, homoarginine, lysine, histidine, and citrulline, have been characterized. Although a transporter for ornithine in mitochondria had already been postulated in 1972 (Chappell et al. 1972), a carrier that counter exchanges ornithine, citrulline, lysine, and arginine was first purified from rat liver mitochondria in 1992 (Indiveri et al. 1992). Based on its substrate specificity, it was suggested to play a role in the urea cycle. The transport kinetics and modes of transport as well as inhibition by cysteine and lysine reactive agents of the purified and reconstituted rat liver ornithine carrier has been characterized (Indiveri et al. 1994, 1997, 2001; Tonazzi et al. 2005). The MC for ornithine in *Saccharomyces cerevisiae*, Ort1p (Arg11p), was expressed recombinantly in *Escherichia coli*, purified and reconstituted into liposomes that were used in transport characterization experiments (Palmieri et al. 1997). Ort1p exchanges ornithine, arginine, and lysine but can also catalyze ornithine/proton exchange, which was also shown to be the case for the purified ornithine carrier from rat liver (Indiveri et al. 1999). The human "ornithine carriers" ORC1 (SLC25A15), ORC2 (SLC25A2), and SLC25A29

(Fiermonte et al. 2003; Porcelli et al. 2014), and the *Arabidopsis thaliana* carriers BAC1 and BAC2 (Hoyos et al. 2003; Palmieri et al. 2006b) were identified and characterized using recombinant expression and reconstitution methods; they were shown to have different substrate specificities. In this review, we summarize and discuss the knowledge about these six biochemically characterized MCs that all transport ornithine and related substrates.

Substrate specificity of ORC1, ORC2, SLC25A29, BAC1, BAC2, and Ort1p

The transport characteristics of yeast Ort1p (Palmieri et al. 1997), Arabidopsis BAC1 (Hoyos et al. 2003), and BAC2 (Palmieri et al. 2006b) as well as of human ORC1, ORC2 (Fiermonte et al. 2003), and SLC25A29 (Porcelli et al. 2014) have been determined by expressing the proteins in bacteria, purifying the resulting inclusion bodies and reconstituting the MCs into liposomes followed by transport assays. The transport experiments were done by loading the MC-containing proteoliposomes with excess cold internal substrate and initiating substrate exchange by the addition of radioactive external substrate. The substrate specificities were determined by measuring the exchange rates using different internal substrates in exchange for one external radioactive substrate. The kinetic parameters were determined by varying the external concentration of substrate in homo-exchange experiments, i.e., with the same substrate inside and outside the proteoliposomes.

The substrates of the six above-mentioned "ornithine carriers" are listed in Table 1. All the substrates transported by these carriers have in common the Cα amino and carboxylic groups, and a hydrocarbon side chain with a terminal amine group. The substrates vary when it comes to the stereochemistry around Cα (L- and D-forms), the length of the hydrocarbon side chain and the nature of the terminal amine group (primary amine, guanidino, carbamoylated amine or imidazole).

The substrate specificities of recombinant ORC1, ORC2, SLC25A29, BAC1, BAC2, and Ort1p are compared in Table 1. Although the relative transport rates for the different substrates vary between these MCs, it is clear that they all transport L-ornithine, L-lysine, and L-arginine. Some of the substrates are clearly not transported by some of the carriers. ORC1 or Ort1p do not transport L-histidine probably because of its bulky side chain. Also, ORC1 does not transport L-homoarginine or the D-forms of ornithine, histidine, and arginine. SLC25A29, BAC1, and Ort1p do not transport L-citrulline, which has a size similar to that of L-arginine but lacks a terminal positive charge on its side chain. From Table 1, it is also possible to deduce that some carriers tend to have preferences among their substrates. ORC1 prefers shorter and non-cyclized side chains. ORC2 is the carrier with the least restrictive substrate specificity, accepting all substrates listed in Table 1. SLC25A29 has a preference for the L-form amino acid substrates with longer side chains.

The kinetic parameters for ornithine homo-exchange were determined for ORC1, ORC2, and Ort1p: the K_M was between 0.11 and 0.40 mM and the V_{max} between 1100 and 3000 μmol/min/g protein at 25 °C (Table 2). The kinetic parameters for arginine homo-exchange were determined for ORC1, ORC2, SLC25A29, BAC1, and BAC2: the K_M was between 0.16 and 1.58 mM and the V_{max} between 38 and 3000 μmol/min/g protein at 25 °C. These K_M values are close to the intracellular concentrations of arginine and ornithine in non-hepatic cells (approximately from 0.5 to 2 mM) but somewhat higher than the arginine concentration in mammalian hepatocytes (50–100 μM range) (Mann et al. 2003; Wu 2013). The values of the kinetic parameters presented in Table 2 are also in the same range that has been found for other MCs with their respective substrates (Palmieri 2004, 2013).

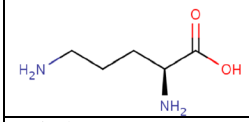
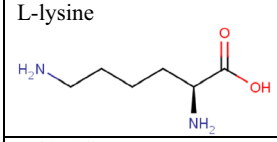
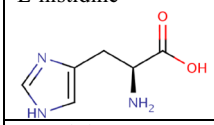
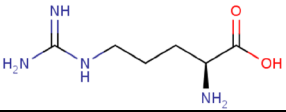
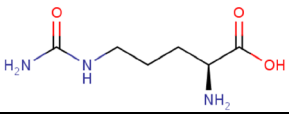
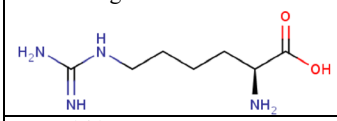
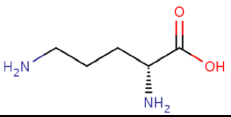
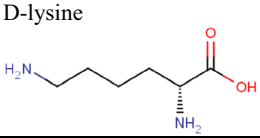
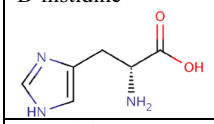
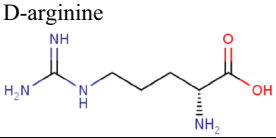
Substrate binding site

The differences in substrate specificity cannot be explained by comparing the sequence identity between the six MCs that transport ornithine and related substrates. ORC1 and ORC2 share 87 % identical residues, while the identity between all other characterized "ornithine carriers" fall between 27 and 32 %, which is not much above the basic level of identity existing among all MCs. Notably, the high similarity between ORC1 and ORC2 is not reflected in more similar substrate specificity as compared with the other four MCs.

The residues determining the different substrate specificity of ORC1 and ORC2 have been investigated by site directed mutagenesis and transport assays (Monné et al. 2012). The results indicated that R179 of ORC1 and the corresponding residue Q179 in ORC2 are mainly responsible for the differences in substrate specificity between the two isoforms. It was suggested that the ORC1 residues R179 and E180 of contact point II on H4 bind the substrate carboxylate and α-amino groups, respectively (Fig. 1). E77 of contact point I on H2 most likely binds the terminal amino group of the substrates in an interaction in which N78 might also play a role. Furthermore, R275 of contact point III on H6 was shown to be crucial for transport; it could be involved in the substrate-induced conformational changes through a contact with R179 mediated by W224. Therefore, the main carrier-substrate contacts are electrostatic interactions.

The residues with side chains protruding into the central pore of the carrier at the level of the contact points

Table 1 Substrate specificity of recombinant ORC1, ORC2, SLC25A29, BAC1, BAC2, and Ort1p reconstituted in proteoliposomes

Substrate in the proteoliposomes	**ORC1** human (Fiermonte et al. 2003)	**ORC2** human (Fiermonte et al. 2003)	**SLC25A29** human (Porcelli et al. 2014)	**BAC1**[a] *A. thaliana* (Hoyos et al. 2003)	**BAC2**[a] *A. thaliana* (Palmieri et al. 2006b)	**Ort1p** *S. cerevisiae* (Palmieri et al. 1997)
None	8	24	19	8	15	7
L-ornithine 	**100**	**100**	41	38	38	**100**
L-lysine 	80	108	112	75	38	73
L-histidine 	5	96	37	30	45	6
L-arginine 	70	89	**100**	**100**	**100**	88
L-citrulline 	38	40	17	10	47	6
L-homoarginine 	8	93	112	n.d.	51	n.d.
D-ornithine 	13	85	n.d.	15	18	64
D-lysine 	21	100	25	31	45	55
D-histidine 	5	85	19	n.d.	n.d.	n.d.
D-arginine 	5	88	28	39	76	n.d.

The numbers indicate the percentage of transport compared with the homoexchange of the radioactive substrate added at a concentration equal to the K_M (**100** %)

n.d. not determined

[a] Not initial rates

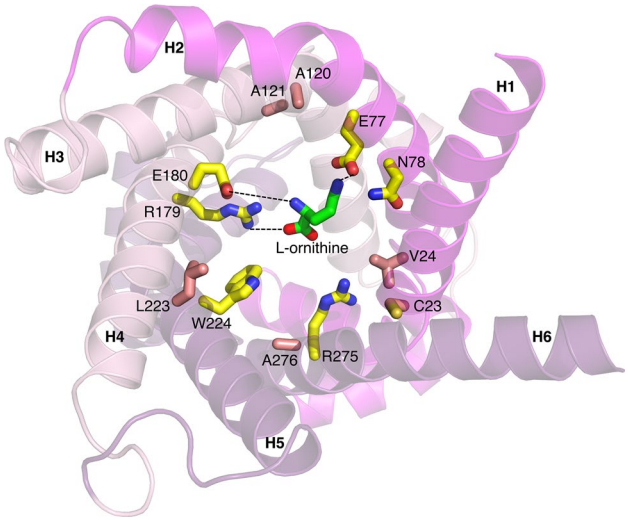


Fig. 1 The human ORC1 substrate binding site. The construction of the structural homology model is described in Monné et al. (2012). The transmembrane α-helices H1-H2 of the first repeat (*pink*), H3-H4 of the second repeat (*light pink*), and H5-H6 of the third repeat (*dark pink*) are represented as cartoon. The substrate L-ornithine (*green*), the residues involved in substrate binding (*yellow*), and residues located at the same level as the binding site (*orange*) are shown as thick sticks

on all six transmembrane α-helices of ORC1, ORC2, SLC25A29, BAC1, BAC2, and Ort1p are summarized and compared in Table 3. Is it possible to find some specific features that would explain the different substrate specificities? The residues in some positions are highly variable such as the first positions in H3 and H5, which might suggest that these residues are not important for function, while the arginine and the alanine in the sixth helix are conserved in all the six MCs implicating that they could be essential for function. The specific determinants for the differences in substrate specificity among the six MCs might be found in sequence features that are specific for one or two of the carriers with respect to the others. For example, ORC2 differs from ORC1 in having a glutamine in position 179, and there is already experimental evidence that this residue is mainly responsible for the difference in substrate specificity compared to ORC1 (Monné et al. 2012). Either the H2 glutamate, which is likely involved in binding the terminal side chain positive charge of the substrate, or the H2 asparagine, which is crucial for transport, is lacking in SLC25A29, BAC1, and BAC2. This feature might contribute to the lower preference of these carriers for the short side-chain substrate L-ornithine in comparison to ORC1, ORC2, and Ort1p that all have glutamate and asparagine in H2. BAC2 has an aspartate instead of glutamate on H4 in a position that has been shown to be crucial for the stereospecificity of the amino acid substrate. It could be that the ability of BAC2 to transport also the D-forms of the amino acid substrates depends on this residue. Only ORC1 and Ort1p have the combination of glutamate and asparagine in H2 as well as arginine and glutamate in H4. It might be that this combination is important for orienting the substrate and is incompatible with L-histidine as a substrate.

Physiological roles of mitochondrial "ornithine" carriers

All characterized cationic amino acid-transporting MCs catalyze bulk transport of amino acids that are transformed, recycled, or used in anabolic and catabolic pathways. The

Table 2 Kinetic constants of recombinant and reconstituted ORC1, ORC2, SLC25A29, BAC1, BAC2, and Ort1p evaluated from homoexchange experiments

Transporter	Substrate	K_M (mM)	V_{max} (μmol/min/g protein)	References
ORC1 human	Ornithine	0.22 ± 0.02	3000 ± 400	Fiermonte et al. (2003)
	Arginine	1.58 ± 0.18	3000 ± 400	
	Lysine	0.80 ± 0.06	3000 ± 400	
	Citrulline	2.52 ± 0.30	3000 ± 400	
ORC2 human	Ornithine	0.40 ± 0.06	1200 ± 200	Fiermonte et al. (2003)
	Arginine	0.71 ± 0.09	1200 ± 200	
	Lysine	0.32 ± 0.05	1200 ± 200	
	Histidine	1.28 ± 0.14	1200 ± 200	
SLC25A29 human	Arginine	0.42 ± 0.04	237 ± 48	Porcelli et al. (2014)
	Lysine	0.71 ± 0.10	237 ± 48	
BAC1 *A. thaliana*	Arginine	0.19 ± 0.05	48 ± 12	Hoyos et al. (2003)
	Lysine	0.68 ± 0.16	48 ± 12	
BAC2 *A. thaliana*	Arginine	0.16 ± 0.02	38 ± 8	Palmieri et al. (2006b)
Ort1p *S. cerevisiae*	Ornithine	0.11 ± 0.01	1100 ± 300	Palmieri et al. (1997)

Table 3 Residues close to the substrate binding site

	ORC1	ORC2	SLC25A29	BAC1	BAC2	Ort1p
H1	C23	C23	G15	T28	G26	G27
	V24	V24	V16	V29	I27	K28
H2 contact point I	**E77**	E77	*I72*	E87	*Q79*	E83
	N78	N78	N73	*S88*	N80	N84
H3	A120	A120	Q106	I127	Q120	A121
	A121	A121	C107	S128	S121	S122
H4 contact point II	**R179**	***Q179***	R160	R185	R169	R178
	E180	**E180**	E161	E186	*D170*	E179
H5	L223	L223	S203	C235	S214	F228
	W224	W224	W204	W236	W215	*N229*
H6 contact point III	**R275**	R275	R257	R287	R264	R275
	A276	A276	A258	A288	A265	A276

Residues in MCs transporting ornithine and related substrates corresponding to those of ORC1 shown in Fig. 1. Residues of ORC1 and ORC2 that have been shown to be important in substrate translocation are shown in bold and variations of these residues in the other carriers are marked in italics. In addition, Q179 is indicated in both bold and italics as it is important in substrate translocation and it is present only in ORC2

Table 4 Evidence for the involvement of the biochemically characterized MCs that transport ornithine and related substrates in specific physiological functions

MC	Major findings linking MCs for basic amino acids to a physiological function	References
ORC1	Mutations in ORC1 cause HHH syndrome, which is classified as a urea cycle disease	Camacho et al. (1999) Tsujino et al. (2000) Salvi et al. (2001) Miyamoto et al. (2001, 2002) Al-Hassnan et al. (2008) Tessa et al. (2009) Ersoy Tunalı et al. (2014) Marobbio et al. (2015)
BAC2	BAC2 expression is induced by salt stress in lateral root development BAC2 expression is induced by hyperosmotic stress and contributes to proline accumulation	He et al. (2005) Toka et al. (2010)
Ort1p	Mutations of *Ort1* reduce ornithine and arginine pools The yeast strain with mutations in *Ort1* does not grow in the absence of arginine	Cabreel et al. (1996) Ersoy Tunalı et al. (2014) Marobbio et al. (2015)

proposed in vivo roles of ORC1, ORC2, and SLC25A29 in humans, BAC1 and BAC2 in Arabidopsis, and Ort1p in yeast are based on their substrate specificities, their expression patterns, and the association of their substrates with known tissue/cell type specific metabolic pathways that are distributed between the cytosol and mitochondrial matrix. However, in a few cases, reports exist on direct links between the biochemically characterized proteins and a physiological process (Table 4). The short list of experimental findings linking the proteins and their in vivo function might be explained by the fact that many cationic amino acid-transporting carriers are likely to have multiple and overlapping physiological roles due to the similarity in substrate specificity and tissue distribution.

Apart from the specific physiological functions discussed below, these MCs probably also play a general role in equilibrating cytoplasmic and matrix pools of arginine, lysine, and histidine by exporting or importing excess amino acids from or to the mitochondrial matrix depending on the rate of mitochondrial protein turnover. It is not yet clear whether mitochondrial translation requires net import of amino acids or the degradation of imported matrix proteins from the cytosol provides a sufficient supply of them. However, if amino acid import is needed for mitochondrial translation, it is likely that ORC1, ORC2, SLC25A29, BAC1, BAC2, and Ort1p all contribute to provide arginine, lysine, and histidine to this process.

Physiological roles of human ORC1, ORC2, and SLC25A29

ORC1 and ORC2 are expressed in liver, pancreas, lung, testis, small intestine, spleen, kidney, brain, and heart

(Fiermonte et al. 2003). In all these tissues the mRNA levels of ORC1 were higher than those of ORC2. Expression of SLC25A29 was found in heart, brain, liver, lung, and kidney (Sekoguchi et al. 2003; Camacho and Rioseco-Camacho 2009).

The mammalian urea cycle, which primarily takes place in liver, serves to remove toxic ammonia by incorporating it into urea that is not toxic at physiological levels. Excess ammonia in the form of non-toxic glutamine and alanine is transferred to liver from other organs. Transaminases in the cytosol of hepatocytes transfer the α-amino groups of amino acids onto 2-oxoglutarate to form glutamate which is translocated into the mitochondrial matrix by the glutamate carrier (Fiermonte et al. 2002) to provide ammonia for the urea cycle. The enzymes that catalyze the first two steps of ammonia detoxification (carbamoylphosphate synthetase I

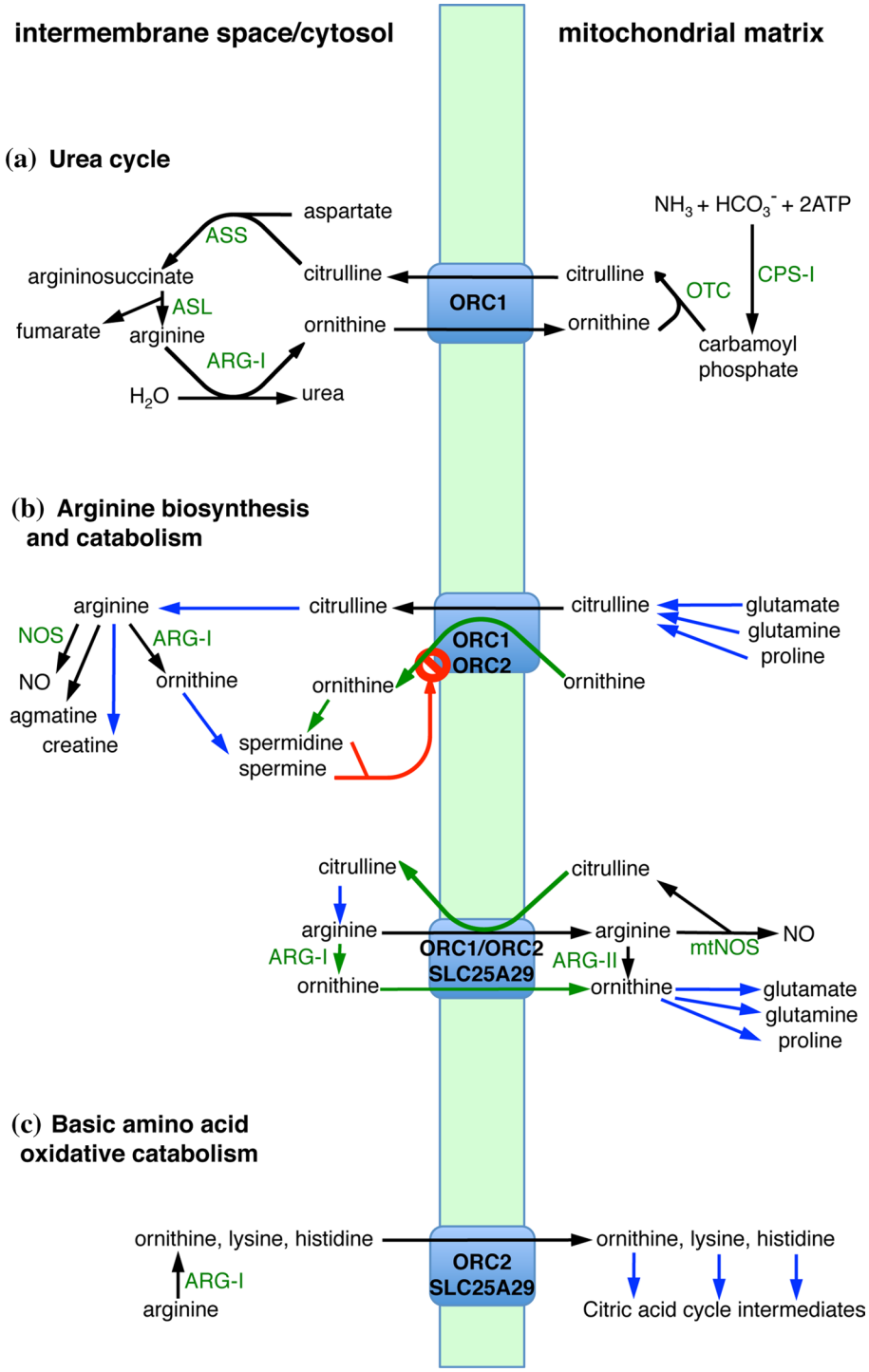


Fig. 2 Functions of the mitochondrial carriers for ornithine and related substrates in humans. **a** Urea cycle, adapted from Palmieri (2004), **b** arginine biosynthesis and catabolism, and **c** basic amino acid oxidative metabolism. *Black arrows* correspond to single reaction steps, *blue arrows* to multiple reaction steps, *red arrows* indicate feed-back inhibition loops, and *green arrows* alternative routes. Abbreviations in *green* of enzymes involved CPS-I, carbamoylphosphate synthetase I; *OTC* ornithine transcarbamoylase; *ASS* argininosuccinate synthase; *ASL* argininosuccinate lyase; *ARG-I* arginase I; *ARG-II* arginase II; *NOS NO* synthase; *mtNOS* mitochondrial NO synthase

and ornithine transcarbamoylase) are located in the mitochondrial matrix and the subsequent enzymes are located in the cytosol (Fig. 2a). The matrix and cytosolic reactions of the urea cycle are connected by the exchange of matrix citrulline + H^+ for cytosolic ornithine$^+$ across the inner mitochondrial membrane by the ornithine/citrulline carrier (Indiveri et al. 1992, 1997; Palmieri 2004). Since ORC1 transports citrulline and ornithine more efficiently than ORC2 and it is much more abundant in liver than ORC2, it is likely that the former carrier plays the major role for the urea cycle. This hypothesis is further supported by the clinical symptoms of hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (Shih et al. 1969; Valle and Simell 2001), which is caused by mutations in ORC1 and is discussed in more detail below. ORC2 might compensate for defective ORC1 in HHH patients to some extent, i.e., ORC2 might have a minor role in the urea cycle at least in special conditions. SLC25A29 has also been suggested to partly complement the lack of ORC1 activity (Camacho 2003; Camacho and Rioseco-Camacho 2009), but this is unlikely because SLC25A29 transports ornithine very poorly and does not transport citrulline (Porcelli et al. 2014).

While lysine and histidine are nutritionally essential amino acids for humans, arginine is conditionally essential, i.e., humans have the capability of *de novo* synthesis of a certain amount of arginine (Wu 2009). In small intestine glutamine, glutamate and proline, which are precursors of arginine biosynthesis, are converted by mitochondrial enzymes to citrulline (Fig. 2b) (Wu and Morris 1998). The citrulline produced in mitochondria needs to be exported to the cytosol by uniport or exchange, most likely catalyzed by ORC1 and ORC2. In small intestine, cytosolic citrulline is directly converted into arginine in neonates, whereas in adults, it is secreted into the blood and transported to the kidney, which is the major organ for endogenous arginine synthesis. Although in liver, arginine synthesis may be accomplished by a truncated urea cycle, it seems that hepatic arginine is recycled in the urea cycle and no net production is observed.

Arginine in the cytosol of non-hepatic tissues serves as precursor for several biosynthetic pathways (Fig. 2b) (Wu and Morris 1998). Cytosolic arginine may be used by the inducible nitric oxide (NO) synthase (NOS) for the production of NO especially in cells of the immune system. This synthesis is important in many biological processes such as nitrosylation, signaling, and many other functions (Kleinert et al. 2003). NO, derived from arginine by neuronal NOS, plays an important role in regulating sympathetic nerve activity and skeletal muscle (Wang and Golledge 2013). Endotelial NOS also uses cytosolic arginine for the production of NO that acts on smooth muscle causing vasodilator effects (Shaul 2002). In brain, kidney, adrenal gland, macrophages, and small intestine, cytosolic arginine may be used for the biosynthesis of agmatine (Morrissey et al. 1995), which has been reported to play a role in the regulation of NO and polyamine production (Galea et al. 1996; Satriano et al. 1998). In many cell types cytosolic arginine is also a precursor for polyamines, such as spermidine and spermine, which regulate DNA synthesis and are necessary for cell proliferation and differentiation. Polyamines might also be synthesized directly from ornithine exported from mitochondria in exchange for protons, a reaction driven by the pH gradient across the inner membrane (Indiveri et al. 1997; Palmieri et al. 1997; Palmieri 2004). ORC1 and ORC2 are inhibited by spermine and spermidine, suggesting that this represents a feed-back mechanism to block mitochondrial export of ornithine that is necessary for polyamine synthesis (Fiermonte et al. 2003). Creatine, of which the phosphorylated form is used as an energy reserve in skeletal muscle and brain, is synthesized from cytosolic arginine and glycine mainly in the renal tubules and pancreas (McGuire et al. 1986).

ORC2 and SLC25A29 may be responsible for importing arginine into mitochondria to serve as a substrate for mitochondrial matrix NOS (mtNOS, Fig. 2b), which is found in non-hepatic tissues. Alternatively ORC1 and ORC2 might catalyze the heteroexchange of cytoplasmic arginine for matrix citrulline formed by mtNOS. Important roles have been attributed to mitochondrial NO, which may reversibly inhibit cytochrome c oxidase and react with mitochondrial thiol-containing proteins and superoxide anion to produce peroxynitrite as well as to act as a signaling molecule involved in apoptosis and metabolic syndrome (Ghafourifar and Cadenas 2005; Ghafourifar et al. 2005; Litvinova et al. 2015). Arginine may be converted to ornithine in the matrix by arginase II, which is the non-hepatic isoform and is localized in mitochondria, or in the cytosol by arginase I that is mainly expressed in liver (Wu and Morris 1998). Ornithine imported into mitochondria is a precursor for the biosynthesis of glutamate, glutamine, and proline, of which some of the enzymatic steps take place in the cytosol (Herzfeld et al. 1977). Notably, arginase II has been suggested to play a role in mtNOS regulation (Lim et al. 2007) as well as in atherosclerosis and endothelial dysfunction (Ryoo et al. 2008).

Oxidative catabolism of amino acids relevant for metabolic energy generation occurs in conjunction to synthesis and degradation of proteins, excess protein content in the diet, and during starvation. Many amino acid degradation pathways, which mainly take place in liver and kidney, are located in both the cytosol and the mitochondrial matrix. In these processes, the amino groups of the amino acids are transferred to 2-oxoglutarate forming glutamate destined to urea cycle (see above); the remaining partly oxidized carbon skeletons are fed into the citric acid cycle as various

intermediates requiring transport by several MCs; ORC2 and SLC25A29 may contribute to the efflux or influx of the basic amino acids from and to the mitochondrial matrix presumably in exchange with protons (Fig. 2c). Arginine is degraded to ornithine that is imported into mitochondria and converted via glutamate to 2-oxoglutarate. The first enzyme of the main pathway for lysine degradation in upper eukaryotes, the saccharopine pathway, is mitochondrial (Blemings et al. 1994; Papes et al. 1999). The enzymes involved in the degradation of histidine in the liver are thought to take place both in the cytosol and mitochondrial matrix (Morris et al. 1972).

HHH syndrome

Mutations in the human ORC1 gene cause the HHH syndrome (OMIM 238970) (Camacho et al. 1999; Tsujino et al. 2000; Salvi et al. 2001; Miyamoto et al. 2001, 2002; Al-Hassnan et al. 2008; Tessa et al. 2009; Ersoy Tunalı et al. 2014). HHH is a rare autosomal recessive disorder characterized by high levels of ammonia, ornithine, and homocitrulline in the blood, and by neuronal complications and sometimes liver failure (Palmieri 2008, 2014; Martinelli et al. 2015). Other urea cycle disorders are linked to mutations in other enzymes of the urea cycle, and they display different but related symptoms.

The majority of the single residue mutations causing HHH syndrome are found along the substrate translocation pathway in the ORC1 homology model (Palmieri 2008; Martinelli et al. 2015). Some of these mutations have been shown to impair or completely abolish ORC1 transport function by direct transport assays of the recombinant mutated proteins (Fiermonte et al. 2003; Tessa et al. 2009; Ersoy Tunalı et al. 2014; Marobbio et al. 2015). Among the disease-causing mutations, E180K and R275Q are found in the substrate binding site and T32R and P126R in the signature motif sequence. It is thought that lack of ORC1 transport activity impedes the removal of excess nitrogen. The deficient ORC1 function diminishes ornithine import into mitochondria and leads to build up of ornithine produced from arginine in the cytosol, which increases polyamine production. The hyperammonemia can be explained by the accumulation of ammonium in the mitochondria (Fig. 2a), which diffuses to the cytoplasm and outside the cell. Some of the ammonia may be transformed to carbamoylphosphate and condensed with lysine forming homocitrulline, the accumulation of which causes homocitrullinuria. Homocitrulline may also enter the pyrimidine biosynthetic pathway leading to increase in uracil and orotic acid excretion. The impaired urea cycle function due to ORC1 deficiency also causes increased levels of glutamine, alanine, and liver transaminases. Patients with HHH syndrome are recommended low-protein diets with citrulline or arginine supplement.

Physiological roles of arabidopsis BAC1 and BAC2

BAC1 and BAC2 are expressed in stems, leaves, flowers, siliques, and seedlings (Hoyos et al. 2003; Catoni et al. 2003). The highest levels of BAC1 transcripts were found in flowers, siliques, and seedlings, whereas BAC2 transcripts were most abundant in flowers.

Unlike in humans, ornithine carbamoyltransferase and argininosuccinate synthase are both localized in the cytosol of plants and fungi, and therefore, arginine synthesis does not require export of mitochondrial citrulline (Shargool et al. 1988). Another difference from humans is that amino acids in plants are rarely, if ever, used for oxidative catabolism to extract metabolic energy but rather for biosynthetic pathways to produce other metabolites. One explanation is that in plants, the nitrogen source is often a rate-limiting factor of growth and nitrogen metabolism is tightly regulated. In plants, arginine and citrulline are used as endogenous nitrogen storage molecules (Ludwig 1993). In seeds, BAC1 and BAC2 have been suggested to play a role in mitochondrial import of arginine. This amino acid represents a nitrogen storage molecule before germination and is utilized by mitochondrial arginase, producing ornithine and urea, after germination (Fig. 3a) (Hoyos et al. 2003; Palmieri et al. 2006b). Overexpression of BAC2 in vivo leads to arginine depletion and urea accumulation in leaves implicating that BAC2 expression is linked to stress recovery in Arabidopsis leaves (Planchais et al. 2014). Furthermore, the activity of arginase has been suggested to be coordinated with that of urease for the use of seed reserve proteins (Thompson 1980).

In plants arginine is also a precursor of many compounds similar to those in humans, although the end products of the plant biosynthetic pathways involving arginine may have different physiological functions (Fig. 3a). Mitochondrial arginine may be utilized by mtNOS for the production of NO, which stimulates seed germination (Kopyra and Gwozdz 2003). In addition, arginine and ornithine may be used as precursors for the biosynthesis of glutamate, proline, polyamines, agmatine, and alkaloids (Hanfrey et al. 2001; Illingworth et al. 2003). Due to the high expression of BAC2 in pollen, this carrier has been suggested to export ornithine from the mitochondrial matrix to the cytosol for proline biosynthesis. Indeed, proline is the most abundant amino acid in this tissue where it plays a role in the protection of drought stress (Mestichelli et al. 1979; Venekamp et al. 1989; Verma and Zhang 1999; Ramachandra Reddy et al. 2004; Palmieri et al. 2006b). This hypothesis has been experimentally supported by mutational studies which

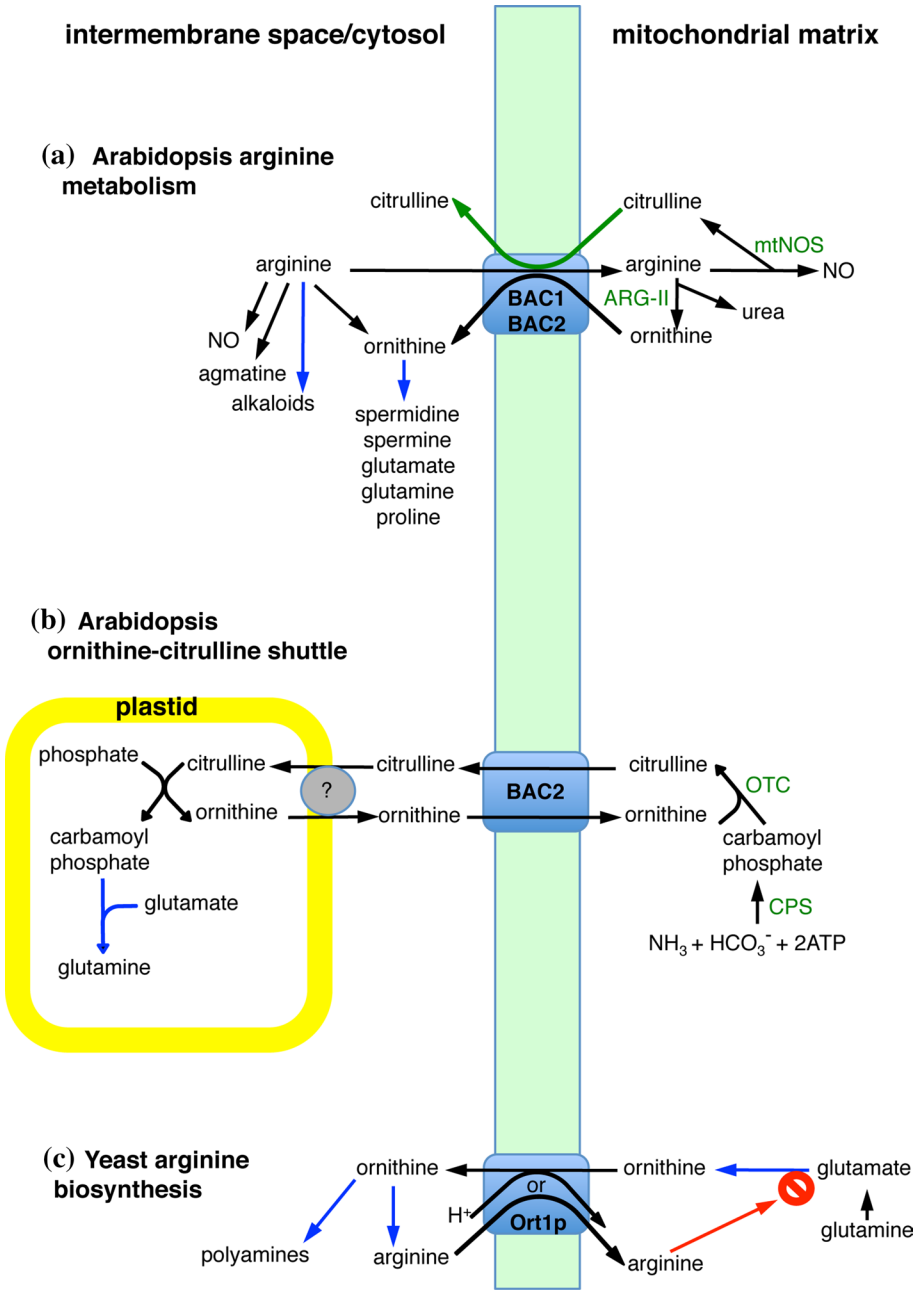


Fig. 3 Functions of the mitochondrial carriers for ornithine and related substrates in Arabidopsis and *S. cerevisiae*. **a** Arabidopsis arginine metabolism, **b** Arabidopsis ornithine-citrulline shuttle, adapted from Linka and Weber 2005, and **c** Yeast arginine biosynthesis. *Black arrows* correspond to single reaction steps, *blue arrows* to multiple reaction steps, and *red arrows* indicate feed back inhibition loops. Abbreviations in *green* of enzymes involved CPS, carbamoylphosphate synthetase I; *OTC* ornithine transcarbamoylase; *ARG-II* arginase II; *mtNOS* mitochondrial NO synthase

showed that the expression of BAC2 is induced by hyperosmotic stress and results in proline accumulation (Toka et al. 2010). Furthermore, in the development of lateral roots the expression level of BAC2 was found to be regulated by transcriptional factors responding to salt stress (He et al. 2005).

Due to its ability to catalyze citrulline/ornithine exchange (Palmieri et al. 2006b), BAC2 in leaf has been proposed to be involved in a shuttle of mitochondrial ammonia, which is produced in large quantities during photorespiration, to plastids where it is assimilated into glutamine (Fig. 3b) (Taira et al. 2004; Linka and Weber 2005). However, the existence of a mitochondrial ornithine carbamoyltransferase has not been proved in Arabidopsis.

Physiological roles of *S. cerevisiae* Ort1p

Ort1p in *S. cerevisiae* is likely to catalyze the transport of ornithine, produced in mitochondria from glutamate or glutamine, to the cytosol where it is used for arginine and polyamine biosynthesis (Fig. 3c). In yeast, the biosynthesis of arginine involves five mitochondrial enzymes that convert glutamate to ornithine and three cytosolic enzymes

that convert ornithine to arginine. There is no urea cycle in *S. cerevisiae* because excess nitrogen is secreted directly as ammonia in line with the fact that citrulline is a poor substrate for Ort1p (Palmieri et al. 1997). Furthermore, it has been shown by the same researchers that Ort1p may exchange ornithine for protons, lysine, or arginine. In the mitochondrial matrix, these two cationic amino acids could be used for mitochondrial translation and arginine to inhibit the first two enzymes of its biosynthetic pathway in a feedback inhibition mechanism (Palmieri et al. 2000). The role of Ort1p in arginine biosynthesis in yeast is further supported by genetic data; in fact, the gene encoding Ort1p was originally isolated from a screen of mutations causing defects in arginine synthesis (Crabeel et al. 1996).

The physiological role of MCs transporting homoarginine

In humans, homoarginine deficiency is associated with cardiovascular mortality as well as renal and heart diseases (März et al. 2010; Tomaschitz et al. 2014). Although it has been demonstrated that ORC2 and SLC25A29 (and BAC2 in plants) have the ability to transport homoarginine (Fiermonte et al. 2003; Palmieri et al. 2006b; Monné et al. 2012; Porcelli et al. 2014), its role in mitochondria is not clear. It could be speculated that homoarginine biosynthesis or degradation takes place, at least in part, in mitochondria because single-nucleotide polymorphisms associated with homoarginine deficiency were found in three genes encoding mitochondrial enzymes linked to arginine metabolism that could be involved in providing precursors for homoarginine biosynthesis (Kleber et al. 2013). Another hypothesis is that homoarginine is imported into mitochondria by ORC2 or SLC25A29 because homoarginine is a substrate of purified mtNOS, leading to the production of homocitrulline and NO (Moali et al. 1998). However, many of the different cytosolic NOS isoforms also have the ability to use homoarginine as a substrate, which might suggest that homoarginine, if it is produced in mitochondria, is exported from the mitochondria.

Conclusions and perspectives

The six biochemically characterized MCs for ornithine and related substrates from *H. sapiens*, *A. thaliana* and *S. cerevisiae* all transport amino acids with side chains terminating with at least one amine group, across the mitochondrial inner membrane. Although their protein sequences are divergent, with the exception of human ORC1 and ORC2, the substrate binding site residues of these transporters are quite conserved. However, the few residues that are different in the substrate binding site may explain the differences in their substrate specificities, as suggested by a detailed study of the substrate binding sites of ORC1 and ORC2. The human ORC1, ORC2 and SLC25A29 are suggested to share some physiological functions related to nitrogen metabolism, such as NO production, amino acid biosynthesis and degradation as well as biosynthesis of agmatine, polyamines and creatine. The fundamental physiological function in the urea cycle of catalyzing ornithine/citrulline exchange across the inner mitochondrial membrane is accomplished almost completely by ORC1 as confirmed by the fact that mutations in this transporter cause HHH syndrome. Arabidopsis BAC1 and BAC2 play specific roles in nitrogen metabolism, amino acid biosynthesis and NO production. The expression of BAC2 is connected to osmotic stress and proline biosynthesis. Yeast Ort1p is mainly involved in arginine and polyamine biosynthesis.

Future work is warranted to identify and biochemically characterize other members of the MC family which are capable of transporting ornithine and/or related amino acids in other species and, unlikely but not entirely excluded, in humans, Arabidopsis or yeast. Another perspective is to find out whether one or more of the six well-characterized MCs for cationic amino acids (or other carriers) transport further physiologically important basic amino acid-related substrates across the mitochondrial inner membrane such as asymmetric dimethylarginine (ADMA), which is a risk factor for cardiovascular disease (Alpoim et al. 2015), and homocitrulline that accumulates in liver mitochondria of patients with HHH syndrome. These findings will help in better understanding the evolutionary relationship of the MCs and the determinants for substrate specificity, which could in turn serve to improve the prediction of the substrates transported by still uncharacterized carriers. Furthermore, as seen in Table 4, there are few examples where MCs that transport ornithine and related substrates are correlated with a specific physiological function. It seems that many of the characterized transport functions of these MCs are redundant and responsible for multiple and overlapping physiological roles. Further investigation is required to establish whether this is the case or each carrier has specific roles.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article is a review summarizing the results and conclusions of available publications that include previously performed studies on human or animal subjects.

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