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Development of an innovative strategy based on engineered autologous erythrocytes as "enzyme replacement therapy" for the treatment of Phenylketonuria

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Alla mia famiglia presente e futura,
perché nonostante tutto c'è

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INTRODUCTION

1. PHENYLKETONURIA: THE EPITOME OF HUMAN BIOCHEMICAL DISORDERS

When in 1934 the Norwegian endocrinologist Dr. Asbjørn Følling first diagnosed the presence of a high concentration of an unknown substance (which he called phenylpyruvic acid) in the urine of two siblings with mental retardation, probably he was not aware of the importance his discovery would have for thousands of people from that moment on. The credit for the discovery was also due to that caring and stubborn mother, who could not resign herself to the mental retardation of her children without having found a reason [http://pkuworld.org/home/history.asp].

By means of a traditional assay of classical chemistry for the detection of ketones, consisting in the addition of ferric chloride to the urine of diabetic patients, Dr. Følling observed the appearance of a deep green colour, which he had never seen before. Further chemical analyses and steps of purification on many other urine samples from patients sharing the same neurocognitive and developmental delays with the first ones, led to the identification of a chemical substance whose empirical formula was $C_9H_8O_3$. The physical and chemical characterization of this molecule revealed that it was acidic, with a molar mass of 164; under slight oxidizing conditions it produced a benzaldehyde-like odour whereas a strong oxidation gave origin to benzoic acid and oxalic acid. All these features permitted to identify the compound as phenylpyruvic acid. What was still to be discovered was the causal relation to the mental retardation. From the analysis of the urine from another 430 mentally impaired subjects, Dr. Følling identified eight patients excreting the same substance and for the first time he understood the correlation between mental impairment and excretion of phenylpyruvic acid, a condition he named “oligophrenia phenypirouvica” (also named after him as Følling’s disease) [1]. Two years later the condition was renamed “phenylketonuria” to link the disease to the metabolic phenotype [2]. When L-Phe is not metabolized by the specific enzyme phenylalanine hydroxylase (PAH), it enters an alternative pathway of transamination and decarboxylation, which leads to the formation of phenylpyruvate, phenyllactate, and o-hydroxyphenylacetate, the metabolites whose excretion in the urine confers to it the typical colour and odour [3]. Further studies of family relationships highlighted an autosomal recessive mechanism of transmission [4]. To explain the causes of the phenylpyruvic acid excretion, he hypothesized some kind of defect in phenylalanine metabolism, with phenylalanine present at high concentration in the blood of such patients if the hypothesis was verified; all this was later on confirmed [5] through a microbiological test developed by Dr. Robert Guthrie which exploited the reversal of growth inhibition observed in *Bacillus subtilis* ATCC 6051 in the presence of a high level of phenylalanine [6].

Phenylketonuria is the first example of impaired cognitive development which was recognized to have a chemical etiology, i.e. hyperphenylalaninemia [2]. Today PKU is considered “the epitome of metabolic disorders” and is often employed as a model to describe and understand many other inborn errors of amino acid metabolism [7, 8].

2. THE DISEASE

Phenylketonuria (OMIM# 261600) is an inborn error of amino acid metabolism, caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH, EC 1.14.16.1) that catalyzes the conversion via para-hydroxylation of the amino acid L-phenylalanine (L-Phe) into L-tyrosine (L-Tyr) [9]. The lack of PAH activity results in phenylalanine accumulation in body fluids (including brain and liquor), reaching neurotoxic levels and thus causing a progressive severe and irreversible neurological and intellectual disability, due to the lack of neurotransmitters for which Tyr is a precursor [10, 11]. PKU is inherited as an autosomal recessive trait [4] with a prevalence varying in a wide range according to geographic region and ethnicity; nevertheless, its rate of 1 out of 10,000 live births in Europe makes the pathology one of the most widespread genetic diseases among Caucasians [10, 12], with peak values in those populations with a high rate of consanguinity [13].

The disease is mainly caused by a mutation in a gene located in chromosome 12 (region 12q22-q24.2, GenBank U49897) encoding the cytosolic hepatic enzyme phenylalanine hydroxylase (PAH, E.C. 1.14.16.1) which catalyzes the irreversible conversion of the amino acid phenylalanine into tyrosine, a limiting step for the complete oxidation of L-Phe to CO_2 and H_2O. To work properly, PAH needs the presence of a pterin cofactor, terahydrobiopterin (BH_4), as well as molecular oxygen and iron (Figure 1). The final concentration of phenylalanine in the body is in fact the result of a finely regulated balance: L-Phe input amount, coming from diet and the endogenous recycling of amino acids, and L-Phe output amount, represented by that fraction integrated in newly synthesized proteins and the one oxidized to L-Tyr through the PAH-mediated reaction [9].

When PAH does not carry out its own activity, phenylalanine accumulates in body fluids, including liquor, giving origin to hyperphenylalaninemia (HPA) and the related wide spectrum of mental disturbances typical of this condition [9, 14].

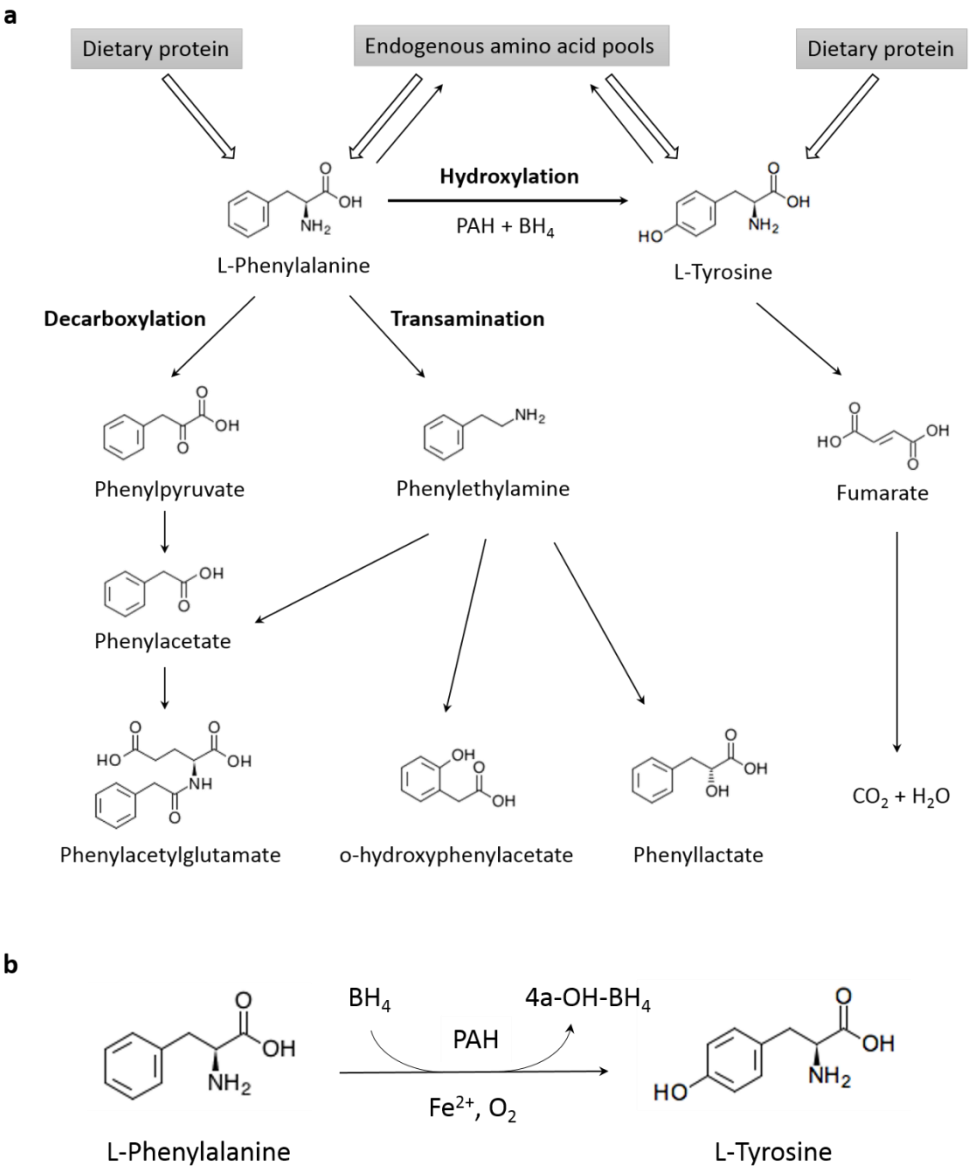


Figure 1. Metabolic pathways involving phenylalanine. a) Phenylalanine metabolisms in normal conditions; b) the conversion catalyzed by Phenylalanine Hydroxylase.

2.1. Clinical manifestations and pathogenic mechanism

The main organic symptom of the disease is hyperphenylalaninemia; L-Phe reaches plasma concentration of up to and over 1200 µM, depending on the severity of the mutation. The high concentration L-Phe is the main agent of the other clinical manifestations, because it acts on different aspects of the brain functioning, as described below.

Patients with PKU in its most severe (untreated) form present with impaired growth and brain development. Symptoms include microcephaly, epilepsy, seizures, motor deficits, severe intellectual disability and behavioral disturbances, including psychotic, autistic, and aggressive disorders [15, 16]. Mental retardation is thought to arise from a decreased myelination that confers a reduced protection from proteolytic degradation [17] in combination with a reduced myelin production [18]. Many studies

have identified problems related to the altered development of the brain architecture due to the exposure to excessive L-Phe, as extensively reviewed by several Authors [16, 18, 19]; these modifications include abnormal myelination, cortical plate width and altered dendritic arborization with a reduced number of synaptic spines. The exposure to high concentration of L-Phe makes the already formed myelin unstable, whereas demyelinated axons undergo a reverse maturation, with consequent neuronal dysfunction [20]. Even in early and well-treated PKU patients dysmyelination still occurs, which may cause some of the above mentioned clinical manifestations [21]. *In vitro* studies have also demonstrated a depressed glutamaergic synaptic transmission in the presence of high L-Phe levels [22], data confirmed by a subsequent *in vivo* study [23]. The reported impaired activity of the glutamate receptor may explain, at least partially, the reduced dendritic arborization and number of spines experienced by untreated PKU patients; this effect was not observed for the transamination products of L-Phe, i.e. phenylpyruvic, phenylactic and phenylacetic acid, further indicating L-Phe as the neurotoxic agent in PKU [24]. The accumulation of potentially toxic L-Phe metabolites in the blood does not reach indeed a level sufficiently high to cause brain injury [18].

A significant inverse correlation has been found between L-Phe levels and IQ score, especially during the critical developmental period (age 0-12 years), even in early treated children: each 100 μM rise in L-Phe concentration corresponded to a 1.3-3.1 IQ point decline [25]. Later, a meta-analysis study identified a larger IQ decline, ranging from 1.9 to 4.1 points per each 100 μM mean rise in L-Phe in children treated since the neonatal age; a similar correlation was also found between lifetime L-Phe levels and IQ scores in early-treated individuals [26].

Moreover, Anastasoaie and colleagues [27] demonstrated that a better explanation of the cognitive outcome be provided by blood L-Phe variability, rather than the mean lifetime blood value as itself in early and continuously treated children, thus suggesting the importance of maintaining an L-Phe concentration as stable as possible within the recommended range of 120–360 μM over time, in order to prevent the deterioration of the cognitive performance.

All the neurological alterations encountered in PKU patients ultimately account for deficits commonly belonging to the field of the executive functioning, including also response speed, academic abilities, language-related tasks (including reading and arithmetic), problem solving ability, attention, interhemispheric transfer of information, and visuo-spatial and visual-motor abilities, as observed by Scriver et al. [14] and then extensively reviewed in the works by Bone et al. [16] and by Huijbregts et al. [28]. In a meta-analysis study, processes such as planning, working memory, inhibition, processing speed, and cognitive flexibility resulted to be impaired in early diet-treated patients, compared to controls [29].

The psychological and psychiatric problems documented in adolescent patients concern the area of social life, with negative findings in terms of autonomy, self-esteem, frustration threshold, school

achievements, attention, mood disturbances, depression and anxiety [30], even in early treated children [31]. Discontinuation of the dietary therapy as well as high levels of L-Phe are of course associated with a higher incidence of behavioral problems. Untreated individuals show more severe symptoms such as autism, hyperactivity, aggression, social withdrawal, anxiety, depression, psychosis, and profound intellectual disability [31], whereas adult patients early treated in childhood present with generalized depressed and anxious mood, lack of autonomy, low self-esteem and a tendency to social isolation. Phobias are also typical, and the most common one is agoraphobia [31-33].

L-Phe accumulation in blood results in its increased accumulation in the brain. This is due to the fact that phenylalanine belongs to the group of the Large Neutral Amino Acids (LNAAs), to which valine, leucine, isoleucine, threonine, histidine, tryptophan, methionine and tyrosine also belong. All these amino acids share a common carrier, the L-amino acid transporter-1 (LAT-1), to cross the blood-brain barrier (BBB) and enter the brain [34]; when there is an excessive circulating amount of L-Phe, this one is thought to compete with the other LNAAs for the available LAT-1 transporter, saturating it and thus resulting in a L-Phe overload in the brain and in a corresponding decreased amount of the other LNAAs, particularly L-Tyr and L-Trp [18, 34]. Moreover, across species LAT-1 appeared to have a higher affinity for L-Phe than the other LNAAs, and this is particularly marked for the human species, making it more susceptible to the negative effects of HPA [35].

Besides being important for protein synthesis, the LNAAs L-Tyr and L-Trp are also precursors for neurotransmitters, namely dopamine (DA, and consequently norepinephrine (NE) and epinephrine) and serotonin (5-hydroxytryptamine, 5-HT) respectively (Figure 2) [18]. Dopamine plays an important role in motor and cognitive functioning; norepinephrine is involved in learning and memorization processes, in the arousal of attention, fear and anxiety, and in the development of the maternal behaviour in females; serotonin is important for neuronal proliferation, synaptogenesis and morphogenesis [36, 37].

Indeed, there are two possible mechanisms by which L-Phe alters brain functioning: if on the one hand the increased L-Phe presence in brain results in a decreased level of the other LNAAs including L-Tyr and L-Trp, as described above, on the other hand it acts as a competitive inhibitor of the other two amino acid hydroxylases, TyrOH and TrpOH [18, 38-41], thus generating a lack of their products. In fact, high brain L-Phe was reported to negatively affect the activity of the other hydroxylases, notwithstanding the increased availability of BH_4 (whose production is stimulated by L-Phe itself [42]) [18].

Before acting as neurotransmitters, biogenic amines also represent fundamental signals for the correct early brain development [43], as demonstrated in experiments in mice whose genes encoding the biosynthetic enzymes TyrOH and dopamine β-hydroxylase (DBH) were deleted [44, 45].

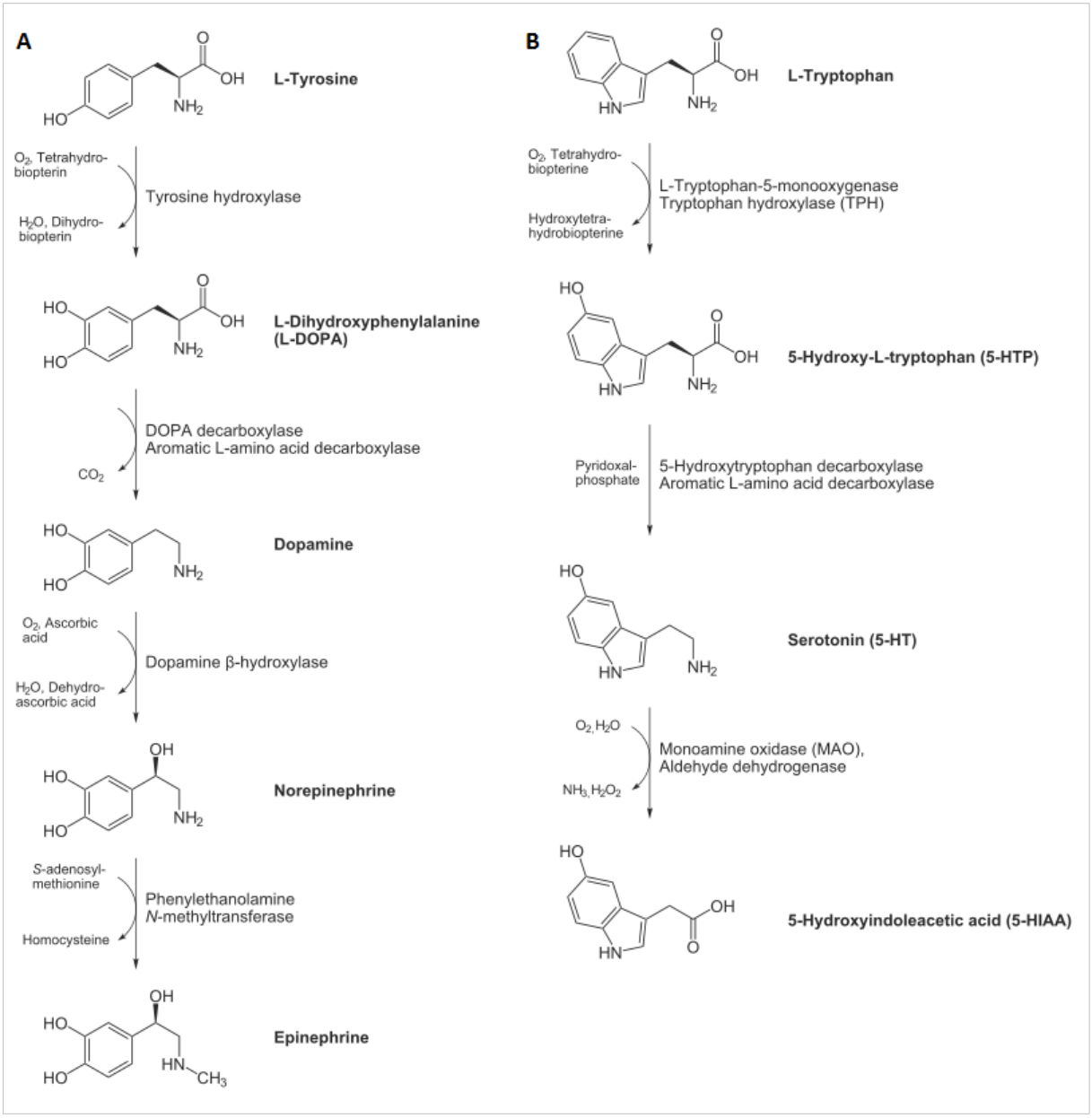


Figure 2. Biosynthetic pathways of neurotransmitters lacking in the brain of PKU patients. (A) Synthesis of catecholamines (dopamine, DA, norepinephrine, NE, and epinephrine); (B) synthesis of serotonin (5-hydroxytryptamine, 5-HT) (adapted from http://www.hdri-usa.com/).

The development of the cerebral cortex occurs following a precise sequence of events, well defined in time and space, also spanning over the post-natal period especially as regards the synapses and dendrites in the prefrontal cortex. These time-windows are characterized by a different availability of brain amines, with peak increases of catecholamines and serotonin during the critical developmental period followed by reduction to adult levels, and are the most susceptible to L-Phe-induced damages, as extensively demonstrated by studies on animal models [36, 37, 41, 44-53]. Low levels of biogenic amines have been found in the cerebrospinal fluid of hyperphenylalaninemic patients [54].

The healthy prefrontal cortex is particularly rich in dopamine, which is fundamental for the executive functions, and PKU children have been found to have an impaired dopaminergic innervation, with subsequent attentional deficits [55, 56]. A work by Burlina et al. [57] confirmed a reduced aminergic

synthesis in the brain of adult PKU patients even if compliant with a free L-Phe diet. Some cognitive and behavioral alterations, significant though subtle, have also been reported in patients with a good control of blood L-Phe levels [29, 58], which could be explained as a consequence of small permanent cortical dendritic changes, secondary to NE depletion in the perinatal period [49].

In a series of *in vivo* studies, Pascucci et al. [41, 51, 59] demonstrated in mice with the BTBR-Pah^{enu2} genotype, the genetic model of human PKU, a severe lack of whole brain serotonin during critical post-natal periods (PND14-21) and deficits in the level of its immediate and limiting precursor 5-hydroxytryptophan (5-HTP), not corresponded by a reduction in its initial amino acidic precursor L-Tryptophan; these evidence support the hypothesis of TrpOH activity inhibition exerted by L-Phe excess, rather than a hampered access of L-Trp across the blood brain barrier (BBB) [40, 51], thus confirming the minor involvement of tryptophan in the L-Phe induced alterations [59]. In addition, Pascucci and colleagues detected a 50% reduction in 5-HT, followed by a 40% decrease in NE levels and a 30% decrease in DA, thus confirming previous observations [47, 51].

As reported, among the considered biogenic amines dopamine and its precursor L-3,4-dihydroxyphenylalanine (L-DOPA) are the less affected by HPA, owing to the fact that when L-Tyr levels are abnormally low and L-Phe is extremely high, the latter can serve as substrate for TyrOH for the production of L-DOPA [60].

All the described pathophysiologic mechanisms involve L-Phe as a single molecule. In the recent few years, a new amyloidosis-like etiology has been proposed: it has been observed that when L-Phe is present at extremely high concentrations, such as in the brain of PKU patients, it self-assembles to form fibrils with amyloid-like morphology and cytotoxicity [61-63]. Such deposits have been found in the hippocampus of model mice and in the parietal cortex tissue obtained from phenylketonuric individuals [61] and their toxicity seems to derive from the fibril arrangement in a hydrophilic core and hydrophobic exterior made up of aromatic side chains of the L-Phe monomers. Therefore the lipophilic character of the fibril outer surface would promote insertion into cell membranes, while the hydrophilic core interacts with ions; the result is ion leakage and a consequent cellular damage [63].

Interestingly, Singh and coworkers demonstrated inhibition of L-Phe fibril formation by D-Phe enantiomer when added at ≥ 8% of L-Phe concentration in solution. The DL-Phe solution formed instead aggregates with a completely different morphology of flat flakes with irregular edges, which proved to be more stable than fibers and, most importantly, unable to propagate further [62]. Supplementation with D-Phe could therefore be a possible strategy to prevent HPA-associated brain alterations both by preventing accumulation of the amyloid-like L-Phe fibers, and by competing with the L-enantiomer for the LAT1 transporter to cross the blood-brain barrier [64].

Another recent hypothesis is that excessive L-Phe impacts on DNA methylation patterns, like other stressors. To verify this idea Dobrowolsky et al. [65] studied the DNA from the frontal cortex of two

PKU patients, and then compared the methylation patterns and subsequent gene expression to those observed in the DNA from leukocytes (chronically immersed in a hyperphenylalaninemic environment) of other PKU patients on diet, with both well-controlled and poorly-controlled blood L-Phe. The Authors found epigenetic alterations both in PKU brains, suggesting neurological involvement, and in patients' leukocytes, with a strikingly higher methylation rate in regulatory miRNA genes in the non diet-compliant group. A subsequent inversely proportional altered expression (down-regulation) was also identified in the miRNA-targeted genes, which, in most cases, code for structural proteins or proteins involved in synapse formation and functioning and in myelination process. These findings confirm and extend to human PKU what had already been observed in a preclinical study on mouse fetuses affected by maternal PKU [66] and suggest DNA methylation pattern in leukocytes as a possible biomarker for historic L-Phe exposure, as well as epigenome as a candidate target for PKU treatment [65].

2.2. The role of BH_4 cofactor in PKU pathogenesis

Hyperphenylalaninemia (HPA) can be caused indeed not only by defects in the PAH gene, but also in those genes encoding enzymes involved in the pathways of *de novo* synthesis or recycling of the PAH cofactor tetrahydrobiopterin. This condition was initially referred to as "malignant phenylketonuria" [67]. The biosynthetic enzymes involved are GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) which catalyze the *de novo* synthesis of BH_4 starting from guanosine triphosphate; on the other hand, the recycling enzymes are pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) which catalyze the reduction of the oxidized cofactor quinonoid dihydrobiopterin (qBH_2) once Phe conversion to L-Tyr has occurred [68]. Deficiencies in the BH_4 synthesis or recycling enzymes are inherited similarly to the PAH mutations as autosomal recessive traits, and account for approximately 2% of HPAs detected in babies by newborn screening [69]. In this case the metabolic block can be overcome by supplementing a biologically active synthetic form of the cofactor, i.e. sapropterin dihydrochloride ((6R)-2-amino-6-[(1R,2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydro-4(3H)-pteridinone dihydrochloride), investigated for the first time by BioMarin Pharmaceutical Inc. (Novato, CA) and commercialized since 2007 both in the U.S.A. and Europe with the name of Kuvan® [https://www.bmrn.com/about-us/history.php#2008]. To date, this is the only drug approved by FDA with the "orphan" designation for the treatment of PKU.
In Figure 3 are represented the metabolic pathways through which the cofactor is produced in the organism.

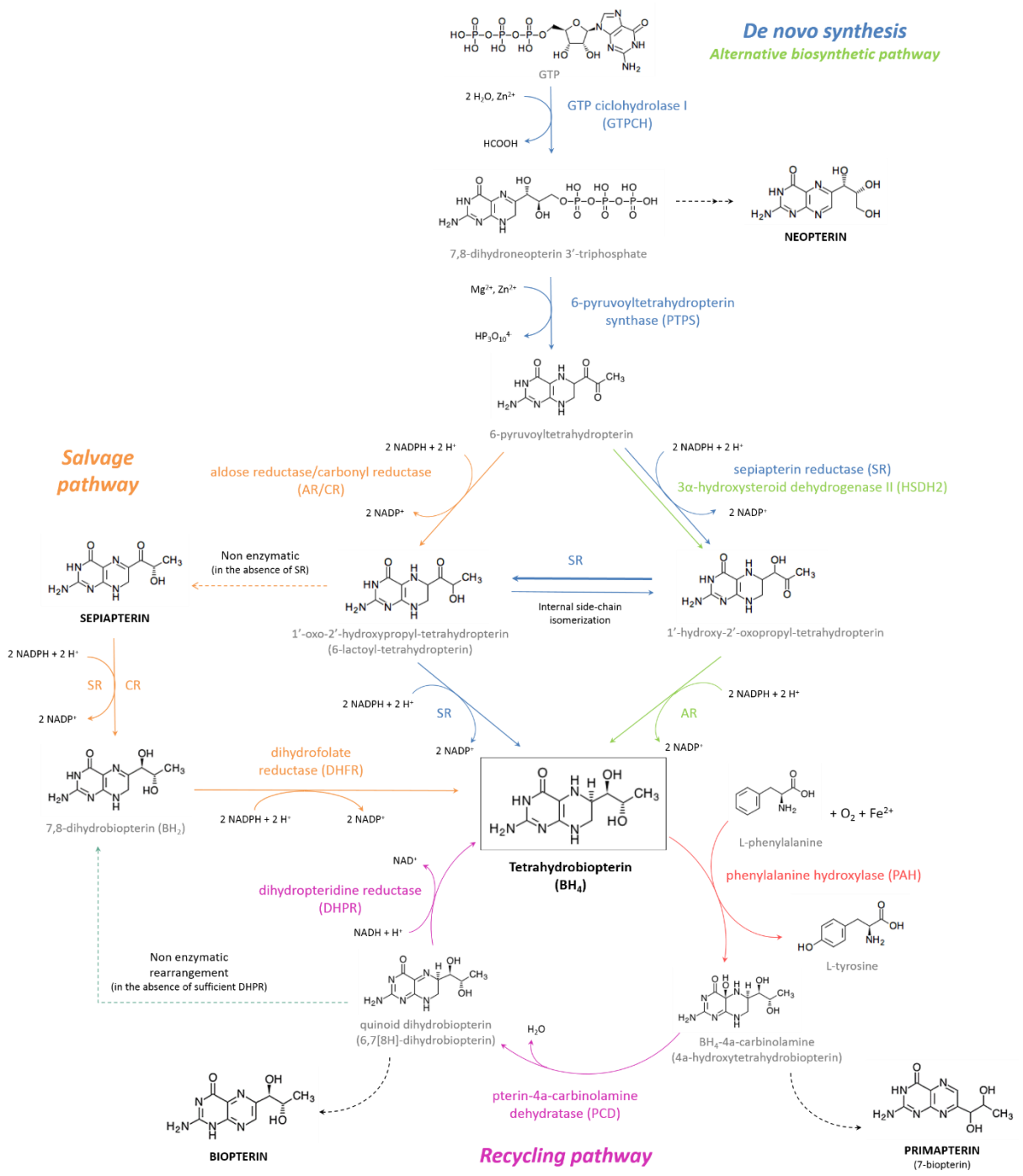


Figure 3. BH_4 metabolism. The different colours identify the three metabolic pathways, with the one in blue being the main anabolism. The "green" enzymes belong to an alternative pathway operating in parallel with the main biosynthetic route (Adapted from Werner et al. 2011 [70]).

The pterinic cofactor BH_4 has a very important role in PKU symptom determination. The lack of tetrahydrobiopterin is one of the causative agents of a group of pediatric disorders named monoamine deficiencies and also known as "atypical PKU" [71, 72]. Such alterations take origin from defects in the neurotransmitter biosynthetic enzymes, as well as in BH_4 availability, which serves as cofactor in the reactions [73]. In fact, apart from Phenylalanine Hydroxylase, tetrahydrobiopterin is a common cofactor for the other two hydroxylating enzymes, TyrOH and TrpOH, meaning that individuals with

enzymatic defects in the BH_4 biosynthetic or recycling pathways suffer not only from the lack of neurotransmitter precursors, but in addition are not even able to synthesize catecholamines and serotonin from tyrosine and tryptophan introduced with the diet [74].

Among the other options, the regulation of BH_4 production relies also on BH_4 itself and blood L-Phe; both molecules act on the first enzyme, GTPCH, via the GTPCH feedback regulatory protein (GFRP), the first one (BH_4) inhibiting it and the second one (L-Phe) activating it. As a consequence, in HPA there is a high plasma concentration of pterin cofactor which, however, cannot be used due to the lack of PAH [75]. On the other hand, Phenyalanine Hydroxylase is not inhibited by the product of BH_4 oxidation, i.e. BH_4-4a-carbinolamine, but rather by primapterin, an isomer of biopterin carrying the dihydroxypropyl chain at position 7 instead of 6 of the pteridine ring. When the genetic defect occurs in the enzyme pterin-4a-carbinolamine dehydratase (PCD), the carbinolamine cofactor can also be non-enzimatically dehydrated to quinonoid dihydrobiopterin, but this reaction is not efficient enough to supply the reduced form of the cofactor in the required amounts [76]. Therefore, a lack of PCD mainly induces BH_4-4a-carbinolamine to chemically rearrange in two steps to dihydroprimapterin (that is, 7-substituted dihydrobiopterin) which is then further converted into primapterin (7-biopterin) and in this form excreted into the urine, thus defining a condition named “primapterinuria” [71, 72, 77-80]. The 7-substituted form of biopterin (7-BH_4) was reported not to be a good cofactor for PAH, due to the uncoupling of the reaction to the production of L-Tyr [81]; moreover, it exerts a potent inhibition on the enzyme already at 1 µM concentration in the presence of physiological concentration of BH_4 and saturating L-Phe, while its oxidation generates hydrogen peroxide, which further inhibits PAH activity [79]. Therefore, the hyperphenylalaninemia observed in patients with normal PAH activity is probably due to the inhibition exerted by the 7-sustituted cofactor generated by an altered BH_4 metabolism [79]; on the other hand, PAH inhibition raises even more L-Phe concentration which, in turn, stimulates the activity of the BH_4-synthesising enzyme GTPCH I through its regulatory protein GFRP [42, 82], thus activating a loop of mutual feedback regulations and making primapterinuria actually dependent on plasma L-Phe levels and liver BH_4 availability, with the possibility to modulate their amounts by means of an L-Phe-restricted diet [80].

When the genetically altered protein is dihydropteridin reductase (DHPR), the quinonoid form resulting from the first step of the recycling pathway is readdressed to a non-enzymatic rearrangement, yielding 7,8-dihydrobiopterin, a substrate for dihydrofolate reductase (DHFR) instead of DHPR. However, this alternative regeneration pathway is not sufficiently effective in restoring the proper concentration of tetrahydrobiopterin, as demonstrated by severe clinical neurological manifestations arising in DHPR-deficient patients [83]. One of the proposed explanation is the accumulation of 7,8-dihydrobiopterin, a recognized strong inhibitor of all aromatic amino acid

hydroxylases, which would further contribute to the diminished synthesis of both L-DOPA and 5-OH-tryptophan [84].

2.3. Other clinical manifestations

The reduced availability of L-DOPA is the factor underlying another hallmark of phenylketonuria, i.e. fair colour of patient hair and skin. It has been demonstrated that tyrosinase (E.C. 1.14.18.1), the Cu^{2+}-dependent oxidoreductase representing the first enzyme in the melanogenesis pathway, works in concert with TyrOH, the latter providing L-DOPA necessary to activate and promote tyrosinase activity and, as a consequence, skin and hair pigmentation [85].

Moreover, a causative relation was discovered between L-Phe and the inhibition of brain and liver β-hydroxy-β-methylglutaryl-coenzyme A reductase (E.C. 1.1.1.88), the rate-limiting enzyme in cholesterol biosynthetic pathway, together with a reduced production of mevalonic acid [86]. The resulting hypocholesterolemia is hypothesized to have a protective effect against cardiovascular diseases in adults [3]. The impairment of the mevalonate pathway also implies a decreased synthesis of coenzyme Q_{10} (ubiquinone-10; CoQ_{10}), actually observed in both serum and lymphocytes of PKU patients, thus supporting a role of excessive L-Phe as an indirect pro-oxidant factor [87, 88].

3. MATERNAL PKU

An adequate control of blood L-Phe levels is particularly important in women willing to become pregnant, since the exposure to elevated L-Phe concentrations is teratogenic for the fetus, causing the so-called “maternal phenylketonuria syndrome” or maternal PKU [34, 89]. Pregnant patients with poor or absent control of HPA or PKU have a high probability to experience pregnancy complications, the most frequent being spontaneous abortion, intrauterine fetal death (IUFD) or growth retardation, and preterm delivery [89-91]. Affected newborns present with low birth weight (and are defined as small for gestational age, SGA), microcephaly, congenital heart disease (CHD), intellectual or developmental disabilities (IDDs), and facial dysmorphism (FD) [90-92]. The incidence of such pregnancy complications and/or neonatal sequelae is strongly correlated with maternal blood L-Phe levels, particularly during the first weeks of embryogenesis, i.e. when organogenesis occurs; this is especially the case for FD [89].

The study of Prick and colleagues [89] clearly demonstrates a significant correlation between the increase in mean L-Phe concentration per trimester in maternal blood and the occurrence of SGA, microcephaly and IDDs, and underlines an increase in CHD as L-Phe per trimester doubled.

Hence, it becomes very important for women with a desire to become pregnant to accurately plan the pregnancy itself and to comply with a strict L-Phe restricted diet also enriched in multivitamin complexes, vitamin B_{12} and folic acid, which had better to be initiated before conception and should

be continued throughout pregnancy, in order to prevent as much as possible fetal damages [89, 90, 93, 94]. During pregnancy maternal L-Phe tolerance increases to some extent thanks to fetal PAH activity, resulting in lower blood levels and in a better cognitive performance; this should permit a relaxation of the strict diet regimen during the second trimester, thus avoiding the nutritional problems that could arise from the adherence to the diet itself [89].

The Maternal PKU Collaborative Study (MPKUCS), which lasted from 1984 to 2002, represented an international effort aimed at assessing the effects of such diet on the offspring of PKU mothers, also in relation to the timing of the treatment [95, 96]. From this study emerged that the main causative agent is the maternal PKU phenotype, determining maternal blood L-Phe concentration and therefore fetal exposure; this could also represent a problem in case of mild HPA, since during pregnancy there is a placental gradient of L-Phe favoring the fetus [97] that can be dangerous if not kept under control [98]. Moreover, the socio-economic status of such women could influence their level of education and subsequently the cognitive outcome [99]. On the other hand, the maternal phenotype becomes determinant only when dietary treatment is discontinued during childhood and adolescence, leading to impaired maternal intelligence and, as a consequence, to the delayed onset of treatment and a poor quality of care both in anticipation of and during pregnancy and the post-natal period [100, 101]. Women should reach a blood L-Phe concentration within the recommended range of at least 120-360 µM (2-6 mg/dl) – better 60-250 µM (1-4 mg/dl) [102] because of the positive gradient across placenta – already before conception or, in any case, not later than the 8th week of pregnancy, and should manage to keep it in this range throughout pregnancy, in order to bear a baby with normal birth parameters (as regards CHD, microcephaly and SGA [103, 104]) and cognitive performance [91, 93, 95, 96, 98]. These reference values origin from the fact that a concentration higher than 120 µM is necessary to avoid the damages produced by hypophenylalaninemia [96, 104], whereas the upper limit comes from the identification of a critical threshold value of 330-360 µM beyond which damages to the fetus occur [105].

PKU mothers bearing non-PKU babies (i.e. healthy carriers of a single mutated allele) are encouraged to breastfeed their children without restriction, since the single non mutated copy of the *PAH* gene is sufficient to metabolize the amount of L-Phe introduced with breast milk [106], also because breast milk contains only 43 mg L-Phe/dl compared to 59-73 mg/dl of infant formulas and 164 mg/dl of cow's milk [107].

Another important finding of the MPKUCS was that L-Phe fluctuations, even within the safe range of concentrations, has a negative effect on offspring cognitive outcome, which on the contrary was not evident for L-Phe mean levels [94]. In fact, Maillot et al. [94] demonstrated a strong negative correlation between the standard deviation (describing maternal blood L-Phe variations within the recommended interval) and offspring IQ measured at the age of 4, 8 and 14 years. Blood L-Tyr does

not seem to have effect on the developing fetus [96]. The additional treatment with BH_4 supplementation was reported to help the development of fetal PAH activity with a protective effect from maternal HPA [99, 108].

In conclusion, the solution resides in the achievement of the proper degree of L-Phe control already before conception; this might prove to be very difficult, though, especially for women with the most severe forms of the disease, who used to have themselves poor L-Phe control throughout their lives (and consequently a higher probability of mental impairment): it has been demonstrated that a maternal IQ <85 is associated with a later achievement of the target L-Phe range and a more negative influence on the fetus [101, 109]. Hence, it is important to provide these and all other patients with special support and education on the importance of diet compliance, together with more frequent monitoring throughout pregnancy [90, 94, 110]. Adherence to such recommendations makes it possible for PKU mothers to have children with the same expectancy of cognitive development as non-PKU people [90].

4. PHENYLALANINE HYDROXYLASE

4.1. Enzyme and gene

Phenylalanine hydroxylase (also named phenylalanine-4-monooxygenase, symbol PAH or PheOH, EC 1.14.16.1) is part of the enzymatic family of pterin-dependent aromatic amino acid hydroxylases. This family also includes two more monooxygenases, i.e. tryptophan hydroxylase (tryptophan-5-monooxygenase, symbol TPH or TrpOH, EC 1.14.16.4) and tyrosine hydroxylase (tyrosine-3-monooxygenase, symbol TH or TyrOH, EC 1.14.16.2); all these proteins share the use of BH_4, molecular oxygen and reduced iron (Fe^{2+}) to carry out their own activity [111, 112].

The human PAH enzyme is encoded by a gene mapped to the long arm of chromosome 12 (locus *PAH* 12q22-q24.2) and expressed in liver and kidney [113-115]; the cDNA of the gene was first obtained and cloned in the 1980s [116-118] (GenBank NM_000277 for PAH mRNA; U49897.1 for PAH cDNA) whereas the full-length genomic sequence of the gene was obtained later (GenBank AF404777) [119]. *PAH* gene is composed of 13 exons and 12 large introns to reach a total length of 121,526 Kbp in the minus strand of the DNA [http://www.genecards.org/cgi-bin/carddisp.pl?gc_id=PAH]. The gene coding sequence (cds, nt 473-1,831) is flanked by a 5'-UTR region (nt 1-472) and a 3'-UTR region (nt 1,832-2,680); it is transcribed into a mature mRNA of approximately 2.6 Kb (2,680 bp), which is in turn translated into a 452 amino acid monomer [http://www.ncbi.nlm.nih.gov/nuccore/U49897.1, last update 1997].

Each polypeptide weighs 51.8 KDa and consists of three domains: an N-terminal regulatory domain (residues Glu19-Leu142, also identified as ACT domain), a catalytic domain (residues Asp143-Phe410), and a short C-terminal tetramerization domain (residues Ser411-Lys452) [3, 120, 121; GenBank

AAC51772.1]. Figure 4 shows the nucleotide cds and the corresponding amino acidic sequence of the *PAH* gene.

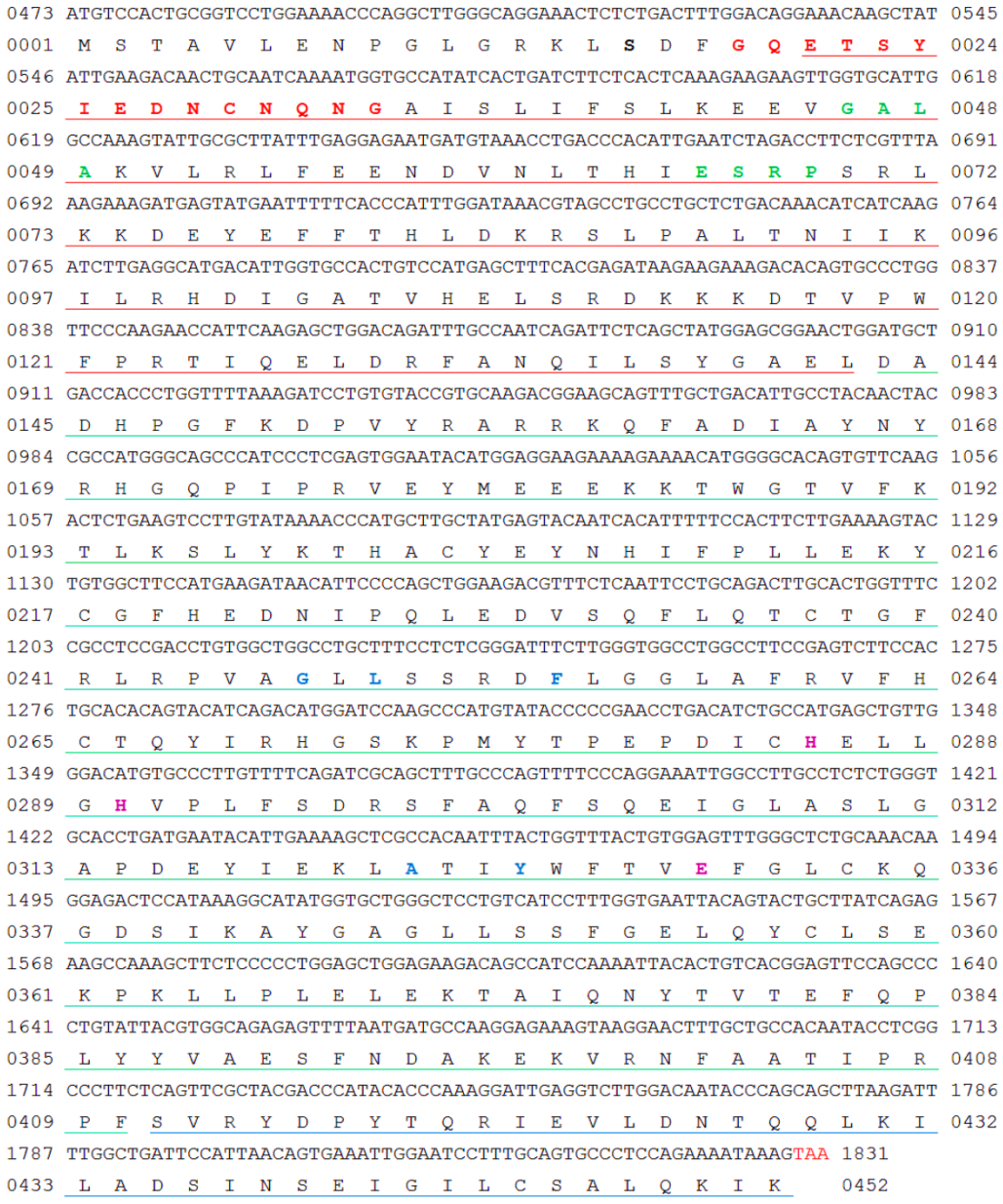


Figure 4. Nucleotide sequence of the coding region (cds, nt 473-1831) of the *PAH* gene (line above) and corresponding amino acidic sequence (line below). Underlined in red is the regulatory domain, in green the catalytic domain and in blue the tetramerization domain. In bold and red are the residues forming the autoregulatory sequence (ARS, Gly19-Gly33); in bold and green the putative L-Phe binding site (residues Gly46-Leu49 and Glu66-Pro69); in bold and blue the amino acids forming the cofactor binding site (Gly247, Leu249, Phe254, Ala322, Tyr325); in bold and purple the metal ion binding site (His285, His290, Glu330); in bold and black the Ser16 residue, the phosphorylation site important for activity regulation.

Despite the great difficulty to obtain the crystallographic structure of the whole enzyme, the availability of the structures of many truncated forms of PAH, containing the regulatory and catalytic domains [122] or the catalytic and tetramerization domains [123, 124], allowed the construction of a full-length crystal model by superimposing the catalytic domains (Figure 5).

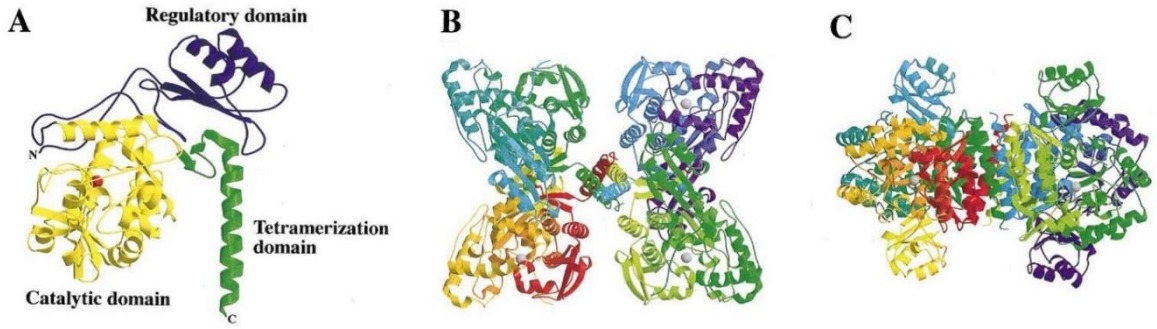


Figure 5. (A) Full-length structure of human phenylalanine hydroxylase monomer obtained by superimposing the catalytic domains of the truncated forms. The red sphere represents iron. **(B, C)** Two perpendicular views of the full-length PAH model structure. The iron is shown as a gray sphere in all four monomers (adapted from Erlandsen and Stevens 1999 [120]).

Full-length PAH has been described to exist in solution both as a functional homodimer and homotetramer, in a pH- and phenylalanine-dependent equilibrium, with a marked shift towards the tetrameric form as pH decreases or L-Phe concentration increases [17, 125, 126]. However both oligomeric forms are functional, as demonstrated by studies on truncated forms of both PAH and TyrOH including only the tetramerization and catalytic domains, which still maintain the enzymatic activity though losing substrate specificity [124], but with the peculiarity that the tetramer formed by PAH is asymmetrical: this is due to the fact that, actually, this tetramer is a "dimer of dimers" [120], where a mechanism of domain swapping between monomers takes place such that secondary elements switch their mutual position in order to promote a stable oligomerization, together with the formation of an antiparallel coiled-coil structure with the other monomers [124, 127].

4.2. PAH regulation

Given the negative effects of an excessive circulating phenylalanine concentration, it is very important to maintain phenylalanine homeostasis *in vivo*; therefore, PAH activity is strictly regulated through different mechanisms, i.e. substrate activation, cofactor inhibition and reversible phosphorylation [19, 126, 128-131; for review see Refs. 132, 133]. The autoregulatory sequence located at the N-terminus of the protein includes a residue of Ser16 which has been demonstrated to be the site of phosphorylation by the cAMP-dependent protein kinase A (cAMP-PKA) [130]. This sequence is named autoregulatory because it sterically limits the access of the substrate to the catalytic site of the enzyme [133]. When the first 30 N-terminal residues are removed, PAH shows a higher affinity and a consequent higher rate of L-Phe conversion [134].

L-Phe acts on PAH as a homotropic allosteric activator with a highly cooperative action which can be exerted only on the tetrameric form with extensive structural changes occurring at the tertiary/quaternary level [17, 131]. In fact, the substrate-mediated mechanism of activation involves all the four monomers, inducing modifications in the monomeric structures such that a stronger interaction at the dimer interface is promoted, whereas the interactions between dimers in a tetramer

are weakened. As a result, the dimer/tetramer equilibrium is shifted towards the tetrameric form of the enzyme upon binding of L-Phe [126], the volume of the tetramer increases and a competent catalytic site is exposed [17]. L-Phe binds in a specific allosteric site located in the regulatory domain [135], different from the active site of the catalytic domain, and the binding on one site do not automatically excludes the binding on the other one, although the affinity for the allosteric site is seven-fold higher [131, 136]. In fact, in each monomer the N-terminal tail stretches over the active site, thus preventing access for the substrate, unless L-Phe binds on the regulatory sequence and causes its displacement [124].

Besides being L-Phe-dependent, the rate of PAH activation by its substrate seems to be also pH-dependent, with less L-Phe necessary for activation at basic values of pH; at low L-Phe concentration the enzyme shows a low activity, in accordance to its allosteric regulatory mechanism [17].

If L-Phe is an allosteric activator of the enzyme, on the other hand its cofactor BH_4 acts as a classic allosteric inhibitor, which binds to the N-terminal autoregulatory sequence, thus preventing the conformational changes necessary for enzyme activation [137] (whereas the alterations induced upon L-Phe binding prevent BH_4 from exerting its negative effect on activity [138]) and moreover closing the access to the active site [139]. Nevertheless, BH_4 is necessary for the primary reduction of the Fe^{3+} ion to Fe^{2+}, an inevitable prerequisite for enzyme activation [136].

Another regulatory element is represented by the unique post-translational modification undergone by the protein, i.e. phosphorylation, which occurs on the Ser16 by cAMP-dependent PKA. Phosphorylation promotes the enzymatic transition from the steady state towards the active state, facilitating L-Phe access to the active site [126]; it is a reciprocal mechanism in that the substrate makes PAH more susceptible to phosphorylation by cAMP-PKA, which in turns sensitizes the enzyme towards allosteric activation by L-Phe by lowering the concentration of substrate needed for activation, but without changing its positive cooperativity [128-130].

Moreover, the phosphoric moiety disrupts the binding site of BH_4, therefore interfering with its inhibitory action [19]; on the other hand, BH_4 is a potent inhibitor of the enzyme in that it prevents the access to Ser16 for phosphorylation. This effect is compensated by L-Phe when L-Phe and BH_4 are simultaneously present [130]. Notwithstanding the main regulatory role exerted by both L-Phe and BH_4 and the more acute response to L-Phe in the presence of BH_4 [140], phosphorylation can also represent another way to modulate the hydroxylase activity. Shiman [141] demonstrated that both phosphorylated and unphosphorylated forms of the enzyme require L-Phe for activation; hence, phosphorylation does not equal allosteric activation, but lowers the energy needed for it to occur. This happens by means of two mechanisms: by promoting the transition to the active state of the protein, and by reducing BH_4 affinity for its inhibitory binding site. These aspects explain why *in vivo* phosphoPAH has a higher affinity for the substrate L-Phe, a higher activation rate and a lower

sensitivity to BH_4-mediated inhibition [19]. In Figure 6 is a schematic representation of the regulatory pathways in which PAH is involved.

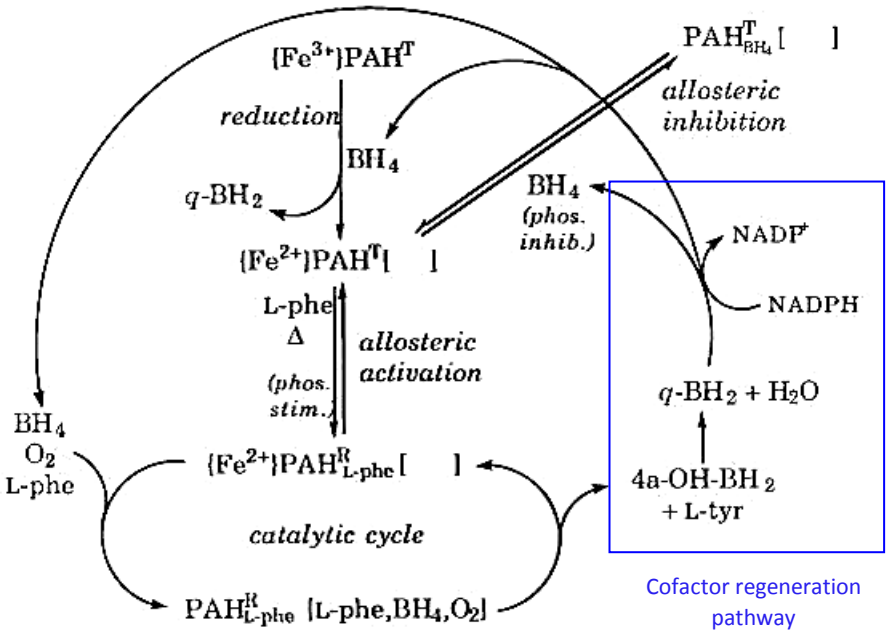


Figure 6. Regulatory pathways of PAH (from Kappock and Caradonna 1996). PAH^T = PAH in the steady state; PAH^R = PAH in the active state; the empty square brackets represent a substrate-binding pocket located in the active site.

4.3. PAH mutations

After Følling's discovery of the disease, many other studies followed and allowed the identification of the underlying metabolic block [142, 143]. The first PAH mutations could be detected after the gene cloning and mapping [116], thus paving the way for structure and functional analyses *in vitro*. The phenylalanine hydroxylase gene is characterized by great allelic diversity, as reported in the *PAH* variation database which currently contains 890 records of gene variants [PAHvdb, Blau N and Yue W, and Perez B, http://www.biopku.org/pah/]. To date, 567 mutations both pathologic and polymorphic have been discovered and are collected in a database named PAHdb created in 1996 (http://www.pahdb.mcgill.ca) [11, 144]. Of these, the most common are missense mutations (60.1%), followed by deletions (13.4%), splice alterations (10.9%) and silent or non-sense mutations (5.6% and 4.9% respectively) (http://www.pahdb.mcgill.ca/cgi-bin/pahdb/mutation_statistics-1.cgi); the final result is a total or partial lack of enzymatic activity that can result in different phenotypic severity. In fact, most patients are not homozygous for a single mutation; they are instead compound heterozygous for two different allelic alterations. Some patients, who are compound heterozygous, are phenotypically functional hemizygous, due to a combination of a severe mutation (such as a null one) with an allele that still allows the production of enzyme, even if only partially functioning: in those cases, the less severe mutation determines the PKU metabolic phenotype [145]. Together with the polymorphic nature of the phenylalanine hydroxylase gene, this is the main reason underlying the

great phenotypic diversity associated with the disease, and which, in addition, makes PKU very widespread in spite of its recessive inheritance pattern [146, 147].

While many of the most severe disease-causing mutations map to exon 7 which encodes part of the catalytic core of the enzyme [17], the most common (missense) alterations occur in the junction between the catalytic and tetramerization domains [124], making PKU a loss-of-function misfolding disease. By means of *in vitro* expression and site-directed mutagenesis, Gersting and colleagues [148] demonstrated how mutations located not only in the oligomerization domain but also in the catalytic and regulatory portions alter protein folding and assembly, with severe effects on allosteric regulation and proteolytic stability. Interestingly, the majority of these mutations did not reduce the catalytic activity below 50% of the wild type protein, strongly indicating that the loss of function is due to a mechanism of increased degradation. To this purpose, Pey et al. [149] proposed an effective model to analyze the destabilizing effect of missense mutations on the native protein's conformation, in order to predict the phenotypic outcome, and those results were then confirmed in the work by Gersting et al. [148].

The identification of the etiologic agent permitted the development of a treatment aimed at limiting the phenotypic effects of the disease: in fact, phenylketonuric patients show a different tolerance with respect to the daily amount of phenylalanine intake, and, on these basis, a dietary therapy has been proposed in the 1950s, with first positive results published in 1953 [150-152].

Indeed, phenylketonuria has often been defined as a disease born from the discordance between nature and nurture [10, 11], where the nurture component is the essential amino acid L-Phe and the nature is represented by the mutation in the *PAH* gene. The result of the discordance is hyperphenylalaninemia, the PKU metabolic phenotype, which leads and is associated with the clinical phenotype of impaired cognitive development and function. The possibility to act externally on the metabolic manifestation of the pathology makes PKU the first genetic disease to have a pharmacological treatment, thus smoothing the negative effects of gene alterations [10].

5. CLASSIFICATION

Given the multiple factors influencing the disease outcome, a classification of the phenotype can be made considering different aspects, first of which is the type and position of the PAH mutation, which determines the rate of enzymatic activity and consequently the level of hyperphenylalaninemia [34]. Hence, the classification is primarily made on the basis of the severity of HPA, considering that the normal L-Phe concentration in the blood of healthy individuals ranges from 50 to 110 μM [34]. Other criteria, i.e. tolerance to dietary phenylalanine intake, clinical course of the disease and BH_4 responsiveness, are also employed to describe the phenotype [34, 153, 154].

5.1. Classification according to blood L-Phe

This classification was introduced in 1980 by Güttler et al. [155] and defines the following phenotypes according to pre-treatment L-Phe levels:

- Classical PKU: pre-treatment L-Phe > 1200 μM
- Variant PKU: pre-treatment L-Phe between 600 and 1200 μM
- Mild HPA or non-PKU HPA: pre-treatment L-Phe between 120 and 600 μM

The class named "variant PKU" was later divided into two subcategories [145, 156], resulting in:

- Classical PKU: L-Phe > 1200 μM
- Moderate PKU: L-Phe between 900 and 1200 μM
- Mild PKU: L-Phe between 600 and 900 μM
- Mild HPA: L-Phe above 110 μM but < 600 μM

The amino acidic levels are currently used by approximately 80% of the PKU centers to identify the patients' phenotype, but this approach can lead to some mistaken evaluations, since blood L-Phe depends on some variables, such as the diet followed before L-Phe assessment, the timing of the analysis and the effect of the neonatal catabolism, which can result in false positive or false negative outcomes [153]. For example, the current practice of screening blood L-Phe in newborns within the third day of life can result in a negative conclusion, due to the fact that the amino acid might not have had time to reach its maximal concentration [34].
Recently, a new term was proposed to describe the intermediate range of values between the critical threshold of 360 μM and 600 μM (6-10 mg/dl); currently, there is still uncertainty about whether L-Phe concentrations comprised in such range may have a negative influence on cognitive and executive functioning, thus requiring treatment [157, 158]: this is the so-called "Mild-HyperPhe-gray zone" [159].

5.2. Classification according to tolerance to dietary L-Phe intake

L-Phe tolerance is defined as the amount of daily L-Phe a patient can introduce with a normal diet without experiencing an increase in blood amino acid levels above the upper limit of the recommended range (360 μM) [153]. This parameter has proven to be stable and reliable to the purpose of PKU phenotyping. The first classification proposed by Güttler et al. [155] included three classes:

- Classic PKU: L-Phe tolerance < 20 mg/kg body weight/day
- Variant PKU: L-Phe tolerance between 20 and 50 mg/kg body weight/day
- Mild HPA: L-Phe tolerance > 50 mg/kg body weight/day

Subsequently, a subdivision with four phenotypes has been adopted [145, 156]:

- Classic PKU: L-Phe tolerance < 20 mg/kg/day, corresponding to 250-300 mg L-Phe/day
- Moderate PKU: L-Phe tolerance of 20-25 mg/kg/day (350-400 mg/day)
- Mild PKU: L-Phe tolerance of 25-50 mg/kg/day (400-600 mg/day)
- Mild HPA: patients not requiring dietary restriction

According to the paper by Camp et al. [159], between mild HPA and mild PKU is the Mild-HyperPhe-gray zone, corresponding to a L-Phe tolerance > 50 mg/kg body weight/day.

The assessment of L-Phe tolerance must be performed under standardized conditions so as to obtain a reliable indication of the phenotype [153]. It is generally performed at the age of 5 years in the majority of the medical centres [145], but van Spronsen et al. [160] have demonstrated that reliable determinations can be made already at the age of 2 years and that L-Phe tolerance at 2, 3 and 5 years correlates well with that at the age of 10 years [160]. On the contrary, L-Phe tolerance must be reassessed in adulthood in relation to body weight in order to satisfy as much as possible the criterion of 9.1 mg L-Phe/kg ideal body weight/day [161]. This kind of classification is currently used by 70% of medical centers [162].

An additional classification based on BH_4-responsiveness has been proposed by Blau and Muntau [163] and consists in BH_4-non-responsive HPA and BH_4-responsive HPA, the latter being further divided into BH_4-responsive PAH deficiency and HPA due to defects in the BH_4 pathway.

5.3. Classification according to clinical course

The parameters to evaluate the clinical course include the intellectual outcome, in terms of patient education and IQ, the maximum L-Phe concentration reached in particular conditions or periods of life (such as non compliance to the restricted diet or the occurrence of infectious diseases) and, most importantly, the variations in blood L-Phe levels and the phenylalanine-to-tyrosine (L-Phe/L-Tyr) ratio [27, 164, 165]. The latter two parameters have been demonstrated to influence the cognitive outcome to a large extent, larger than blood L-Phe level itself, and therefore their determination may allow a simplified classification of the phenotypes according to the need of treatment:

- PKU: patients who need a strict dietary control of L-Phe levels
- Non-PKU HPA: patient who do not need any dietary treatment to keep L-Phe under control
- BH_4-responsive PKU: patients who may take advantage from BH_4 supplementation.

6. DIAGNOSIS

The identification of PKU or HPA should be performed as early as possible, so as to introduce an opportune treatment and thus limit, if not completely avoid, all the neurological and metabolic consequences of a too high uncontrolled hyperphenylalaninemia [34, 153]. For this reason, now all

newborns are routinely tested for PKU/HPA soon after birth according to national screening programs [166]. Blood samples are drawn for analysis between the 2nd and the 5th day of life in most centers [167]. Today blood sampling is considered suitable for analysis within 24h-48h from birth when L-Phe/L-Tyr ratio is employed for the diagnosis [168].

Usually, affected babies born from healthy mothers do not display physical alterations, except for a tendency to a reduced body weight and head circumference [169]. Therefore, diagnosis mainly consists in the biochemical assessment of blood L-Phe and L-Tyr, biopterin and neopterin content in blood or urine, and the measurement of specific enzyme activities [153]. All forms of the disease reveal upon neonatal screening a common pattern of blood L-Phe higher than 120 μM, normal or reduced L-Tyr concentrations (with a L-Phe/L-Tyr ratio > 2) and normal values for the remaining amino acids [34]. The analytical methods employed to assess blood L-Phe include:

- Guthrie's test or bacteriological inhibition assay (BIA): first proposed in 1963 by Robert Guthrie [6], it is based on the inhibition of *Bacillus subtilis*, which requires L-Phe for its growth. A small amount of peripheral blood is drawn from the patient (usually from the heel) and collected on a standardized filter paper, the so-called "Guthrie's card". The dried blood spot (DBS) obtained is then submitted to the analysis. This test was introduced for mass screening and nowadays is being increasingly replaced by more modern techniques (e.g. tandem mass spectrometry) characterized by improved precision, sensitivity, practicability, and faster time of analysis;
- Fluorimetric assay: a simplified and automated method yielding a lower rate of false positive results compared to the Guthrie's test [170, 171];
- Analysis by reverse-phase liquid cromatography [172, 173];
- Enzymatic colorimetric assay: based on the reaction catalyzed by phenylalanine dehydrogenase, it was employed to detect L-Phe in plasma samples [174, 175];
- Tandem mass spectrometry (TMS): this procedure is able to simultaneously identify even small amounts of amino acids (L-Phe and L-Tyr) in dried blood spots collected on Guthrie's cards, providing the L-Phe/L-Tyr ratios and yielding a low rate of false positive results [176-179].
- *PAH* locus sequencing: the genetic characterization allows the detection of all the subjects bearing a mutation in one or both *PAH* alleles with a high degree of certainty, also revealing the number and nature of the alterations and thus permitting to evaluate the potential residual enzymatic activity. This approach is particularly useful during prenatal screenings to identify healthy carriers and to recognize those genotypes resulting in a milder phenotype, also presenting a higher probability of BH_4-responsiveness [153].

The early determination of blood L-Phe levels if on the one hand allows the early introduction of the opportune treatment, on the other hand may present some problems regarding the certainty of

patient identification. In newborns, the liver enzymes involved in amino acid metabolism might not have reached complete maturity, especially in pre-term babies, who therefore display a transient hyperphenylalaninemia that is detected as pathological and represents a false positive outcome. Moreover, babies fed cow's milk experience a protein overload that can result in positive tests; false positives may also origin from an improper preparation of the sample or a too thick blood spot, or a combination of two or more of these factors [15], whereas false negatives may origin from analyses performed in extraordinary conditions, such as sickness, parenteral nutrition or blood transfusions [153]. Temporarily higher levels of L-Phe may be due to possible heterozygosity for PAH deficiency [180], as well as to maternal PKU or other non-PKU disorders; for all these reasons, the test on dried blood spot must be repeated a second time in order to confirm the first result [3]. Analysis of L-Phe metabolites (mainly pterin compounds) in urine is not accepted as a diagnostic tool on its own, since their levels vary considerably between blood and urine, and excretion greatly depends upon transaminase activity, which might be reduced in newborns [181].

6.1. Differential diagnosis

Once hyperphenylalaninemia has been detected, it is necessary to distinguish, among the different forms, those originating from disorders of BH_4 metabolism, which are responsible for about 2-3% of the reported cases of HPA [9, 153, 182]. The differential diagnosis involves the measurement of urinary neopterin and biopterin, as well as the activity of the enzymes of BH_4 metabolism in blood, with particular attention to DHPR [9, 15, 153, 183, 184]. Quantification of folates and neurotransmitter metabolites 5-hydroxyindoleacetic acid and homovanillic acid (deriving from serotonin and dopamine respectively) in the cerebrospinal fluid, together with a *BH_4 loading test*, provide further information on the disease, thus enabling a correct differentiation among the various severity forms of PAH or BH_4 deficiency [183-185]. All the determinations requiring blood samples can be performed on a single dried blood spot by means of tandem mass spectrometry [179, 183].

Nowadays, the so-called *BH_4 loading test* is integral part of the neonatal screening tests. It was initially performed to distinguish the cases of HPA due to BH_4 deficiency from those caused by PAH defects, but now it is also employed to identify patients affected by PKU variants responsive to BH_4 supplementation [186, 187]. In Figure 7 is reported the flow-chart commonly followed to perform the differential diagnosis of PKU or BH_4 deficiencies, once HPA has been detected.

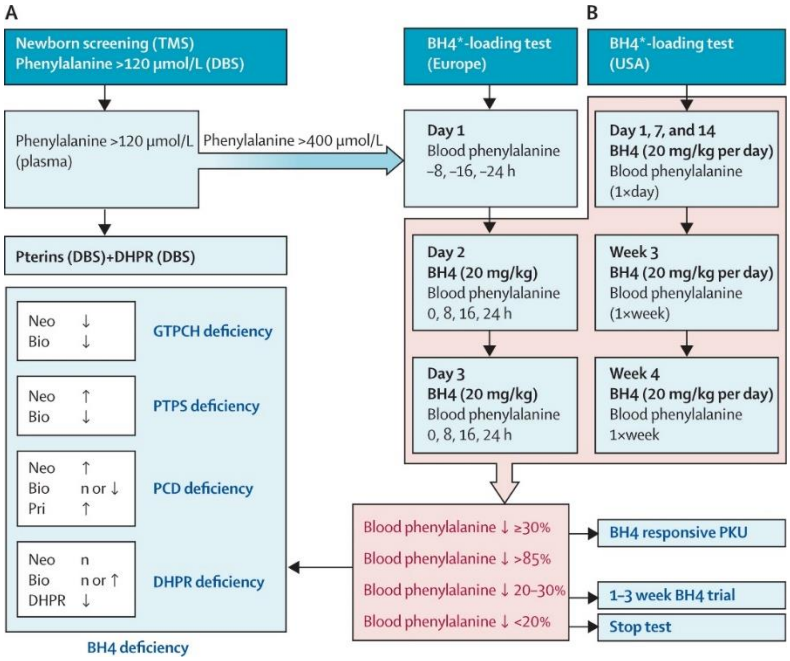


Figure 7. A) Diagnostic flow-chart employed to distinguish the different disorders of PAH and BH_4 metabolism that can result in hyperphenylalaninemia. **B)** BH_4 loading test protocols in Europe [according to Blau et al. 2009] and USA [according to Levy et al. 2007]. DBS = Dried blood spot; n = normal; Neo = neopterin; Bio = biopterin; Pri = primapterin.*BH_4 can be either tetrahydrobiopterin or sapropterin (Kuvan®) (From Blau et al. 2010 [34]).

6.2. The BH_4 loading test

Many of the PAH mutations catalogued in the BIOPKU database, are responsible for mild PKU or mild HPA phenotypes: this means that the protein originating from those altered sequences still retains partial hydroxylating activity that can be stimulated by BH_4 supplementation, with a consequent reduction in measured L-Phe levels. The first observations of such response date back to 1999 [154] and patients with up to 800 µM L-Phe are most likely to respond to the treatment [190, 191].

There is lack of consensus as regards the definition of BH_4 responsiveness, since various criteria can be considered for definition. A dose of 10 mg BH_4/kg body weight (BW) has been used for the analysis [192], otherwise 20 mg BH_4/kg BW [193] or a combination of 20 mg BH_4/kg BW and 100 mg L-Phe/kg BW has also been employed to challenge patients over a period of 24 hours [194, 195]. Sometimes variations exceeding normal individual variability in L-Phe levels have also been considered as a criterion [193] as well as a 1- or 2-fold increase in patient tolerance to daily L-Phe intake [34].

The most widely accepted definition of BH_4 responsiveness consists in a blood L-Phe reduction of at least 30% of the pre-treatment value upon a single administration of 20 mg BH_4/kg body weight [163, 190, 191]. A reduction ≥ 20% may also be considered of clinical relevance [189].

Test procedures for BH_4 responsiveness are different between Europe and USA [34] and there are many protocols, differing from one another as for timing and dose administration and total duration of the analysis, with longer procedures being more common in the USA [189]. The “24 h protocol” commonly employed in European centers [163] consists in the administration *per os* of 20 mg BH_4/kg

body weight and in the subsequent blood L-Phe and pterin measurement at 0, 4, 8 and 24 hours after treatment. Since the response is dose- and time-dependent, some patients may not be identified by this test, if it takes them a longer time to show a sufficient effect on L-Phe; therefore an extended version of the test has been proposed, the "48 h loading test", with a second administration of 20 mg BH_4/kg BW 24 hours after the first one, followed by additional L-Phe assessment at 32 and 48 h [196, 188]. When a protocol involving L-Phe loading is selected, administration of ^{13}C-L-Phe enables subsequent evaluations to be performed non-invasively on patient breath (Phenylalanine breath test); in this case, the amount of exhaled $^{13}CO_2$ is measured, obtaining indications on PAH activity and on the overall condition of the L-Phe catabolic pathway of the organism (L-Phe oxidation capacity), which is also expected to reflect the clinical phenotype [194, 195, 197].

On the basis of the percentage reduction in blood L-Phe, patients can be divided as follows:

- ✓ Rapid responders: patients experiencing a decrease in L-Phe ≥ 30% after 8 h and ≥ 50% by 24 h from BH_4 administration;
- ✓ Moderate responders: patients showing a decrease rate ≥ 20% after 8 h, ≥ 30% after 24 h and ≥ 50% after 48 h;
- ✓ Slow responders: patients whose plasma L-Phe levels decrease of ≥ 20% by 24 h and ≥ 30% after 48 h.

When L-Phe reduction overcomes 85% upon BH_4 treatment, the condition is identified as BH_4 deficiency [34].The *BH_4 loading test* should be performed early after birth and before the introduction of the low L-Phe diet, so as blood L-Phe variations upon BH_4 treatment are more evident. Blood L-Phe must be over 400 μM, otherwise older patient who are already on dietary regimen must increase the protein intake before and during the testing period, or are submitted to a concomitant phenylalanine load, consisting in a single administration of 100 mg L-Phe/kg BW [34, 198]. Although the test is effective at all ages, its sensitivity in newborns has been questioned due to liver immaturity and to the fact that only 24 h protocols can be employed at this age [199]. Performing the analysis early allows the early introduction of the restricted diet in non-responders, but at the same time, implies the possibility to miss slow responders (who are mistakenly considered negative to the test). Therefore, it is advised to repeat the analysis according to longer protocols after 3 months of life, that is when the liver has reached complete maturity and longer testing protocols can be applied [199].

7. THERAPEUTIC APPROACHES TO PKU

PKU is the first genetic disease to have an effective treatment, though not decisive. The main aim of all available therapeutic approaches is to restore at least near-physiological levels of circulating L-Phe and L-Tyr, so as to remove as much as possible the biochemical and neurological consequences

resulting from their imbalance. There is great lack of consensus among medical centers worldwide as regards the threshold value beyond which treatment must be introduced. The most common concentrations employed to this purpose are 360 μM, 400 μM and 600 μM [34] and the range of L-Phe concentrations considered safe is between 120 and 360 μM, at least until 12 years of age [106, 200], with the upper limits rising up to 900 μM after the 12th year of age [106]. However, there is great inconsistency about the target range to be reached in adolescence and adulthood, resulting in a wide spectrum of disease management and outcomes [201, 202].

7.1. L-Phe restricted diet

The chief treatment for the disease is a low-phenylalanine diet, introduced for the first time in the early 1950s [151, 152] and still remaining the mainstay of the available approaches. Dietary treatment must be adopted as soon as possible after birth [34]. Since genotype is not always a reliable predictor of the patient phenotype, diet should be individually tailored in order to reach the target concentration, with particular attention to the specific needs of every age and condition [15].

Patients must exclude foods rich in protein [15, 34] and pay particular attention to those containing the artificial sweetener aspartame (L-aspartyl-L-Phe methyl esther) which releases L-Phe, L-aspartic acid and methanol when metabolized [3]. Low-Phe containing natural foods such as potatoes and most vegetables can be consumed only in small amounts, whereas low-protein versions of some foods (i.e. bread and pasta) also exist [34]. The foods allowed in this approach make the diet appear as a medically prescribed vegetarian or vegan diet, but it implies even more food restrictions [203]. This regimen alone does not enable the achievement of the target L-Phe level, as well as sufficient protein and energy intake; hence, it must be integrated with commercially available phenylalanine-free medical foods and protein substitutes [15, 34]. Moreover, the diet must be carefully monitored, so as to adapt it to individual L-Phe tolerance, age and growth requirement, illnesses, physical activity and pregnancy in females [15, 109]. Breastfeeding is encouraged in infants in combination with the medical formula [34]; the strict dependence (approximately 85% [204]) of the required protein intake on semi-synthetic formulas is likely to introduce many nutritional deficiencies, involving also L-Phe and L-Tyr [15]. Even too low concentrations of L-Phe (< 30 μM) must be avoided in order not to impair development [205, 206], but levels between 60 and 120 μM should not be regarded as too low especially in patients with more relaxed adherence to diet [109]. Two systematic reviews by Demirkol [203] and Singh [204] report that the most frequent nutrient deficiencies experienced by PKU patients on-diet concern essential long-chain polyunsaturated fatty acids (particularly arachidonic acid and docosahexanoic acid, DHA) and micronutrients, such as minerals (zinc, copper, manganese, selenium, calcium, iron) and vitamins (A, C, E, B_2, B_6, B_{12}, D), as well as other metabolically important compounds (i.e. CoQ10,

carnitine) [207]. Tyrosine supplementation of medical formulas is also common to ensure its correct supply in the absence of L-Phe, but its actual efficacy has not been demonstrated [208].

Despite in the past many centers allowed for diet relaxation after adolescence, today there is controversy about the concentration range to be maintained in adulthood; on the whole, there is general agreement on the need for a "diet-for-life" approach, based on the observation that if mental disability does not occur when patients have been well controlled during infancy, many adverse manifestations can develop upon diet relaxation [209]. The closer L-Phe is to normal values, the better the overall individual well-being [15, 106, 203, 210].

During infancy compliance to diet is achieved thanks to the fact that the disease management is completed demanded to parents [34]; however, as children grow up the risk of diet relaxation or even discontinuation becomes very high. Adolescents face the normal challenges provided by social environment, with increased desire for independence, social acceptance and rebellious behaviour that can ultimately lead to loss of metabolic control and of all the benefits gained during early treatment [109, 211]. Many factors negatively influence adherence to diet: the difficulty of the diet itself which is usually poorly palatable, the psychological and emotional burden implied for patients and families, the high cost of the medical formulations, together with the lack of knowledge of the disease and follow-up by medical centers have been reported as common reasons for poor treatment compliance [34].

Current efforts are focused on the development of a dietary treatment which ultimately results not only in L-Phe control but also in preservation of the patients' quality of life [109].

7.2. Glycomacropeptide (GMP)

Glycomacropeptide (GMP) is a natural protein deriving from cheese whey, rich in essential amino acids but devoid of phenylalanine, tyrosine and tryptophan [212]. When it is used on its own it must be integrated with the lacking essential amino acids Tyr, Trp, His and Leu, but it can also be added to other foods with the additional effect of ameliorating the palatability and, indirectly, the diet compliance [213, 214].

7.3. Large neutral amino acids (LNAAs)

This class of amino acids includes tyrosine, tryptophan, threonine, methionine, valine, isoleucine, leucine and histidine [215]. All of them are essential except for tyrosine which, however, becomes so in PKU patients. The rationale underlying this approach is to reduce L-Phe absorption in the intestine and brain uptake by exploiting the competition between L-Phe and the other LNAAs for the transporter LAT-1, present both in the gut and in the blood-brain barrier [34, 216-218].

Such approach has in addition the advantage of increasing brain availability of the amino acidic precursors of neurotransmitters, thus being also helpful for the treatment of the disorders of BH_4 metabolism [3]. Treatment with LNAAs is currently limited to adolescent and adult patients and is not advised during pregnancy, due to limited knowledge on the effect on fetal development [109].

7.4. Supplementation with BH_4

Many mild PKU genotypes have proven to respond to BH_4 supplementation. An explanation is that these patients have mutations allowing a sufficient residual PAH activity to be maintained. Pharmacological doses commonly ranging from 5 to 20 mg BH_4/kg BW/day are able to stimulate PAH to an extent that is sufficient to reach the target range of circulating L-Phe [154, 162; for a review of clinical studies see Ref. 219]. The mechanism underlying BH_4-responsiveness is multifactorial [190], being determined by phenomena such as negative allelic complementation which makes a genotype result in a phenotype different from what expected [189, 220]; the main action operated by BH_4 is the stabilization of the molecular structure, thus preventing misfolding, subunit aggregation, thermal inactivation and proteolytic degradation, therefore functioning as an actual molecular chaperone [70, 148, 221]. BH_4 supplementation has also been used in disorders involving BH_4 metabolism, but many patients respond to this treatment even if they do not present with alterations of the cofactor metabolic enzymes [154, 189].

Two pharmacological formulations of BH_4 have been developed: 6R-BH_4 dihydrochloride (Schircks Laboratories, Jona, Switzerland) and sapropterin ((6R)-2-amino-6-[(1R, 2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydro-4(3H)-pteridinone) dihydrochloride, (BioMarin Pharmaceutical Inc, Novato, CA), either of which have also been clinically tested with positive results and few adverse events of mild severity [189, 222, 223; Clinicaltrial.gov website]. To date, of the two formulations only sapropterin dihydrochloride from BioMarin Pharmaceutical has obtained the authorization for therapeutic use by the US Food and Drug Administration, the European Medicine Agency and the Japanese Pharmaceutical and Medical Devices Agency with the commercial name of Kuvan® [34].

The main advantages deriving from the treatment with the cofactor in PAH-deficient patients is represented by an increase in L-Phe tolerance, which enables people to introduce even substantially larger amounts of natural proteins in their diet with a consequent upgrade in their quality of life [109]. An improvement in neuropsychiatric symptoms or an increase in L-Phe tolerance with or without a decrease in blood L-Phe levels are always considered sufficiently relevant effects to justify treatment continuation [109]. The disadvantages of the therapeutic approach with sapropterin concern its half-life (about 6.5 hours) and its rapid elimination with the urine when administered orally [224]. Another major limitation is represented by the high costs of the daily therapy with high doses in comparison with the costs of the dietary regimen, which are also high [69].

7.5. Gene therapy

The general aim of this approach is to restore PAH activity by administering a correct copy of the mutated coding gene to the affected organ. PAH cDNA is obtained and included in a vector which is then directed to the liver or muscle, where it directs the production of the native protein. First attempts were made in the 1980s on PAH-deficient mice and led to the discovery that even only 10% total activity is sufficient to yield a complete normalization of L-Phe [225, 226]. Since then many other attempts have been made [227-229]. Gene transfer has been accomplished by means of both non-viral and viral vectors, the latter yielding better results in terms of transfer efficiency and expression stability [230]. Vectors deriving from retroviruses, adenoviruses and most recently adeno-associated viruses have been exploited. Adenoviral vectors are able to infect a wide range of host cells with an extremely high efficiency of transduction, and they also infect non-dividing cells, but the effect in this case lasts only a few weeks due to the episomal mechanism of viral replication, which does not include DNA integration in the host cell. The immune response elicited by the expression of viral proteins produces neutralizing antibodies, thus preventing subsequent treatments from being effective [230, 231]. Retroviral vectors allow a prolonged expression of the gene by integrating it in the cell genome, but they proved to cause leukemia-like disorders and therefore have been completely abandoned [230, 231]. Adeno-associated viruses are currently the safest vectors available for gene transfer because they elicit minimal immune response while allowing prolonged gene expression without DNA integration [232]. When targeted to the liver as in the case for PKU, the main drawback is that a little cell turnover exists, and the acquired gene copies are lost as hepatocytes divide. This has led to switching the target to the skeletal muscle that is characterized by a lower regeneration rate and is more easily accessible [233]. In this case, however, it is necessary to bring also the complete set of cDNAs of the enzymes involved in BH_4 synthesis, since the muscle does not produce this cofactor [228]. If the researchers manage to develop a sufficiently safe gene therapy approach, this will represent a really revolution for PKU patients, since it would enable complete discontinuation of the dietary regimen, with great improvements of the quality of life.

7.6. Cell therapy

Liver transplantation would be a definitive approach to restore the normal PAH activity. As an alternative to whole organ transplantation, liver repopulation with donor-derived wild-type hepatocytes could be more feasible. This approach exploits the natural liver capability to regenerate after a damage, but to be effective the donor-derived cells should enjoy a selective growth advantage over native hepatocytes [234]. Both transplantation and liver repopulation would require life-long treatment with immunosuppressant to contrast the host rejection. Potential solutions to the need for chronic immunosuppression might be represented by hepatocytes deriving from embryonic stem cells

or from autologous adult stem cells genetically modified to produce PAH [235]. Preclinical studies have been carried out to test this therapeutic strategy, which proved to be effective in restoring L-Phe levels when at least 10% PAH-deficient hepatocytes were replaced by wild-type cells [236]. Despite being a viable option, the application of therapeutic liver repopulation to humans is mainly limited by the scarce availability of sources of wild-type human hepatocytes, on which this approach depends [235]. Another future potential alternative that can be considered half-way between gene therapy and cell therapy would be to extract hepatocytes or even precursor stem cells from PKU patients, treat them *ex vivo* by permanently introducing a normal copy of the PAH cDNA, reimplant the modified cells into the patient and then allow them to repopulate the liver.

7.7. Enzyme replacement or substitution therapy

Given the several difficulties of adherence to the strictly low L-Phe dietary regimen, and the non-responsiveness to BH_4 treatment by patients suffering from the most severe forms of PKU (but also by some of the non-PKU HPA patients), there has been increasing interest in the enzyme therapy, whose action is not dependent on the patient genotype.

Enzyme therapy can be done either as a replacement therapy (ERT) or a substitution therapy (EST), depending on whether PAH or another L-Phe-metabolizing enzyme is used. Employment of human PAH, which would theoretically be the first choice, presents multiple difficulties, i.e. the protein's intrinsic instability that prevents large-scale isolation and purification, its need for the BH_4 cofactor, the complex enzymatic activity due to the protein tetrameric structure in addition to susceptibility to proteolytic degradation. The last but not the least factor making its utilization problematic is represented by its potential immunogenicity when administered to people lacking the native enzyme [237]. Attempts have been made to stabilize the protein by truncating it or introducing chemical modifications (i.e. PEGylation) to protect the enzyme from the host immune system [238].

A better strategy is provided by the use of another L-Phe-metabolizing enzyme, Phenylalanine Ammonia Lyase (PAL, E.C. 4.3.1.24) as an enzyme substitution therapy [239]. This enzyme has been thoroughly studied and has entered clinical investigations, after having been conveniently modified to overcome stability and immunogenicity issues [ClinicalTrials.gov, NCT00634660].

7.7.1. *Phenylalanine Ammonia Lyase*

Phenylalanine ammonia lyase (PAL, E.C. 4.3.1.24) is an autocatalytic protein of plant and bacterial origin. In plants PAL is involved in the anabolic pathways leading to the formation of various polyphenyl compounds [240], whereas in bacteria it catalyzes the non-oxidative deamination of L-Phe to *trans*-cinnamic acid and trace amounts of ammonia (Figure 8). *Trans*-cinnamate in humans is converted in the liver into benzoic acid and then excreted in the urine as hippurate [241]. The estimated amount of approximately 3 g of *trans*-cinnamate that would be produced daily by PAL-mediated conversion of

dietary L-Phe intake is thought to be harmless to the organism, as well as the ammonia produced is metabolically insignificant [242]. *In vivo* studies on mice also demonstrated no embrytoxic effect of *trans*-cinnamate [241].

PAL enzyme has been isolated and biochemically characterized from different bacterial sources [243]. To the purposes of enzyme substitution therapy, phenylalanine ammonia lyase extracted from the blue-green algae *Anabaena variabilis* proved to be the one with the best characteristics in terms of catalytic activity (K_m for L-Phe 0.045 mM) and protein stability [239, 243, 244].

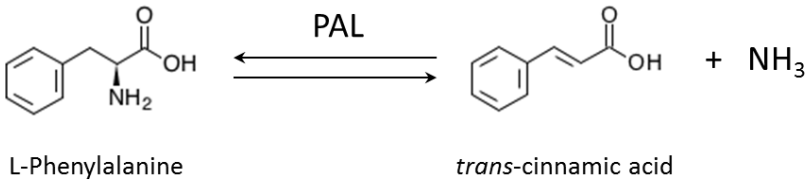


Figure 8. The reaction catalyzed by phenylalanine ammonia lyase (PAL).

PAL from *Anabaena variabilis* (*Av*PAL, Figure 9) is a homotetrameric protein (each monomer being 64 kDa) composed of 567 amino acids; its catalytic activity requires the electrophilic prosthetic group 4-methylideneimidazole-5-one (MIO), which is formed autocatalytically by cyclization and dehydration of three conserved residues, Ala167-Ser168-Gly169 [243]. The enzyme does not require any external additional cofactor.

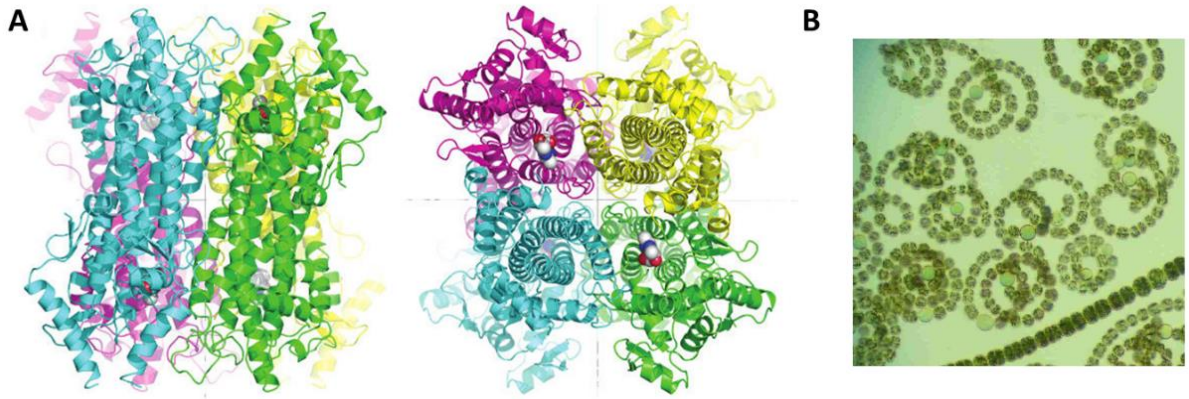


Figure 9. A) Side view and top view of the crystal structure of wild-type phenylalanine ammonia lyase from *Anabaena variabilis* (from Moffitt et al. 2007 [243]); the spheres represent the atoms of the four MIO prosthetic groups. **B)** *Anabaena variabilis.*

The use of PAL in enzyme substitution therapies presents some advantages over PAH: it is structurally and metabolically more simple, not requiring the BH_4 or any other cofactor to the reaction; it is stable in a wide range of temperatures [245], more resistant to proteolytic degradation and less susceptible to aggregation [239].

The first attempts to employ PAL in an enzyme substitution therapy approach for PKU have been done in the early 1980s [241, 242, 246]. PAL has been administered in various routes, both enterally and

parenterally. The oral administration of PAL inside enteric-coated gelatin capsules was a viable and effective option, able to reduce blood L-Phe levels [246]. Giving PAL orally as itself was ineffective due to intestinal proteolysis and sensitivity to the acidic pH of the upper gastrointestinal tract [245]. Different strategies to protect the enzyme from protease-mediated degradation have been developed, such as immobilization on artificial cells [247] or encapsulation inside silk fibroin fibers [248]. The high costs of PAL production at those times led to the abandonment of all these techniques [237], moreover such solutions were all applicable only as supporting therapies to the dietary treatment in patients with the milder forms of the disease, since they were not able alone to guarantee a sufficient L-Phe reduction [245, 247]. The parenteral administration of PAL via endovenous injection elicited a great host immune response whereas the protein showed a short half-life in circulation [249]; the application of an external PAL-containing reactor demonstrated to overcome the immunity issue while depleting L-Phe, but it was not a long-term viable solution [250]. The need for life-long therapy of PKU patients would require the development of an oral formulation of the therapeutic enzyme [251], but unfortunately the current preotease-protected forms show a lower specific activity, meaning that a larger amount of enzyme is needed to lower blood L-Phe levels, and moreover they require a longer time of contact with the substrate during the passage through the gut [237]. For all these reasons, other administration routes, such as subcutaneous injections, are currently more feasible.

Whatever administration route is considered, there is need to protect the enzyme from proteolytic degradation and to reduce its immunogenicity. DNA engineering techniques have made it possible to isolate and express the recombinant form of PAL, as well as the introduction of specific alterations in the amino acidic sequence by means of site-specific mutagenesis, so as to mask the cleavage sites recognized by human proteases and allow an oral delivery of the enzyme [251].

Both cleavage and antigenic sites can also be altered by chemical derivatization with other molecules, particularly polyethylenglycole (PEG). PEGylation occurs on superficial lysine residues and is directed to those residues which are known to be part of antigenic portions of the molecule or to cleavage sites. PEG binding on these sites produces an increase in half-life and duration of activity of the enzyme, and reduces the activation of the host immune system [252, 253]. Site-specific mutagenesis and PEG derivatization studies have been performed [254, 255]. Currently the best results have been obtained with C503S/C565S double mutant recombinant AvPAL, modified with PEG; in this variant the double mutation showed no significant alteration of the catalytic activity while reducing aggregation upon purification [255], while the PEG molecules allowed the masking of antigenic epitopes [253].

This double-mutant rAvPAL-PEG has been produced by BioMarin Pharmaceutical Inc. (Novato, CA), and after preclinical studies [244], it is currently under investigation in clinical trials in which it is administered subcutaneously. Results from the phase I trial (ClinicalTrials.gov ID: NCT00634660) have been recently published [256] and show a marked dose-dependent reduction in blood L-Phe levels

after a single s.c. injection. The treatment was generally fairly well tolerated, however patients experienced some adverse events of mild intensity classified as injection-site reactions, and all participants developed anti-PEG antibodies by the end of the study. Phase II clinical trials to test safety, efficacy and tolerability of multiple administrations of rAvPAL-PEG have already been completed (ClinicalTrials.gov ID: NCT01212744, NCT00925054, NCT01560286, NCT00924703) while phase III trials (including a substudy to evaluate the executive functioning of treated patients) are currently ongoing (ClinicalTrials.gov ID: NCT01819727, NCT01889862, NCT02468570).

Despite the positive results obtained with rAvPAL-PEG, adverse injection-site reactions and most importantly the production of anti-PEG antibodies still represent a major concern; in fact, PEG is widespread in the environment and commonly used in many pharmaceutical formulations, meaning that PKU patients might have already been made sensitive to this compound [256, 257], thus enhancing the potential immune reaction, especially if considering the need for life-long treatment of PKU patients with repeated administrations of the enzyme. The high costs of some PEGylation procedures and the potential enzymatic alterations negatively affecting the catalytic activity are other reported drawbacks of this approach [258].

The application of an opportune delivery system able to carry the enzyme while providing protection form proteolytic degradation and from immune inactivation could be an effective alternative. Many drugs have successfully been administered through different delivery systems, thus overcoming many of the previous limitations [259].

8. DRUG DELIVERY SYSTEMS: ERYTHROCYTES AS THE BEST CHOICE

The most common limitations to the efficacy of drugs are represented by the premature degradation, inactivation or elimination from the body, as well as the potential undesirable immune response elicited by the external agent. Drug bioavailability in the desired site of action at the proper concentration, along with possible interactions with other plasmatic proteins are also additional major issues limiting the systemic use of many drugs. These concerns have led to an increasing interest in drug delivery systems able to target the therapeutic agents as much uniquely as possible to the desired site of action and with minimal side effects [260, 261].

To this purpose many carriers have been proposed, either simple soluble molecules, such as for example monoclonal antibodies and biodegradable polymers, or more complex structures, such as microcapsules and particles, cells, liposomes and erythrocytes [260, 261]. Among all possibilities, erythrocytes (red blood cells, RBCs) proved to be so far the best delivery system currently available, in that they display unique characteristics which make them ideal carriers for a potentially infinite number of pharmaceutical compounds. Such qualities include [260-267]:

- Biocompatibility, especially if autologous erythrocytes are used;

- Biodegradability, without formation of toxic by-products;
- Rapid availability in large amounts (the human body normally possess 2-3 x 10^{13} RBCs continuously produced at a rate of 2 million per second);
- A long *in vivo* life-span of 100-120 days, the longest among delivery systems, which is transferred to the encapsulated agent;
- An average cellular volume of approximately 90 fl being mostly available for large amounts of drugs to be encapsulated, since mature RBCs lack nucleus and organelles;
- The biconcave shape, enabling great flexibility and membrane deformability which make RBCs able to travel across small undamaged capillaries (until 2-3 μm in diameter) avoiding unwanted extravasation;
- Ease of *ex vivo* handling by means of several already existing procedures allowing the reversible opening of pores on the cell membrane (from 10 nm up to 500 nm diameter) by exploiting RBC ability to behave like an osmometer, shrinking or swelling according to the salt content of the external medium, thus permitting many non-diffusible large compounds (such as proteins and peptides) to be loaded inside the cell, maintaining morphological, biochemical and immunological properties similar to those of native cells;
- Ability to protect the encapsulated agent from the premature degradation or inactivation by immune reactions, since it is a completely natural carrier, not recognized as non-self by the host immune system (in contrast with most of the other delivery systems);
- Possibility to modulate the drug pharmacokinetics, thus enabling a sustained release of the therapeutic agent in circulation while providing protection to the organism from the potential negative effects of peak concentrations of drugs;
- Possibility to use erythrocytes not only as passive carriers but also as actual bioreactors thanks to the presence of the intrinsic pool of enzymes able to process entrapped pro-drugs and to convert them into active drugs [268];
- Possibility to selectively target macrophage cells and the reticulo-endothelial system (RES) by exploiting the natural mode of macrophage-mediated erythrocyte removal from circulation, thus eliminating drug toxicity to other body districts [269, 270].

However, like all therapeutic agents, erythrocytes as drug delivery system show some drawbacks [266]: the macrophage-mediated removal of senescent or damaged RBCs can be an undesired side-effect, shortening the half-life of the encapsulated drug; some molecules may alter RBC physiology or rapidly leak from them, thus being lost in the bloodstream; given their natural origin, erythrocytes present an intrinsic variability that may lead to different rates of success in the loading procedures; in addition, being erythrocytes viable cells, they need special attention during storage so as not to compromise their action once reinfused *in vivo*; many strategies have been adopted to overcome this problem, such

as isotonic buffers containing essential nutrients and low temperatures. Finally, a major concern is the potential contamination due to blood origin, the loading equipment used and the environment where the procedure is carried out; strict controls are therefore needed to guarantee a correct collection and handling of the erythrocytes, as well as the safety of the final product.

The above mentioned positive features still overcome the negative drawbacks and erythrocytes as drug carriers have been studied since the mid-1970s [271] and have been employed for different purposes, as extensively reviewed by many Authors [260, 261, 266, 272-274].

Different protocols, based on either physical or chemical techniques, have been studied to load various substances into RBCs [264, 275]. The most common procedures are based on osmosis and include hypotonic hemolysis [276], hypotonic dilution [277, 278], hypotonic dialysis [263, 278, 279] and hypotonic preswelling [280]. Briefly, all of them consist in causing erythrocytes to swell by putting them into a hypotonic medium; after a period of equilibration with a solution of the molecule to be entrapped, RBCs are washed and resealed by adding a hypertonic solution, which restores the physiologic isotonic environment thus permitting closing of pores and membrane reannealing.

Many different agents including antiviral and antineoplastic drugs, therapeutic proteins and peptides (i.e. vaccines and enzymes), cytokines, oligosaccharides, nucleic acids, anti-inflammatory drugs (like the glucocorticoid dexamethasone, DEXA) and contrasting agents for diagnostic purposes, have been efficiently delivered by RBCs [261, 263, 272]. As an example, many clinical studies have been performed with RBCs loaded with DEXA-21-phosphate as prodrug of DEXA in the treatment of various inflammatory diseases, without manifestation of negative side effects attributable to long-term administration of glucocorticoids [281-284 ClinicalTrials.gov ID NCT01171807].

8.1. Carrier erythrocytes in enzyme substitution therapies

This approach has also been successfully employed in *in vivo* studies as enzymatic therapy for many diseases. The first attempt was made by Beutler and colleagues [285] who aimed at treating Gaucher's disease by means of erythrocytes loaded with glucocerebrosidase. Up to now, RBCs loaded with L-asparaginase have been employed in clinical trials (ClinicalTrials.gov ID NCT01523782) to remove L-asparagine, a non-essential amino acid necessary for lymphoblastic proliferation in acute lymphoblastic leukemia (ALL) [279, 286-288]; adenosine deaminase (ADA) is another therapeutic enzyme encapsulated both in its native and PEGylateg form for the treatment of ADA deficiency [289-292]. Thymidine phosphorylase has been studied in animal models and also in patients as enzyme replacement therapy for the rare disease MNGIE (mitochondrial neurogastrointestinal encephalomyopathy) [293-295].

As for phenylketonuria, the first attempts to produce enzyme-loaded erythrocytes have been made in 1990 by Sprandel and Zöllner [296] who entrapped PAL in human RBCs. A recent work by Yew et al.

[297] was aimed at evaluating the *in vivo* pharmacokinetic profile of PAH from *C. violaceum* enclosed in erythrocytes after a single i.v. administration to normal mice. Even if they found an improved pharmacokinetics of the enzyme, its ability to metabolize circulating L-Phe when administered to hyperphenylalaninemic mice was not so pronounced, perhaps due to a too low specific activity of the enzyme in respect to the large amount of L-Phe to be removed. Therefore, erythrocytes loaded with PAL represent a more feasible alternative compared to PAH-encapsulated RBCs, also because PAL administration does not require the concomitant injection of a cofactor.

The current availability of an electromedical apparatus named Red Cell loader® (RCL) produced and commercialized by the biotechnology company EryDel SpA (www.erydel.com) could be exploited for the passage from bench to bedside of this therapeutic approach. Indeed the RCL is a fully automated apparatus conceived to function with a disposable, CE marked kit, and both are designed to reproducibly load human autologous erythrocytes with different drugs, in safe, sterile and apyrogenic conditions, as required for the performance of clinical studies, so as to yield a final product suitable for the reinfusion into patients with several pathologic disturbances [298]. Up to now the apparatus has undergone a process of upgrading, leading to the full-optional machine currently available. The device has been included in several clinical studies both with healthy volunteers (ClinicalTrials.gov IDs NCT01925859, NCT02380924) and with patients, where it was employed to load DEXA-21-P for the treatment of inflammatory diseases (ClinicalTrials.gov ID NCT01277289). The so-called EryDex system has been used in clinical trials for the treatment of ataxia-teleangiectasia (ClinicalTrials.gov ID NCT01255358; EU Clinical Trial Register number 2010-022315-19), it has received the Orphan Drug designation by European and US Authorities and is now entering a pivotal phase III study (http://www.erydel.com/en/erydex).

AIM OF THE WORK

The aim of our work was to develop an enzyme substitution therapy for the genetic metabolic disease phenylketonuria by means of erythrocytes employed as circulating bioreactors to metabolize excess L-Phe, in order to overcome all the bioavailability and immunogenicity issues risen by administration of the free enzyme. To this purpose we utilized the best performing variant of phenylalanine ammonia lyase currently available, i.e. C503S/C565S double mutant recombinant PAL from the cyanobacterium *A. variabilis* (rAvPAL) [255], kindly provided by BioMarin Pharmaceutical Inc. (Novato, CA) in its chemically unmodified (non PEGylated) form.

Our project consisted in different phases:

1. Optimization of the loading procedure in murine erythrocytes obtained from wild type BTBR mice, the genetic background of the most diffuse PKU mouse model (i.e. BTBR-Pah^{enu2} mice);
2. *Dose finding study*: first preclinical study aimed at evaluating the efficacy of a single infusion of three different doses of rAvPAL-loaded RBCs in reducing blood L-Phe levels in BTBR-Pah^{enu2} mice. Anti-rAvPAL IgG levels have also been assessed;
3. *Repeated administration study*: preclinical evaluation of the long-term efficacy of repeated infusions of a selected dose of enzyme (resulting from the *dose finding* study) in lowering and maintaining L-Phe levels near the physiologic condition. This study was divided in two steps:
    a. *Step 1*: the selected dose was administered three times every 18-19 days to assess the best time-span between infusions; the immune response has also been evaluated;
    b. *Step 2:* the selected dose was administered seven times at time intervals resulting from Step 1; evaluation of anti-rAvPAL IgG production has been performed.
4. Optimization of a protocol to load proteins in human erythrocytes to be performed by means of the Red Cell Loader® device and evaluation of the best storage conditions, in the perspective of a possible future clinical application.

On the whole, this project will desirably lead to the optimized production of processed rAvPAL-loaded erythrocytes with features as similar as possible to those of native untreated RBCs, but with the additional ability to efficiently metabolize L-Phe for the long-term repeated treatment of hyperphenylalaninemic patients.

MATERIALS

AND

METHODS

1. Enzymes

1.1. Recombinant AvPAL

Recombinant Phenylalanine Ammonia Lyase from *Anabaena variabilis* (rAvPAL) was prepared by the BioMarin Pharmaceutical Inc. (Novato, CA) clinical manufacturing group. Briefly, the protein, which contains two point mutations to prevent aggregation, Cys503→Ser and Cys565→Ser, was cloned in a pIBEX7 plasmid, expressed in the *E. coli* strain BLR (Novagen) and purified by anion exchange chromatography followed by hydrophobic interacting chromatography. Purified rAvPAL was concentrated and buffer exchanged by ultrafiltration/diafiltration to a final concentration of approximately 100 International Units (IU)/ml. Final concentration was determined by Bicinchoninic acid (BCA) assay and activity assayed as previously described by Wang et al. [255]. Recombinant AvPAL was provided in a liquid form, dissolved in buffered saline solution and in two separate lots of production, the first one used for the *in vitro* study and the second one employed in all *in vivo* studies. The first preparation was concentrated 120 IU/ml with a specific activity (SA) of 1.8 IU/mg protein; the second lot had a concentration of 105 IU/ml and a SA of 1.54 IU/mg.

1.2. Hexokinase (E.C. 2.7.1.1)

Hexokinase (HK) type III from *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich S.r.l. (Milan, Italy) and employed as model protein in the loading studies performed on human RBCs by means of the Red Cell Loader® device. This decision was made owing to temporarily unavailability of rAvPAL. HK was provided as lyophilized powder, of which an opportune amount was weighed and dissolved in 18 ml water for injection (WFI) corresponding to a total nominal amount of 25,000 IU enzyme. An aliquot of each protein solution employed was frozen for actual HK activity assay, which was carried out as described later.

2. Human blood

Whole blood (WB) was obtained from healthy volunteers included in the Italian blood donor registry (registered A.V.I.S. donors) who signed an informed consent form. Blood was collected and provided by the “San Salvatore” Hospital Blood Bank in Pesaro, Italy, in bags containing 400-450 ml including a fixed 63 ml volume of CPD (citric acid/sodium citrate, sodium dihydrogen phosphate, dextrose) as anticoagulant. Each bag was tested for the presence of microorganisms and viruses (HIV, HBV and HCV) and was defined as virus-free. Blood whose hematological parameters were out of the range regarded as normal was excluded from the experiments; no further exclusion criterion was adopted. The blood used in the experiments with the RCL® device was processed within 7 hours from withdrawal and prior

standardization to a fixed 40% Ht. Hematocrit standardization was carried out by varying the blood volume to be processed (minimum 50 ml), calculated as follows:

$$\text{WB volume (ml)} = \frac{50 \text{ ml (min volume)} \times 40\% \text{ Ht (desired value)}}{\text{WB Ht (actual value)}}$$

3. Animals

Adult homozygous BTBR-Pah^{enu2} and BTBR wild type (BTBR-WT) mice were employed in this study. Animals involved for the *dose finding* study were bred in "Santa Lucia" Foundation (Rome, Italy), those involved in the *repeated administration* studies were provided by Plaisant s.a.s. (Castel Romano, Rome, Italy).

The genetic enu2 modification is chemically induced after treatment of BTBR wild type mice with ethylnitrosurea (hence the name). The treatment causes an A>T835C missense mutation in exon 7 of the *PAH* gene, resulting in a phenylalanine-to-serine amino acid substitution in position 263 of the protein chain (F263S). These mice present a biochemical and neurological phenotype strictly resembling the human PKU disease, with serum L-Phe 10- to 20-fold higher on a normal diet, increased L-Phe concentration in the cerebral cortex with a concomitant 70% reduction in brain serotonin levels, microcephaly at birth and hypopigmentation (Figure 10) [299, 300].

Figure 10. BTBR wild type (left) and Pah^{enu2} (right) mice. The lighter fur of the mutated mouse is particularly evident from the picture.

Homozygous mice were issued from heterozygous mating, and the presence of the enu2 mutation was determined by PCR amplification of exon 7 of the *PAH* gene on DNA obtained from tail tissue, according to Pascucci et al. [41]. Animals were housed in standard cages, 3 to 6 mice per cage, on a 12 h light:dark cycle and in controlled conditions (temperature 22 ± 1°C, humidity 60%, air change every 12 hours). All mice were fed on Teklad global 18% protein rodent diet (Harlan Laboratories Inc., Madison, WI) and water *ad libitum*. BTBR-WT mice were used as blood donors for the loading procedure, while BTBR-

Pah^{enu2} mice received r*Av*PAL-RBCs. Their use was approved by the Ethical Committee for Animal Experiments of the University of Urbino "Carlo Bo" - Italy (Prot. CESA 3/2012).

4. *In vitro* studies

4.1. Development and optimization of murine rAvPAL-RBCs for *in vitro* studies

Blood was collected from CO_2 anesthetized control BTBR-WT mice by puncture of the retro-orbital sinus in heparinized tubes. rAvPAL from the first lot (SA 1.8 IU/mg) was loaded into mouse RBCs by means of a procedure of hypotonic dialysis, isotonic resealing and "reannealing", essentially according to Magnani et al. [301]. Whole blood was centrifuged to remove plasma and washed twice by 10 min centrifugations at + 4°C, at 900 *g* and 1500 *g* respectively, in a physiological saline solution with 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES, pH 7.4), 154 mM NaCl and 5 mM glucose (henceforth named Hepes solution). In the attempt to identify the best loading conditions maximizing the amount of rAvPAL loaded into RBCs, the procedure was carried out with 24 IU, 42 IU and 54 IU of enzyme (SA 1.8 IU/mg) being added to RBCs suspended in Hepes solution at 60%, 50% and 40% hematocrit (Ht) respectively. It must be considered that only by decreasing RBC Ht a greater volume is available for enzyme addition. Each condition (1 ml final volume) was dialyzed 1 h at + 4°C in a cellulose tube (14 kDa MWCO, Roth, Karlsruhe, Germany) *vs* 50 ml of hypotonic dialysis buffer optimized for murine RBC loading, containing 15 mM NaH_2PO_4, 15 mM $NaHCO_3$ (pH 7.4), 20 mM glucose, 4 mM $MgCl_2$, 3 mM glutathione, and 2 mM ATP. The final osmolality of the hypotonic solution was 85 mOsm, measured by Osmometer Fiske Associates, Model 210 (Norwood, MA, USA). After dialysis, the cells reached about 105 mOsm (the opening of membrane pores in murine RBCs starts at values ≤ 150 mOsm). Subsequent resealing and reannealing steps were carried out by incubating the dialyzed RBC suspension 5 min at + 37°C; after that PIGPA solution (10% v/v) was added to the RBCs to restore isotonicity (300 mOsm) and the suspension was incubated another 25 min at + 37°C under gentle stirring, to allow pore closure. Final washing steps were performed by 10 min centrifugation at 300 *g* and + 4°C to avoid cell lysis, while the amount of entrapped enzyme was determined according to Wang et al. [2008] and briefly summarized later on. Hematological parameters were measured by an automatic ABX Micros® 60 cell counter (Horiba Medical, Irvine, CA) and percent RBC recovery was calculated from the number of RBCs submitted to the dialysis step and those recovered at the end of the loading procedure.

4.2. L-Phenylalanine *in vitro* metabolism by murine rAvPAL-RBCs

The evaluation of L-Phe consumption by rAvPAL-loaded RBCs in the preliminary *in vitro* study was performed by incubating the erythrocytes at + 37°C for up to 60 min in Hepes solution containing 2 mM L-Phe to a final 40% Ht. At planned time points (0, 30 and 60 min) 40 µl RBC suspension (in

duplicate) was spotted on filter paper (Whatman 903), dried and processed for L-Phe determination by tandem mass spectrometry (MS/MS) in the Department of Experimental Medicine of the "Sapienza" University of Rome. Whole blood was used as negative control.

5. Phenylalanine Ammonia Lyase activity assay

The kinetic assay was performed essentially as reported by Wang et al. [255]: rAvPAL activity was determined spectrophotometrically on an RBC aliquot (diluted 1:500 in distilled water, dH_2O) withdrawn from the dialyzed suspension before washing steps to measure the actual total amount of enzyme units added to the dialysis step. The assay was also performed on an aliquot of final loaded RBCs (1:100 in dH_2O) to evaluate the quantity of encapsulated rAvPAL.

A sample volume in the range 10 – 50 µl was added to 950 µl of a 100 mM Tris-HCl solution (pH 8.5) containing 22.5 mM L-Phe. A proper volume of 100 mM Tris-HCl (pH 8.5) without L-Phe was added when needed to reach 1 ml final volume. Activity was assayed by monitoring the formation of *trans*-cinnamic acid (tCA) at 290 nm wavelength for 20 minutes at + 30°C (tCA molar extinction coefficient ε_{290nm}: 10.238 $\mu mol^{-1}\ cm^{-1}\ ml^{-1}$).

6. *In vivo* studies

6.1. Loading procedure and *in vivo* efficacy of rAvPAL-RBCs: *dose finding study*

In the first preclinical study, 600 µl packed RBC suspension was dialyzed as previously described with 42 IU rAvPAL (corresponding to 400 µl of enzyme solution with SA 1.54 IU/mg) to reach 1 ml final volume at 50% Ht inside the dialysis membrane. This procedure was carried out for 15 separate tubes; at the end, all RBC suspensions were pooled and allowed to equilibrate 5 min at + 37°C under gentle stirring. At this stage the cells reached 102 mOsm. After resealing and reannealing steps, loaded erythrocytes were washed twice in Hepes solution at 450 *g* for 10 min to remove the unentrapped enzyme. An aliquot of RBC suspension was retained immediately after the end of the loading procedure to evaluate the expression of phosphatidylserine (PS) on RBC surface by Annexin V binding, as a marker of cell damage. Loaded erythrocytes were then resuspended to 36% Ht and the amount of entrapped enzyme was assayed before infusion into BTBR-Pah^{enu2} mice (mean body weight 25.64 ± 9.47 g). rAvPAL-RBCs were prepared at 36%, 18% and 9% Ht (corresponding to approx. 4.75, 2.37 and 1.18 IU rAvPAL/ml, respectively) by scalar dilution in Hepes solution, in order to administer the scheduled doses in the same volume (250 µl).

Three different doses of rAvPAL-RBCs were administered to three cohorts of BTBR-Pah^{enu2} mice by intravenous infusion, so as mice of each cohort received 0.25 IU (n = 5 mice), 0.5 IU (n = 6 mice) and 1 IU (n = 3 mice) rAvPAL, respectively. Treatment efficacy was evaluated by biochemical monitoring of blood L-Phe, sampling blood at time 0, and then 1, 2, 5, 8, 12, 16, and 21 days after a single injection.

Whole blood (40 µl) was collected from the submandibular vein by special animal lancets (Goldenrod 5.5 mm, Braintree Scientific Inc., Braintree, MA) after 2 h of food deprivation, spotted on filter papers and analyzed by MS/MS for amino acid levels. On day 21 after treatment blood was also collected to perform a preliminary assessment of anti-rAvPAL IgG levels. Plasma was obtained and frozen until use.

6.2. Loading procedure and *in vivo* efficacy of rAvPAL-RBCs: *repeated administration study*

In this second preclinical study, 42 IU rAvPAL (SA 1.54 IU/mg) was added to 600 µl packed RBC suspension (85.5 ± 7.1% Ht) to obtain 1 ml at 49 ± 2% Ht. Dialysis was carried out for 75 min at + 4°C *vs* dialysis buffer for murine use 81.75 ± 2.31 mOsm, thus obtaining an osmolality of 108.5 ± 5.9 mOsm. The final resealing and reannealing steps, as well as enzyme activity assay, were performed as described above. This study was divided in the following steps:

Step 1 – Assessment of the efficacy of three subsequent infusions of rAvPAL-RBCs performed in 5 BTBR-Pah^{enu2} mice at 18-19 day-intervals; the main purpose of this step was to identify the most appropriate time lag between subsequent injections allowing to keep blood L-Phe within a potentially safe range, and to perform a preliminary evaluation of anti-rAvPAL IgG production;

Step 2 – Assessment of the efficacy of seven infusions of loaded RBCs administered to 8 BTBR-Pah^{enu2} mice at time intervals defined according to Step 1; this second part aimed at evaluating the longer-term therapeutic capacity of a higher number of repeated infusions of rAvPAL-RBCs, as well as the host immune response (which was assayed by indirect ELISA).

Final packed rAvPAL-loaded RBCs were resuspended in Hepes solution at approximately 20% Ht in order to administer 0.67 ± 0.07 IU/mouse in a final volume of 180 ± 43 µl (Step 1) or about 200 µl (Step 2). The amount of enzyme administered in this study was in agreement with the one suggested by the previous *dose finding in vivo* study. In both Step 1 and Step 2 of the *repeated administration* study, BTBR-WT (n = 5) and BTBR-Pah^{enu2} (n = 5) mice were used as healthy and PKU controls respectively, and submitted to the same series of injections as the RBC-treated mice, but they received i.v. infusions of Hepes solution. Blood L-Phe concentration was measured by MS/MS on dried blood spots (DBS); collected as previously described from all animals, including control healthy and BTBR-Pah^{enu2} mice. L-Phe monitoring was performed at time 0 before each administration and then 4 or 5, 9 or 10 and 13 or 14 days after each infusion in Step 1; at time 0 before and 4 or 5 days after each injection in Step 2. Blood L-Tyr was evaluated on the same blood samples as well.

Blood samples (100 µl) for anti-rAvPAL IgG analysis were withdrawn as well in heparin from the submandibular vein at different time points from RBC infusions, i.e. at time 0 (before each

administration) and then 9–10 and 13–14 days post i.v. (Step 1), only at time 0 (before each rAvPAL-RBC injection) for Step 2. In both steps, plasma was collected 21 days after the last infusion, as well. Mice were photographed throughout the experimental period to visually monitor fur colour changes due to restoration of L-Tyr metabolism.

7. Tandem mass spectrometry

RBC suspensions from the *in vitro* study and mouse whole blood were collected on Schleicher&Schuell 903 grade filter paper, dried at room temperature and stored at + 4°C in plastic bags until use. L-Phe analysis in DBS was performed in the Department of Experimental Medicine of the "Sapienza" University of Rome, essentially according to a previous method proposed by Chace at al. [177] with some modifications. Three millimeter diameter dots were punched out from DBS and eluted in 100 µl of methanol/water (80:20) solution spiked with labeled amino acid internal standards (CIL, Andover, MA, USA). The samples were shaken 30 min at + 30°C, then 65 µl of supernatant was dried under nitrogen flow at + 45°C. The residues were derivatized by treatment with 50 µl of 3 M HCl in n-butanol solution at + 60°C for 30 min. After derivatization, the samples were dried under nitrogen flow at + 45°C and recovered in 70 µl of acetonitrile/water (80:20) containing 0.1% formic acid. A 20 µl volume was injected into a LC-MS/MS system (API 2000, Sciex, Toronto, Canada) equipped with a Series 200 micro pump (Perkin Elmer, Norwalk, CT, USA) and a Series 200 autosampler (Perkin Elmer) for solvent delivery and automated sample loading. The mobile phase was acetonitrile/water (80:20) pumped at a flow rate of 50 µl/min. Neutral loss scan of 102 Da fragment and a total acquisition time of 2 minutes were used to detect L-Phe.

8. Evaluation of plasma anti-rAvPAL IgG titers

The immune response against rAvPAL administered through erythrocytes was evaluated by standard indirect ELISA. Blood samples (100 µl) collected in heparin from the submandibular vein were centrifuged 5 min at 1050 *g*, plasma was harvested and frozen until use. ELISA analysis was performed as follows: 2HB flat bottom 96-well plates (Immulon® microtiter plates, Thermo Scientific, Rochester, NY) were coated overnight at + 4°C with 100 µl of rAvPAL dissolved 1 µg/ml in 50 mM carbonate buffer, pH 9.7. Plates were washed four times with 400 µl/well of PBS solution added with 0.05% (v/v) Tween 20 (TPBS), blocked with 100 µl/well of TPBS plus 2% (w/v) fat free dry milk (blocking solution) and maintained 1 h at + 37°C. The plates were finally washed four times with TPBS. Plasma was thawed, serially diluted in blocking solution in the range 1:200 – 1:25,600 for plasma of the *dose finding* study, in the range 1:50 – 1:200 for pre-treatment samples of the *repeated administration* study and 1:400 – 1:409,600 for post-treatment samples, and dispensed 100 µl/well in duplicate. Plasma antibody binding was allowed by incubating plates 90 min at + 37 °C. After four washes with TPBS, goat anti-

mouse IgG-HRP (Bio-Rad, Hercules, CA) diluted 1:1,000 in blocking solution was added 100 µl/well and plates incubated again 60 min at + 37 °C. After another four washes in TPBS, the immune complexes were revealed by adding 100 µl/well of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Roche, Indianapolis, IN) as a chromogenic substrate dissolved in 50 mM sodium citrate solution, pH 3, containing 1 µl/ml of H_2O_2 (35 wt. -% in H_2O, Sigma-Aldrich, Milan, Italy). Plates were incubated 30 min at room temperature protected from light, then absorbance at 405 nm was read by an automated Microplate reader (Bio-Rad, Hercules, CA).

9. Annexin V staining

Annexin V binding on RBC surface was used as a probe to detect cells exposing phosphatidylserine (PS), a phospholipid usually kept on the cytosolic side of the cellular membrane. PS exposition on the extracellular side is associated with cell death or damage, or it is a consequence of membrane components in/out translocation. This event has been employed to evaluate the percentage of erythrocytes that underwent alterations during the loading procedure. Positive RBCs were counted by flow cytometry by colleagues in the Department of Earth, Life and Environmental Sciences of the University of Urbino "Carlo Bo", according to a previously published method [302]. Briefly, RBC samples were diluted to 1.0 x 10^6 RBCs/µl in the binding buffer for annexin V (100 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$, pH 7.4), then fluorescein isothiocyanate (FITC)-conjugated annexin V and anti-CD41 phicoerythrin (PE)-conjugated mAb were added to RBCs in an appropriate volume as suggested by the manufacturer, incubated for 15-20 min at room temperature and finally washed. Annexin V positive RBCs were counted using a fluorescence-activated cell sorter (FACS) equipped with a 15 mW 488 nm, air-cooled argon-ion laser and a second red diode laser, 635 nm (Becton Dickinson, San Jose, CA). Data analysis and quantification were performed using the CellQuest™ Software.

10. Loading of HK with Red Cell Loader®

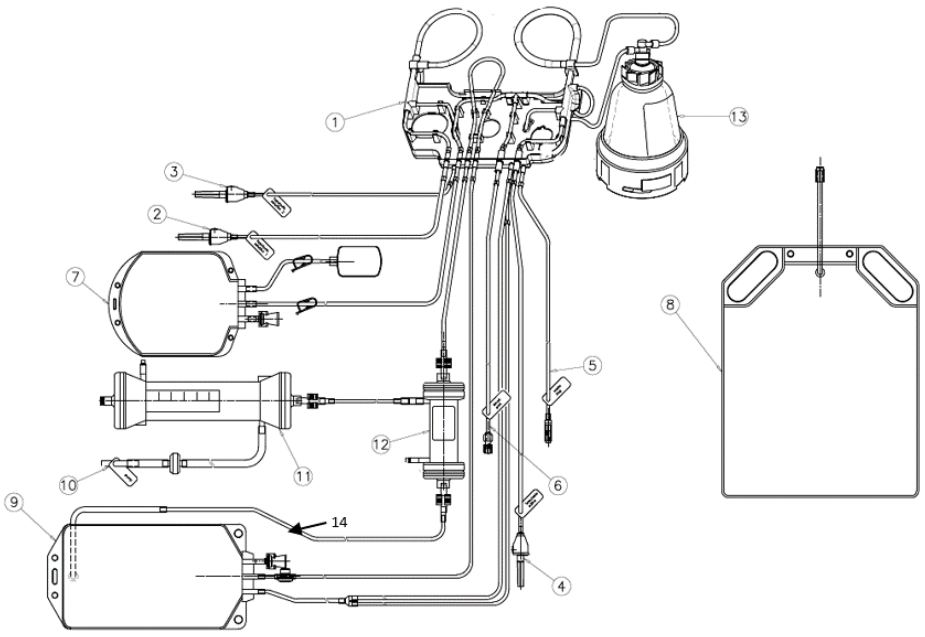
10.1. The Red Cell Drug Loading System

A fully automated proprietary device named Red Cell Loader® (RCL®) was employed in this part of the study, along with a sterile disposable kit (EryDel S.p.A., Urbino, Italy, Figure 11). Both electromedical apparatus and disposable kit (EryKit_01) received CE marking and authorization by European and US Authorities for the loading of drugs (particularly dexamethasone-21-P) in red blood cells for clinical use. The completely automated loading procedure, named EryDex, performs the reversible hemolysis according to the method of "pre-swelling" hypotonic dilution with concentration (which will be discussed later) and is carried out under the control of a proprietary software.

Figure 11. The Red Cell Loader® (left) and disposable EryKit_01 (right).

The Red Cell Loader® is composed of different mechanical parts serving the different steps of the procedure. It comprises three peristaltic pumps for liquid transfer, a heater/shaker plate able to reach + 37°C temperature for RBC incubation with the various processing solutions, a centrifuging unit and a vacuum pump to perform hemoconcentration.

EryKit_01 is a single-use, non-pyrogenic, sterile kit provided in individual packages and sterilized with ethylene oxide. The kit is composed of different parts (Figure 12) linked together and to the RCL® mechanical apparatus through a series of plastic pipes, mediating blood and solution transfer between the different parts. Along the circuitry, a series of *clamps* localized in several sites open or block specific tracts of the pipeline to address the processed RBC suspension and/or the opportune solution to the correct site, according to the protocol step carried out. The software checks the correct functioning of the system and the integrity of the kit at each system start-up, before the beginning of each procedure. User-friendly instructions are displayed on the RCL® touch screen monitor, guiding the operator during the different phases of the process; a set of sensors constantly monitors every step, immediately alerting the operator with visual and acoustic signals in case of any technical problem, thus allowing a successful completion of the loading procedure.



Legend

1. *Cassette to organize and secure the tubing*
2. *Spike connector for Hypotonic Solution 1*
3. *Spike connector for Hypotonic Solution 2*
4. *Spike connector for injectable saline solution (Washing solution)*
5. *Waste bag connector*
6. *Luer-lock connector for 50 ml syringe*
7. *Final collection bag*
8. *Waste bag*
9. *Transfer bag*
10. *Vacuum pump connector on Red Cell Loader*
11. *Reservoir for filtered liquid collection*
12. *Hemoconcentrator filter*
13. *Bowl for RBC washing and separation (Latham's model)*
14. *Septum for drug and hypertonic solution (PIGPA)*

Figure 12. Schematic representation of the EryKit_01 components and pipeline.

Blood is automatically conveyed inside the pipeline from a 50 ml luer-lock syringe where it has to be transferred from the first collection bag. The EryKit_01 is endowed with a bowl where washing steps are performed on the initial whole blood to separate the erythrocytes, and on the RBCs to remove the different solutions added in sequence to the suspension; waste material is collected in the *waste* bag. Incubation steps with hypotonic, hypertonic and drug solutions are carried out in the *transfer* bag. Hemoconcentration occurs by means of a Hemocor HPH® Junior ultrafilter (Minntech, Minneapolis, MN) made of polysulfone fibers with 65 KDa MWCO, coupled to a *reservoir* for ultrafiltered liquid collection. A vacuum pump forces erythrocytes to pass through the filter until the desired final volume of supernatant liquid is removed by ultrafiltration. Both drug and PIGPA resealing solutions are manually added by the operator through an injection port located in the pipe entering the *transfer* bag, immediately before the bag. These are the sole two operations that have to be carried out manually; otherwise, the entire process is automated and software-controlled. Loaded erythrocytes are finally conveyed in the final collection bag, connected to a smaller *satellite* bag available for any sample withdrawal from the main bag in sterile conditions.

10.2. Processing solutions

Washing steps are performed with injectable saline solution 0.9% (w/v) NaCl (Industria Farmaceutica Galenica Senese, Siena, Italy). The kit is supplied with two hypotonic solutions employed in the procedure (Hypo 1 and Hypo 2, respectively, Haemopharm Biofuids, Tovo S. Agata, SO, Italy) and the hypertonic resealing solution PIGPA (COC Farmaceutici Srl, S. Agata Bolognese, BO, Italy). Hypo 1 is a 0.563% ± 5% (w/v) NaCl solution in water for injection (WFI) with an osmolality of 180 ± 9 mOsm, while Hypo 2 is 0.372% ± 5% (w/v) NaCl in WFI (120 ± 6 mOsm). Both hypotonic solutions had a pH ranging from 4.5 to 7.0. PIGPA is a WFI solution containing 33 mM $NaH_2PO_4 \cdot H_2O$, 1.666 mM KCl, 190 mM NaCl, 100 mM inosine, 20 mM ATP disodium salt, 100 mM anhydrous glucose, 100 mM sodium pyruvate and 4 mM $MgCl_2 \cdot 6H_2O$, to a final osmolality 3,785 ± 285 mOsm and pH 7.4 ± 1.2.

10.3. The EryDex System applied to proteins

All the experiments with human blood were performed at EryDel S.p.A. laboratories. The protocol employed to load proteins inside RBCs is essentially based on the EryDex system, currently used to load DEXA-21-P. The software version 3.2.0 used in the experiments with hexokinase has been specifically developed to the purpose of protein loading, and optimized to adapt to the different structural and size characteristics of this class of molecules. The whole process of pre-swelling hypotonic dilution is composed of 17 steps described below:

1. Whole blood previously adjusted to 40% Ht (50 ml minimum volume) is transferred from the syringe into the rotating bowl;
2. The RBCs in the bowl are separated from plasma and platelets by washing with saline solution; plasma and waste saline are immediately collected in the *waste* bag;
3. Washed RBCs are transferred from the bowl to the *transfer* bag;
4. Hypotonic solution 1 is added to RBCs in the *transfer* bag;
5. The *transfer* bag with RBC suspension plus Hypo 1 is incubated 5 minutes at room temperature on the RCL® shaker plate (*pre-swelling* step);
6. Pre-swelled RBCs are concentrated in the rotating bowl and Hypo 1 is removed;
7. RBCs are transferred back from the bowl to the *transfer* bag;
8. Hypotonic solution 2 is added to RBCs in the *transfer* bag;
9. The RBC suspension plus Hypo 2 is incubated 5 minutes at room temperature on the RCL® shaker plate (further erythrocyte swelling step);
10. *a* – first, pre-swelled RBCs are conveyed into the hemoconcentrator filter for Hypo 2 removal, with ultrafiltered liquid being sucked into the *reservoir* by the vacuum pump;
    b – the concentrated RBCs are returned to the *transfer* bag.

These two operations are repeated cyclically until 83 ml ultrafiltered volume is collected in the *reservoir*;

11. Hexokinase dissolved in a proper amount of WFI is manually added with a syringe to the concentrated RBCs via the injection port. The osmolality of the protein solution is such that final opening of pores on the RBC surface is completed and protein equilibration between the inner and outer cellular environments can occur. This operation, whose moment is signaled by the software with a an acoustic alarm, must be done within 5 minutes;
12. RBCs are incubated with HK solution (commonly 10 minutes) at room temperature on the RCL® shaker plate
13. PIGPA hypertonic solution is manually added to the erythrocytes with a syringe via the injection port. Again, the operator is alerted by an acoustic signal and must do the operation within 5 minutes;
14. Erythrocytes are incubated 30 minutes at + 37 ± 2°C on the heated RCL® shaker plate (*resealing* and *reannealing* step);
15. The resealed RBCs are transferred from the *transfer* bag to the rotating bowl;
16. RBCs in the bowl are extensively washed with saline solution;
17. Protein-loaded RBCs are transferred into the final collection bag.

As a whole, the procedure takes approximately 1 hour 30 minutes. At the end, the final collection bag is removed and the rest of the kit is disposed of as biological hazardous waste. The loaded RBCs obtained as final product must be reinfused into the patient within 30 minutes from process completion. In Figure 13 is a schematic graphical representation of the procedure.

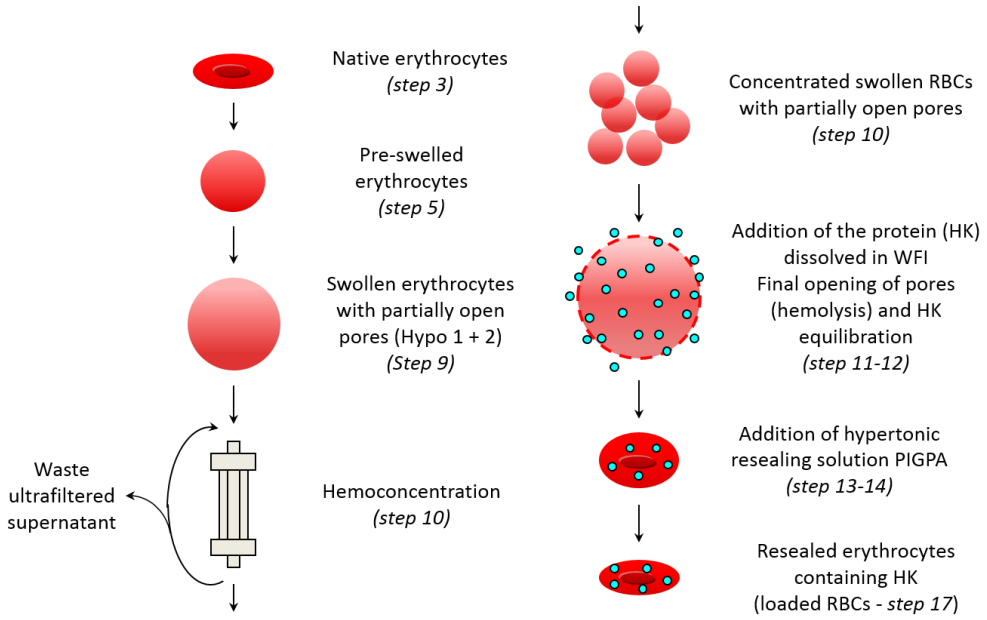


Figure 13. Graphical representation of the main steps of the loading procedure carried out by the Red Cell Loader® and software version 3.2.0.

10.4. Optimization of the loading procedure and RBC membrane stability test in different buffers

To test whether the rate of protein encapsulation might depend on the contact time between the pre-swelled RBC suspension and the protein solution, step 12 of the EryDex procedure was modified to study three different conditions, i.e. 3 min, 5 min and 10 min incubation times, considering the 10 min-condition as the reference standard. Incubation times were shortened by early addition of the resealing solution PIGPA. No other step was changed. The three procedures were carried out on blood from a single donor and within 7 hours from withdrawal, to exclude inter-patient variability and possible RBC alteration due to long maintenance periods at + 4°C. A fixed amount of 25,000 IU HK was used in each loading procedure.

The final bags from the three procedures were analyzed for complete blood count (CBC) and hematological parameters by an automatic Coulter AcT 5 Diff Hematology Analyzer (Beckmann, Miami, FL) and aliquots were frozen for subsequent evaluation of the total amount of encapsulated HK.

A stress test was carried out to verify whether RBC membrane integrity of loaded erythrocytes could be preserved for long periods. To date, previous repeated *in line* determinations of Hb and loaded drugs on waste saline solution deriving from the final washing step have revealed a very low presence of both drug and Hb, the latter being often undetectable (EryDel internal Study: EryDel 2014-P03).

The present test aimed at assessing whether addition of different buffers (or varying concentrations of the same buffer) in final bags could extend membrane resistance to high-speed centrifugation after room temperature (RT) incubation, without leakage of Hb or loaded HK from RBCs.

To this purpose, loaded erythrocytes were split in three vials containing a proper volume of a different buffer each, namely 0.9% NaCl solution (saline, as control), 1 M $NaH_2PO_4 \cdot 2H_2O$, pH 7.4 (phosphate buffer), and 1 M $NaHCO_3$, pH 8.3 (bicarbonate buffer), to reach a final 20 mM concentration following RBC addition (except for saline solution). CBC analyses were performed on aliquots immediately after RBC addition and the three conditions were then incubated 2 h at room temperature. After 30 min and 2 h incubation times, CBC analyses were performed again and two aliquots from each condition (1 ml each) were centrifuged 5 min at 5000 *g*. Supernatants were collected from one aliquot and immediately assayed for free Hb content, measured by HemoCue® Plasma/Low Hb System (HemoCue, Ängelholm, Sweden), while the remaining volume was frozen for HK activity assay. The second aliquot was resuspended and analyzed again for CBC, to verify any RBC lysis induced by the *stress test* (i.e. RT incubation followed by high-speed centrifugation). Time 0 samples (i.e. immediately after RBC addition to the buffers) were not analyzed, assuming that no free Hb nor free HK was present in the supernatant fractions, on the basis of previous EryDel's data.

A subsequent set of procedures was performed in triplicate with 25,000 IU HK to be loaded and step 12 fixed on 10 min incubation time, which resulted to be the best one from the previous preliminary analyses. The final bags obtained were analyzed for CBC and aliquots were frozen for total HK

determination. Since from the previous experiments NaH_2PO_4 solution proved to be the best one in stabilizing RBC suspensions under stress conditions, we verified if different final concentrations of such buffer had the same effect on the final product. Loaded erythrocytes from the three procedures were therefore split in four vials containing an opportune volume of 1 M NaH_2PO_4 to reach a final concentration of 10, 20 and 30 mM after RBC addition; the fourth vial contained 0.9% NaCl solution as control. After RBC addition, CBC analysis was performed again and the conditions incubated 2 hours at room temperature; at 30 min and 2 h incubation times the samples were processed as described above. In this experiment as well, time 0 values of both supernatant free Hb and free HK were assumed to be null and therefore not analyzed.

11. Hexokinase activity assay

All the material was purchased from Sigma Aldrich (Milan, Italy) and the activity assay was performed essentially according to the manufacturer with some modifications. Frozen RBCs and supernatants were thawed and diluted 1:4,000, 1:100 and 1:30 respectively in dH_2O, to reach an HK concentration within the linearity range of 0.3 - 0.45 IU/ml.

A reaction mix for the activity assay was prepared from stock solutions of the single reagents, as reported in Table 1. Glucose-6-phospate dehydrogenase (G6PD, E.C. 1.1.1.49) was added immediately before use. Sample volumes and dH_2O to 1 ml final volume were put into quartz cuvettes before addition of the reaction mix. The assay was performed on 2.5, 5, 10, 15 and 20 µl volume for each diluted sample. After mixing by inversion, the samples were analyzed by a Shimadzu UV-1800 double beam spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a CPS-240A Cell Positioner endowed with thermoelectrical temperature control, previously set at + 37 °C. HK activity was determined indirectly by monitoring NADPH formation at 340 nm for 20 min (NADPH molar extinction coefficient ε_{340nm} 6.2 $\mu mol^{-1}\ cm^{-1}\ ml^{-1}$). Data were collected and analyzed by means of UVProbe software. Kinetic curves were considered only in their linear tract, excluding plateau portions when present; generally, values approximately within 5 – 15 min range were considered for ΔAbs/min computation.

Table 1. Reaction mix for HK assay (reported volumes are for a single analysis).

Reagent	Volume
Glycylglycine 0.25 M, pH 8.1	500 µl
Glucose 50 mM	100 µl
$MgCl_2$ 50 mM	100 µl
ATP-$MgCl_2$ 0.1 M	50 µl
$NADP^+$ 5 mM	50 µl
G6PD (stock solution 1:100 in dH_2O)	10 µl
dH_2O	190 – X µl
Unknown sample	X µl (range 2.5 – 20 µl)
Total reaction volume	1000 µl

12. Statistical Analysis

12.1. *Dose finding study*

Blood L-Phe levels of mice from each cohort involved in the *dose finding* study (1, 0.5 and 0.25 IU/mouse) were analyzed by two-way ANOVA followed by a proper *post-hoc* test: Dunnett's test was employed to compare every time point from each group to time 0 (i.e. L-Phe before treatment); Tukey's test was used when groups were compared within the same experimental time point.

12.2. *Repeated administration study*

Blood L-Phe of mice involved in the Step 1 were compared at each time point to their initial pre-treatment values by one-way Anova followed by Dunnett's test. In Step 2, L-Phe levels observed during the whole experimental period were compared to pre-treatment values by non parametric one-way ANOVA followed by Dunn's test.

12.3. Stability of human erythrocytes loaded by RCL®

Human erythrocytes obtained by EryDex system (sw 3.2.0) and tested in stress conditions with different buffers were analyzed by two-way ANOVA, considering Time and Buffer as independent variables; Tukey's *post hoc* test for multiple comparisons was adopted to compare to one another both the effect of buffers within a single time point, and different time points within each assay condition.
RBC count before and after centrifugation were compared by two-way ANOVA and Dunnett's *post hoc* test for multiple comparisons, with saline as control when the analysis was made considering only the samples after centrifugation.

RESULTS

1. *In vitro* studies

1.1. Development of murine rAvPAL-RBCs

These analyses were performed to identify the best loading conditions with maximum yields in terms of entrapped enzyme and RBC corpuscular indices. RBCs loaded with increasing amounts of rAvPAL could be obtained by simultaneously varying both RBC hematocrit and enzyme units during the dialysis step. The results are summarized in Table 2a.

Table 2a. Optimization of rAvPAL loading into murine RBCs and confirmation of the selected condition.

RBC dialysis Ht	Added rAvPAL (IU)	Loaded rAvPAL (IU/ml RBCs 100% Ht)	RBC recovery (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)	RDW (%)
a) 60%	24	3.15	28	43	16.6	38.3	18.4
b) 50%	42	8.06	29	40	18.5	34.6	18.8
c) 40%	54	15.62	18	42	9	21.3	20.1
Reference values for murine RBCs (range)[a]				48	16.8 – 18.1	34.7 – 37.7	14.5 – 15.2

Table 2b. Confirmation of the selected loading condition.

RBC dialysis Ht	Added rAvPAL (IU)	Loaded rAvPAL (IU/ml RBCs 100% Ht)	RBC recovery (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dl)	RDW (%)
b) 50%	42	6.02	7.7	40	14.7	36.8	17.5
Reference values for murine RBCs[b]				48	18.2	38.1	14.6

[a,b]Reference ranges are the minimum and maximum values observed in murine blood before submission to the loading procedure. Results are from a single experiment.

RBC recovery was lower than reported in previous experiments with human RBCs [303], indicating a greater fragility of murine cells. The corpuscular indices of murine rAvPAL-RBCs were not significantly different from reference values as for dialysis conditions at 60% and 50% Ht, whereas the dialysis condition at 40% Ht yielded cells whose parameters were not comparable to the reference values. This confirmed what already suggested by the recovery rate, i.e. that this conditions is too strong for murine erythrocytes and therefore it is not applicable if aimed at obtaining cells with features as similar as possible to the native ones. Condition b) of this experiment has been repeated for confirmation and annexin V staining was evaluated. The results reported in Table 2b show that although the amount of loaded rAvPAL and RBC parameters were comparable to those of the first experiment, RBC recovery was much lower. We attributed this fact to the intrinsic variability of murine cells. In the second experiment, annexin V binding was also evaluated. The percentage of positively stained cells increased from 0.7% of whole blood to 4.9% of loaded RBCs, a broadly acceptable value.

1.2. *In vitro* L-Phe metabolism by murine rAvPAL-RBCs

In vitro L-Phe consumption by rAvPAL-RBCs obtained from the two experiments was assessed as described in Materials and Methods section, and the results are reported in Figure 14A and B. The time

0 has not been determined for loaded RBCs because the reaction is very rapid in the presence of rAvPAL and alters the result itself. The starting value of the negative control (whole blood) can be considered as a valid time 0.

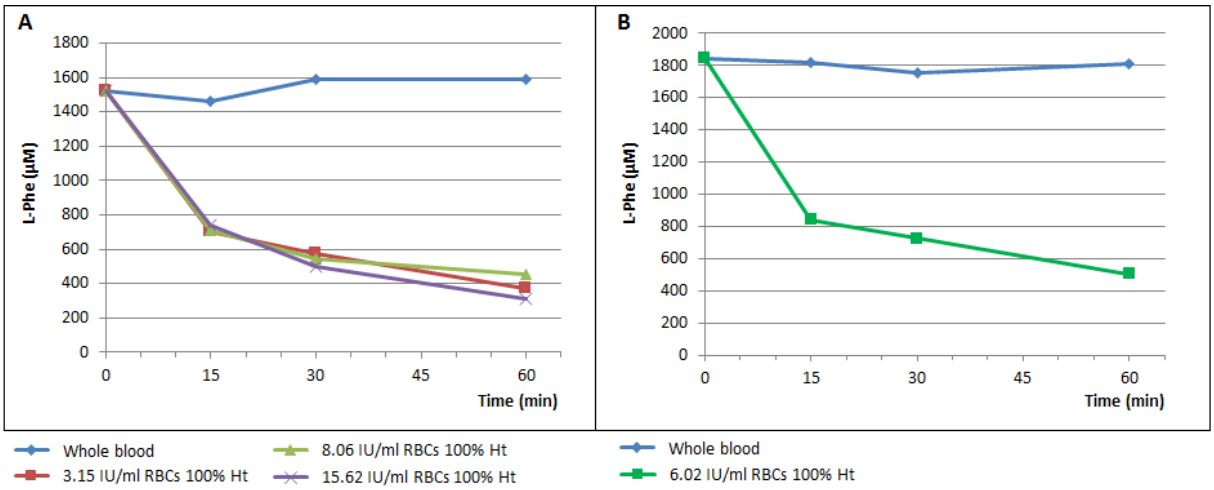


Figure 14. *In vitro* L-Phe consumption by murine RBCs loaded with different amounts of rAvPAL. A. Values corresponding to conditions a), b) and c) of the first experiment; B. Second loading experiment to confirm condition b).

Murine rAvPAL-RBCs from the first experiment were able to metabolize 72–80% L-Phe after 1 h incubation at + 37°C, with no difference among the three tested conditions (A). L-Phe consumption rate of condition b) was confirmed in the second experiment (B).

2. Preclinical studies

2.1. Efficacy of rAvPAL-RBC treatment: *dose finding study*

According to the results from the *in vitro* experiments, condition b) was selected to perform this study. A bulk loading procedure was used, obtaining at the end 2.34 ml of RBCs at 54% Ht loaded with 16.7 IU rAvPAL (corresponding to 13.2 IU rAvPAL/ml RBCs 100% Ht). The actual initial amount of rAvPAL dialyzed with the RBC suspensions resulted to be 41 IU, not different from the nominal value of 42 IU. Table 3 summarizes the results and RBC parameters of the loading procedure. Differences between loaded RBCs of *in vitro* and *in vivo* studies are likely due to intrinsic cell variability among animals.

Table 3. Loading results and RBC parameters of the procedure performed for the *dose finding* study.

RBC dialysis Ht	Added rAvPAL (IU)	Loaded rAvPAL (IU/ml RBCs 100% Ht)	RBC recovery (%)	MCV (µm³)	MCH (pg)	MCHC (g/dl)	RDW (%)	AnnexinV (%)
50%	42	13.2	19.2	43	12.2	28.4	21.3	1.5
Control native RBCs[d]				48	19.2	40.4	13.8	0.3

[c]rAvPAL entrapment was calculated in respect to the actual total initial amount of enzyme IU measured after dialysis. [d]Reference values belong to RBCs prior to be submitted to the procedure.

Loaded RBCs had a final Ht value of 54%. The suspension was serially diluted in Hepes solution to 36%, 18% and 9% final Ht, corresponding to 4.75, 2.37 and 1.18 IU rAvPAL/ml, respectively, in order to administer the scheduled doses in the same volume of 250 µl. Mean basal L-Phe values before

treatment (± standard deviation, SD) were 1,186 ± 342 µM, 1,221 ± 149 µM and 1,292 ± 211 µM in groups that received 1, 0.5 and 0.25 IU/mouse respectively, and were not significantly different ($p > 0.05$ by ANOVA).The treatment with different doses of rAvPAL-RBCs yielded the results shown below.

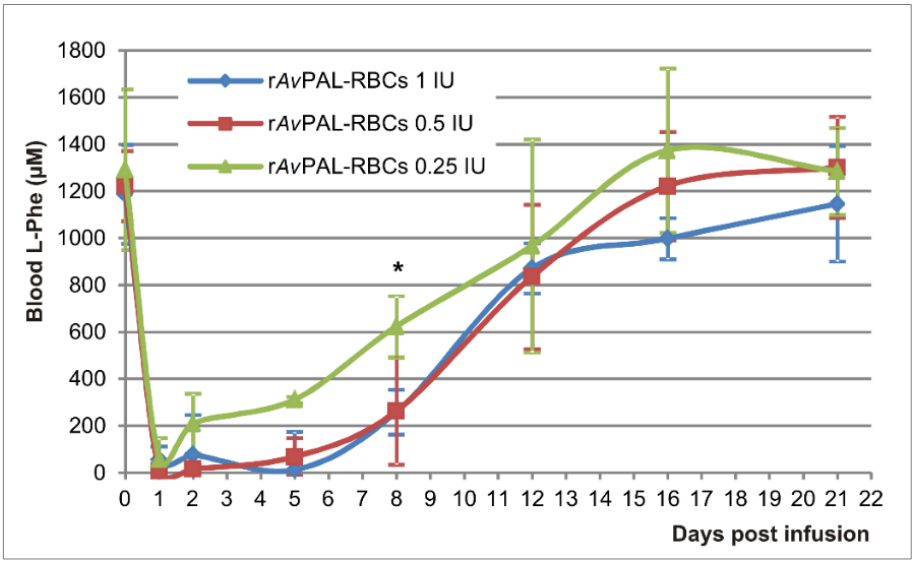


Figure 15. Blood L-Phe concentrations (mean ± SD) before and after rAvPAL-RBC injection.

All the three doses were successful in causing blood L-Phe to decrease steeply in the first hours after treatment, with low peak values reached 24 h from rAvPAL-RBC injection. In fact, after one day, injection of RBCs with 1, 0.5 and 0.25 IU enzyme/mouse produced L-Phe values of 54.8 ± 90.5 µM, 5.6 ± 6 µM and 57.1 ± 56 µM respectively. The lowest tested dose, however, was not able to maintain the reduced amino acid levels beyond the first day. As shown in Figure 15, L-Phe slowly returned towards basal levels, reaching on day 8 after rAvPAL-RBC administration roughly 50% of the pre-treatment values (622.7 ± 95.46 µM), and raising up to 74% (966.6 ± 107.03 µM) on day 12.

The two higher doses had a longer duration of action; their effect remained maximal for the first 5 days after infusion, with slower return to basal levels. Blood L-Phe in groups receiving 0.5 IU/mouse and 1 IU/mouse reached on day 8 after infusion 25% and 21% of their respective starting concentration, and then raised up to 68% and 73% on day 12, respectively. All doses were significantly effective until 8 days after treatment (by two-way ANOVA followed by Dunnett's test, $p < 0.05$ *vs* pre-treatment); however, the reduction in blood L-Phe produced by the two highest doses of enzyme was statistically superior (*$p < 0.05$) to the one resulting from the lowest tested dose on day 8 after administration. Moreover, there was no significant difference between 0.5 IU and 1 IU rAvPAL on day 8 after treatment (*$p < 0.05$, by two-way ANOVA followed by Tukey's test). The results obtained from the evaluation of blood L-Phe over time suggest a minimal dose of 0.5 IU/mouse as the best one to perform the subsequent preclinical study. Time-dependent L-Phe values of the single groups of mice involved in the *dose finding* study are reported in Figure 16.

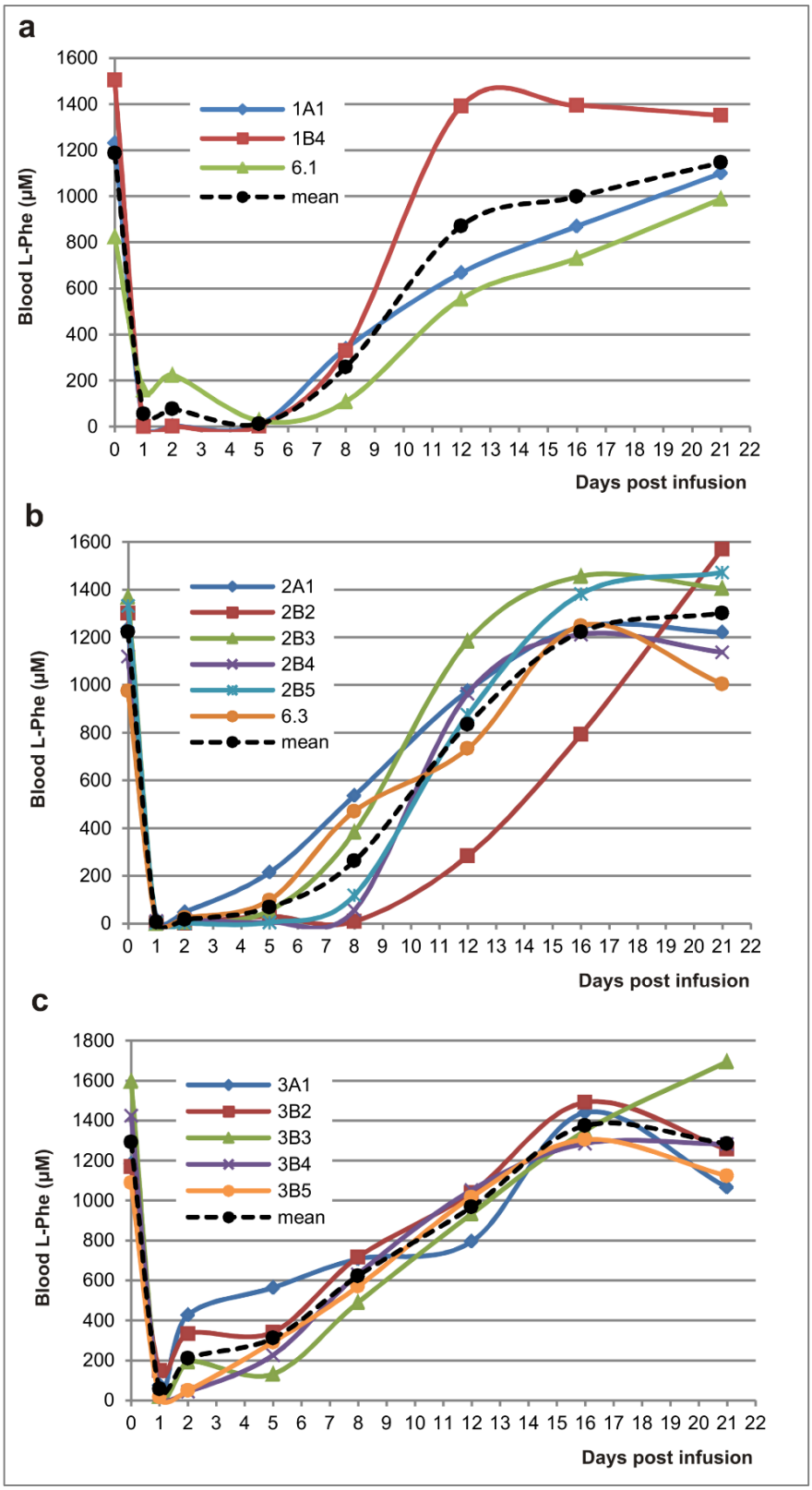


Figure 16. Blood L-Phe concentrations before and after administration of rAvPAL-RBCs (1, 0.5 and 0.25 IU/mouse) in three separate groups of BTBR-Pah^{enu2} mice. Individual data of mice receiving **a)** 1 IU/mouse (n = 3), **b)** 0.5 IU/mouse (n = 6) and **c)** 0.25 IU/mouse (n = 5). Acronyms refer to mice identification numbers.

The variability encountered within groups was in part due to the technical difficulty in performing the intravenous injection in the tail vein of this particular mouse strain. This fact might have influenced the treatment outcome, with mice possibly receiving a partially reduced dose.

2.2. Preliminary evaluation of anti-rAvPAL IgG concentrations

The production of anti-rAvPAL IgGs was evaluated in plasma samples 21 days after treatment by standard indirect ELISA. The results are reported in Figure 17.

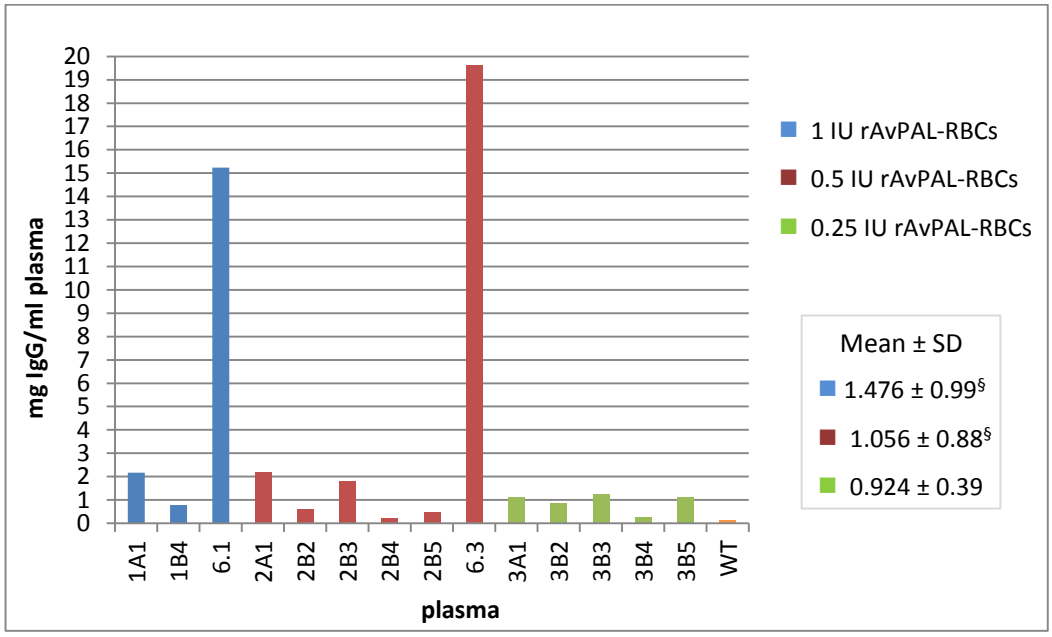


Figure 17. Anti-rAvPAL IgG concentrations of individual mice from the *dose finding* study. Plasma from a BTBR wild type mouse was included as negative control. §Mean ± SD values of the first and second group do not include the outlying concentrations measured in mice 6.1 and 6.3, respectively.

Two mice included in this study, (namely mouse 6.1 in the 1 IU/mouse group and mouse 6.3 in the 0.5 IU/mouse group) had already been treated with a single i.v. infusion of free rAvPAL approximately 6 months earlier, during a previous *in vivo* study; therefore, measured IgG titers in these cases were many fold higher than the remaining "naïf" mice of the groups they belonged. However, the higher IgG titers did not seem to affect the efficacy of the second treatment even in these sensitized mice. Figure 18a and b shows a comparison between L-Phe levels of these mice and the mean value calculated from the remaining mice of their respective groups. As concerns all the other mice treated for the first time, a very low antibody titer was detected, whose level seemed to depend on the dose of rAvPAL-RBCs they received.

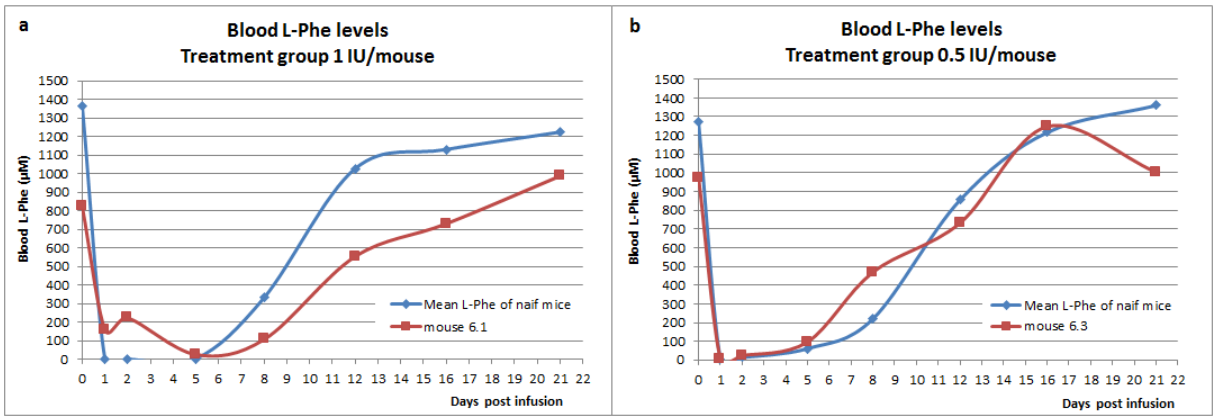


Figure 18. Comparison between L-Phe values of sensitized mice and the mean values of their respective groups. a) mouse 6.1 *vs* mean L-Phe 1 IU/mouse group (n = 2); b) mouse 6.3 *vs* mean L-Phe 0.5 IU/mouse group (n = 5).

These mice could be involved in a second *in vivo* study since they had previously been submitted to veterinary medical evaluation and were judged healthy and suitable for other experiments. We have decided to include them in our study since our main purpose was to assess the efficacy of the treatment, regardless of antibody production. IgG titers have been evaluated just to have a preliminary indication for the subsequent *repeated administration* study, where quantification of antibody production was one of the main aims in the experimental design.

2.3. Efficacy of rAvPAL-RBC treatment: *repeated administration* study

With this study, we aimed at assessing the long-term efficacy of rAvPAL-RBC administrations of a minimum dose of 0.5 IU/mouse in keeping blood L-Phe as near as possible to physiologic values. We performed two series of loading procedures whose results are summarized in Table 4. It is to be noted that annexin V binding maintained the 5-fold increase between basal values and loaded RBCs, indicating that approximately 95% administered erythrocytes would have circulated *in vivo* as long as native cells.

Table 4. Hematological parameters of rAvPAL-RBCs of the *in vivo repeated administration* study, Step 1 and Step 2.

Loaded rAvPAL (IU/ml RBCs 100% Ht)		RBC Recovery (%)	MCV (µm³)	MCH (pg)	MCHC (g/dl)	RDW (%)	Annexin V (%)
Step 1 (n = 3)	17.2 ± 5.69	49 ± 10.45	37 ± 1.83	12.2 ± 1.47	32.88 ± 2.95	17.53 ± 0.47	5.03 ± 1.55
Step 2 (n = 7)	17.97 ± 4.2	45.79 ± 11.3	37.14 ± 1.86	12.69 ± 2.12	33.79 ± 4.34	18.01 ± 0.91	4.6 ± 1.1
Control erythrocytes (range)[e]			48–50	16.5–22.2	33.7–45	13.6–15	1.3 ± 0.4

[e]Reference ranges are the minimum and maximum values observed in overall murine blood before being submitted to the loading procedure, except for Annexin V, for which the mean ± SD value is reported. Data are means ± SD. n = number of independent loading procedures.

2.3.1. *Step 1* – rAvPAL-RBC infusions at 18-19 day intervals

Five BTBR-Pah^{enu2} mice were treated with three i.v. injections of 0.67 ± 0.07 IU/mouse rAvPAL-RBCs, an amount of enzyme selected according to the *dose finding* study, which suggested a minimum dose of 0.5 IU/mouse. We decided to test a time span between injections similar to the duration of the pervious preclinical study. Blood L-Phe levels obtained in this first step are shown in Figure 19a. The treatment was able to decrease the amino acid levels to values near normality on the 4th – 5th day after each injection; the subsequent determination on day 13-14 revealed values back to pre-treatment condition. The second infusion probably went wrong, in the sense that some of the mice might have received a partially or completely subcutaneous rather than intravenous injection; this would explain the higher L-Phe levels and the broader standard deviation obtained following this treatment.

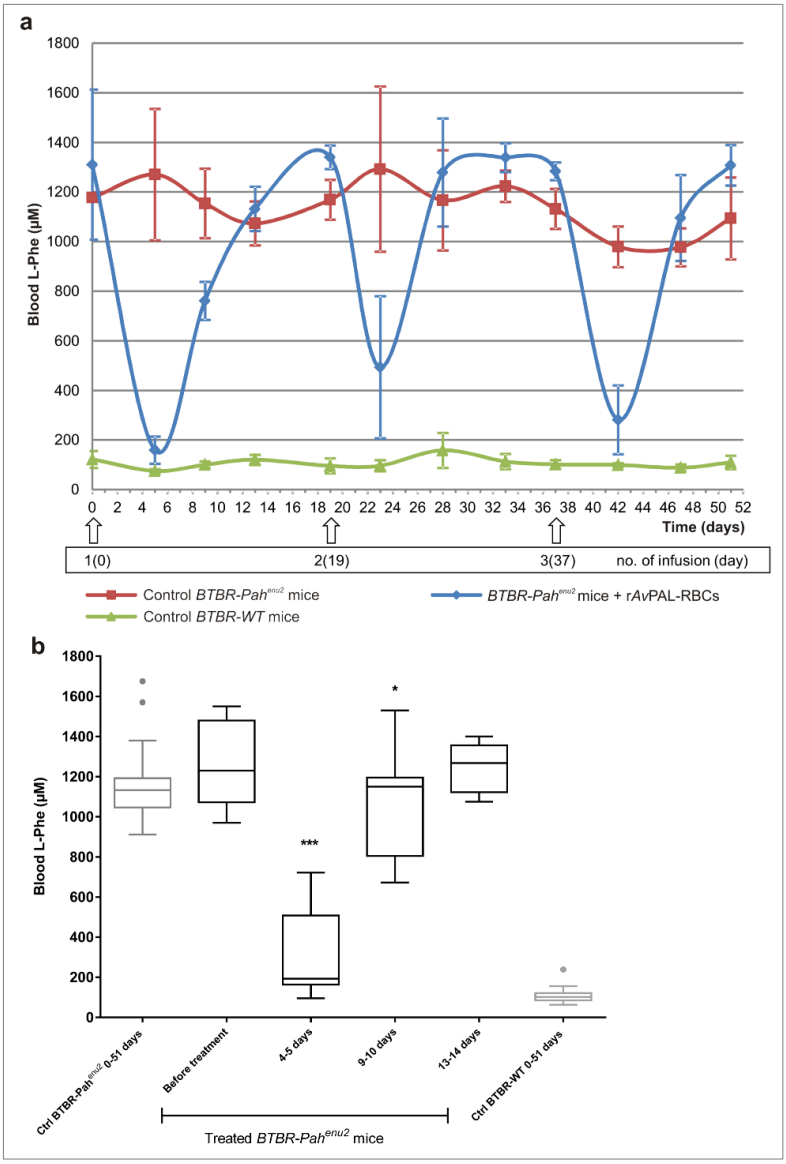


Figure 19. Blood L-Phe levels in control mice and BTBR-Pah^{enu2} mice treated three times with rAvPAL-RBCs every 18–19 days. **a)** Time-course representation of mean L-Phe ± SD values of control (n = 5 for both control groups) and treated mice (n = 5). **b)** Box-and-Whiskers plot of L-Phe values in control (Ctrl) and treated mice; the effect was significant by one-way ANOVA followed by Dunnett's test for multiple comparisons ($p < 0.05$ *vs* before treatment) up to 9–10 days after rAvPAL-RBC administrations. Control mice received i.v. injections of Hepes solution.

For a better understanding of significance, data were grouped by day after administration and also graphed by Box-and-Whiskers plot (Fig. 19b); from this plot, it is evident that 9 – 10 days is the longest possible time interval between administrations capable of maintaining blood L-Phe at concentrations significantly lower than pathological.

The treatment efficacy was also phenotypically evident from the colour change (darkening) of mouse fur (Figure 20).

Figure 20. Fur pigmentation of a BTBR-Pah^{enu2} mouse involved in Step 1 of the *repeated administration* study. Pictures were taken at time 0 before infusions (left), 9 days after the 2nd i.v. (middle) and 20 days after the 3rd i.v. administration of rAvPAL-RBCs (right).

As it is known that excess L-Phe also inhibits L-Tyr conversion to melanin besides its transformation into neurotransmitters, blood L-Tyr levels were also assessed in parallel with L-Phe on the same DBS by tandem mass spectrometry, to verify if RBC administrations could have increased L-Tyr levels and therefore determined the darker fur we observed in treated mice. Blood L-Tyr resulted to be constant and superimposable to those of control, Hepes-receiving BTBR-Pah^{enu2} mice (Figure 21).

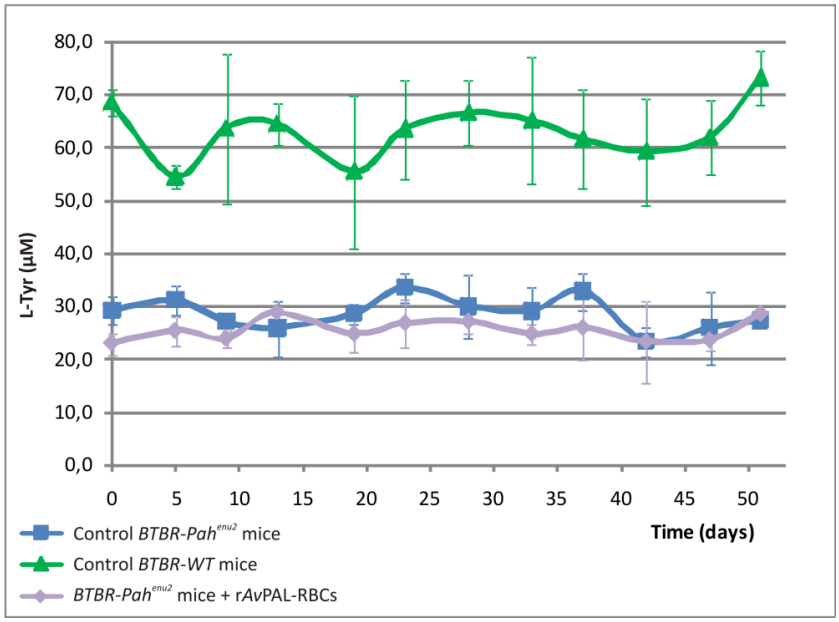


Figure 21. Time course of blood L-Tyr levels in both control and treated mice included in Step 1 of the *repeated administration* study.

2.3.2. *Step 1* – anti-rAvPAL IgG production

The evaluation of anti-rAvPAL plasma IgG titers (Figure 22) revealed an increasing presence of antibodies following the repeated injections. As expected from previous results, the first infusion had minimal effect on IgG production, while subsequent administrations resulted in a strong and increasing boost in the immune response, which however was not able to affect the ability of loaded erythrocytes to lower blood L-Phe.

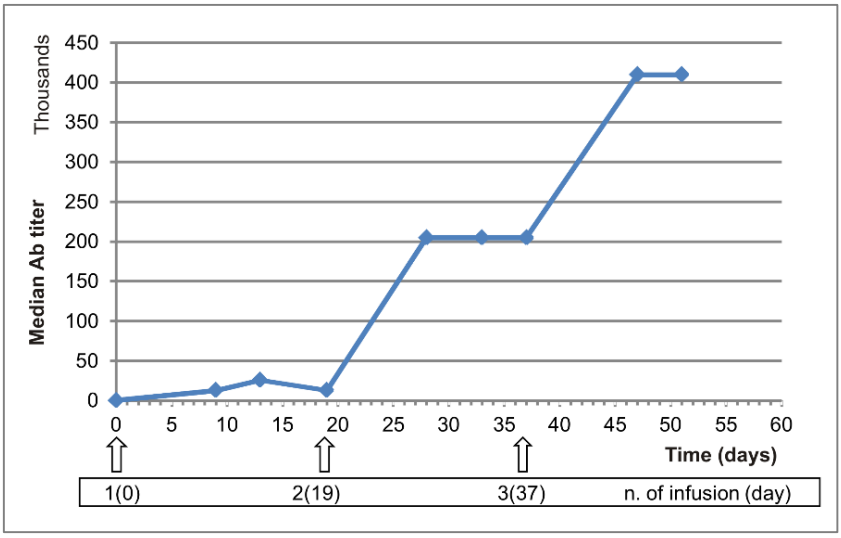


Figure 22. Median antibody titers of BTBR-Pah^{enu2} mice treated with rAvPAL-RBCs every 18 – 19 days (Step 1, n = 5). Blood samples were collected at time 0 before each infusion and at time 9 – 10 days and 13 – 14 days after each infusion. Samples were collected on day 21 after the last treatment, too.

2.3.3. *Step 2* – rAvPAL-RBC infusions at 9-10 day intervals

As a result of the first step of the *repeated administration* study, a time lag of 9 – 10 days between injections was selected for Step 2. Eight BTBR-Pah^{enu2} mice were treated seven times with i.v. injections of 0.67 ± 0.07 IU/mouse rAvPAL-RBCs and L-Phe biochemically monitored throughout the duration of the experiment. The values are reported as time-dependent variable in Figure 23a; the seven administrations managed to keep blood L-Phe within the range formed by the values measured in control BTBR-Pah^{enu2} and control BTBR-WT mice (mean ± SD = 1,137.99 ± 127.19 µM and 115.30 ± 50.40 µM, respectively). All the infusions were able to significantly decrease the amino acid levels to a similar extent, reaching values near those of healthy mice 4 – 5 days after each treatment, and then returning to 65% of pre-treatment amounts 9 – 10 days after each administration, thus confirming what previously observed in Step 1.

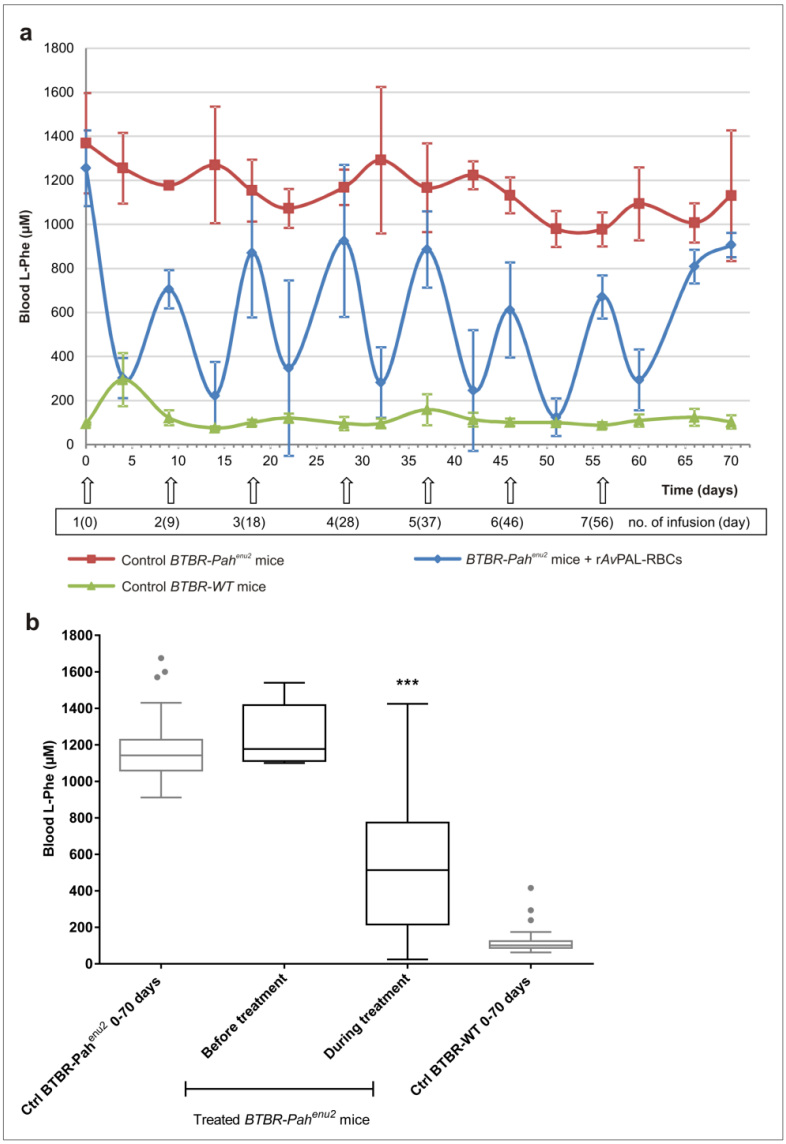


Figure 23. Blood L-Phe levels in BTBR-Pah^{enu2} mice treated seven times with rAvPAL-RBCs every 9 – 10 days. **a)** Time-course representation of mean L-Phe values ± SD of control (n = 5 for both groups) and treated mice (n = 8). **b)** Box-and-Whiskers plot of L-Phe values in control (Ctrl) and treated mice; for the whole duration of the study (10 weeks), rAvPAL-RBC injections maintained L-Phe significantly below the starting condition (by non parametric ANOVA followed by Dunn's test, $p < 0.05$). The set named "During treatment" comprises all values from day 4 after the 1st i.v. to day 14 after the 7th administration. Control mice received i.v. injections of Hepes solution.

Like for the previous step, a Box-and-Whiskers plot was made grouping all values obtained from treated mice in a single data set; the reduction observed in the whole mean L-Phe resulted to be statistically significant. A supplementary confirmation of the efficacy of this treatment was provided by the estimation and subsequent comparison of the Area Under the Curves (AUCs) representing L-Phe values during the entire experimental time (70 days). From this evaluation, a 51.6% reduction in blood L-Phe concentration could be detected in treated mice as compared to BTBR-Pah^{enu2} controls. Fur darkening was observed in these mice as well (Figure 24), confirming the effect already seen in the previous Step 1.

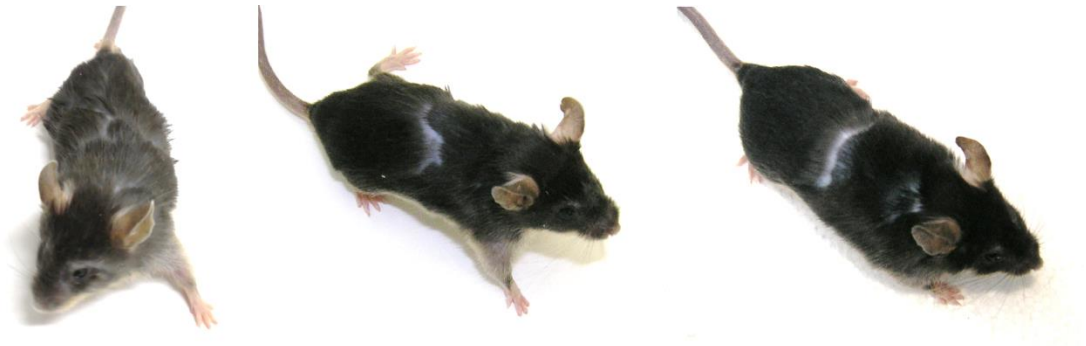


Figure 24. Fur pigmentation of a BTBR-Pah^{enu2} mouse involved in Step 2 of the *repeated administration* study. Pictures were taken at time 0 before infusions (left), 9 days after the 4th infusion (middle) and 10 days after the 7th infusion of rAvPAL-RBCs (right).

Mean blood L-Tyr levels were comparable between control and treated BTBR-Pah^{enu2} mice, thus excluding again any contribution by the erythrocyte content in blood tyrosine concentration (Figure 25).

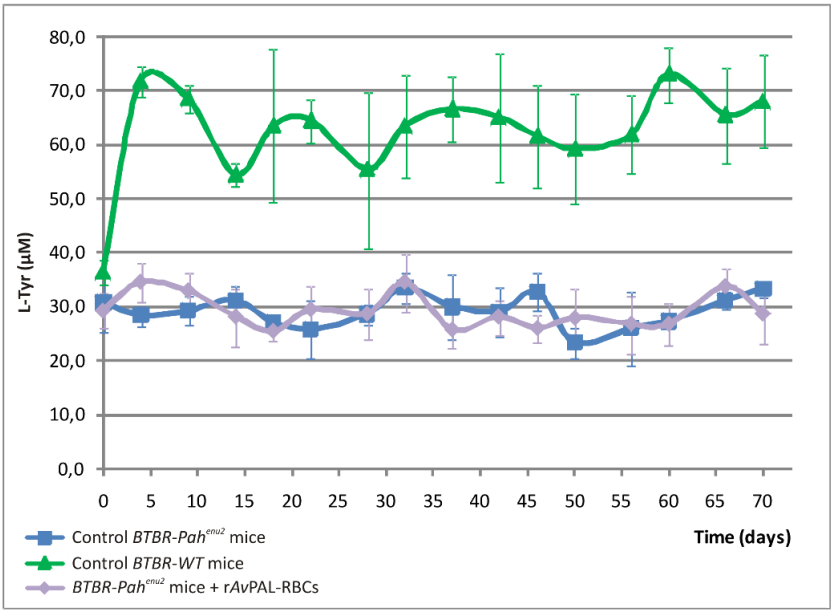


Figure 25. Time course of blood L-Tyr levels in both control and treated mice included in Step 2 of the *repeated administration* study.

2.3.4. *Step 2* – anti-rAvPAL IgG production

Plasma anti-rAvPAL IgG levels were determined for the whole duration of the study, withdrawing blood samples before each infusion and 10 and 21 days after the last administration. Like previously observed in Step 1, plasma IgG titers rose increasingly following the repeated injections (Figure 26), peaking at a similar titer as seen before. However, the steep IgG increase did not affect the treatment effectiveness, since the last infusions lowered blood L-Phe to the same extent of the first ones.

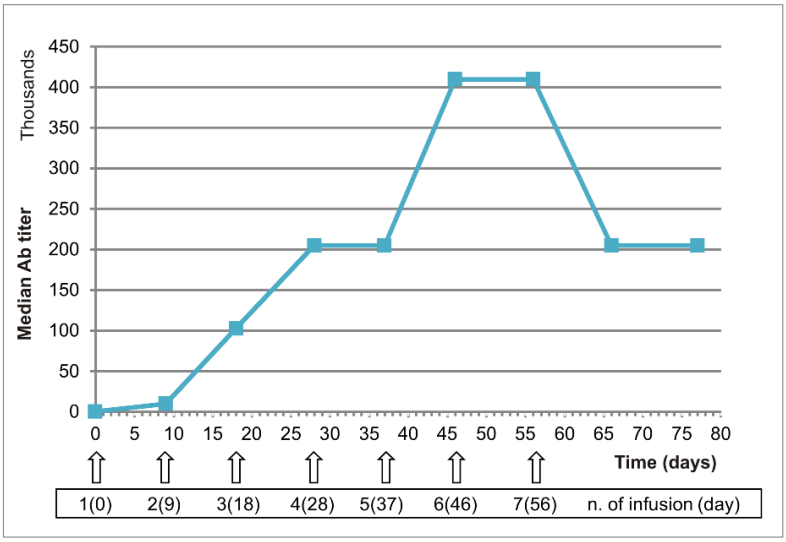


Figure 26. Median antibody titers of BTBR-Pah^{enu2} mice treated with seven rAvPAL-RBC infusions at 9 – 10 day intervals (Step 2, n = 8). Blood samples were collected at time 0 before each infusion and on days 10 and 21 after the last administration.

3. Loading of hexokinase in human RBCs with the Red Cell Loader®

3.1. Optimization of the loading procedure

Three loading procedures were performed, which changed only in their contact time between erythrocytes and hexokinase. The main hematological parameters and results of the three procedures are reported in Table 5.

Table 5. Results and hematological parameters of three different loading procedures with a fixed total nominal amount of HK (25,000 IU).

RBC-HK contact time	Actual initial HK (IU)	Loaded HK (IU/ml RBCs 100% Ht)	HK entrapment (%)	RBC recovery (%)	MCV (µm^3)	MCH (pg)	MCHC (g/dl)	RDW (%)
3 min	24,524.2	399.0	12.7	38.6	87	21.4	24.6	18.4
5 min	28,882.3	371.9	10.9	43.1	86	19.7	23.0	19.1
10 min	29,543.8	411.4	12.5	46.0	84	19.6	23.4	18.5
Whole blood (mean ± SD)[f]					86.3 ± 0.6	32.3 ± 3.2	35.4 ± 0.1	12.7 ± 0.4

[f] Reference values are calculated on the basis of three CBC analyses performed on blood from a single donor.

The best RBC recovery could be obtained with the standard procedure (10 min RBC-HK contact time), whereas HK entrapment was comparable among the three conditions. RBC parameters show a worsening tendency as contact time increases, probably due to the longer incubation in hypotonic environment. All these values were obtained by single procedures (except for the third condition which had been tested several times in past studies), which can only give preliminary indications to orient future experiments. On these bases and while waiting for future confirmation, we decided to maintain the standard procedure (i.e. 10 min RBC-HK contact time) to perform the subsequent experiments.

3.2. Loaded RBC membrane stability in different storage buffers

RBC stability was evaluated by measuring free Hb and free HK activity in the supernatants of final bags buffered with different solutions after 30 min and 2 h incubation at room temperature, followed by 5 min high-speed centrifugation at 5000 *g* (*stress test*). The analyses yielded increasing values for both parameters (Figure 27). Phosphate buffer resulted to be the best one to prevent RBC membrane damages in stress conditions, as suggested by a very low leakage level of both Hb and HK.

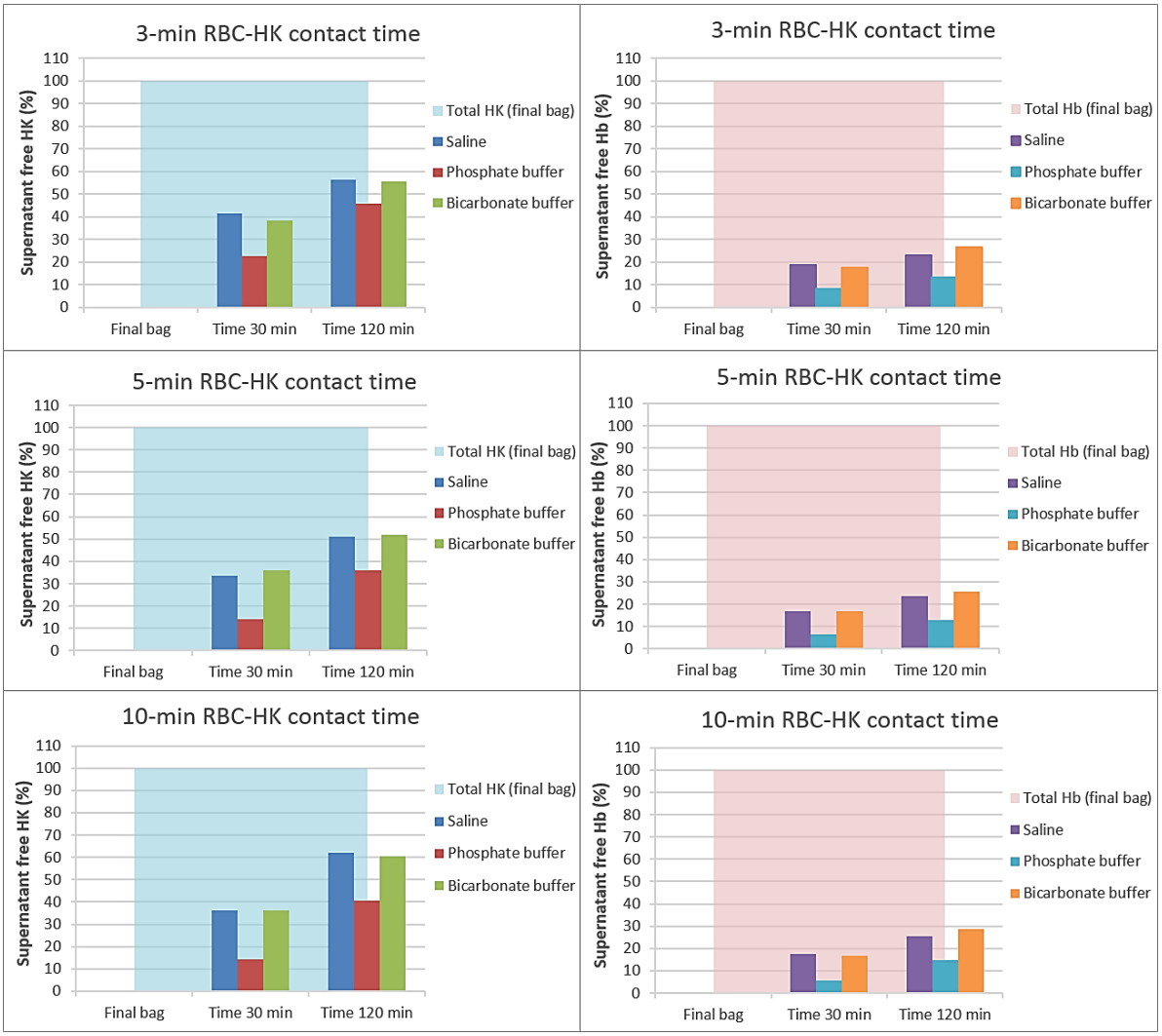


Figure 27. Percentage free HK activity (left column) and free hemoglobin (Hb, right column) measured in supernatants of final bags from the three procedures buffered with 0.9% NaCl (saline), 20 mM NaH_2PO_4 (phosphate buffer) and 20 mM $NaHCO_3$ (bicarbonate buffer) after the stress test. The shaded areas representing total HK and total Hb refer to values analyzed in final bags before splitting in the three conditions. Percentage Hb values were calculated by comparing supernatant free Hb of each condition to the total Hb value resulting from CBC analysis of each respective sample before centrifugation. Data sets named "Final bag" indicate free Hb and HK amounts in the aqueous phase of final bags, which were assumed to be both null based on previous EryDel's tests.

Given the better performance of phosphate buffer in reducing supernatant HK and Hb independently of the loading conditions, we decided to perform a stability test on final bags incubated with increasing concentrations of such solution.

3.3. Loading of HK in standard conditions

In this second part, the loading procedure was carried out in triplicate with 10 min RBC-HK contact time. Whole blood was from a single donor, and was submitted to CBC analysis before each process. The results of the procedures are reported in Table 6, while time-dependent mean hematological parameters of final bags and tested conditions are shown in Table 7a and b.

Table 6. Results of HK loading in standard conditions (mean ± SD of 3 procedures).

RBC-HK contact time	Total nominal HK (IU)	Actual initial HK (IU)	Loaded HK (IU/ml RBCs 100% Ht)	HK entrapment (%)	RBC recovery (%)
10 min	25,000	22,277.07 ± 3,274.25	354.5 ± 33.75	14.2 ± 2.1	46.9 ± 2.2

Table 7a. Hematological parameters (MCV and MCH) of whole blood, final bags and loaded RBCs in four different buffers. Values are mean ± SD of 3 procedures.

	MCV (µm³)			MCH (pg)		
	Time 0[h]	Time 30 min	Time 120 min	Time 0[h]	Time 30 min	Time 120 min
Whole blood[g]	84.3 ± 0.6	-	-	29.2 ± 0.1	-	-
Final bags	79.7 ± 3.5	-	-	15.9 ± 0.5	-	-
Saline	79.3 ± 2.1	81.7 ± 1.5	87.5 ± 2.1	15.6 ± 0.5	16.1 ± 0.5	15.8 ± 0.5
Phosphate buffer 10 mM	80.7 ± 3.2	87.0 ± 3.6	91.0 ± 1.0	15.6 ± 0.5	15.7 ± 0.6	16.0 ± 0.7
Phosphate buffer 20 mM	79.3 ± 2.9	86.7 ± 4.2	93.0 ± 2.0	15.7 ± 0.6	15.6 ± 0.5	16.2 ± 0.6
Phosphate buffer 30 mM	78.0 ± 3.5	84.7 ± 4.2	89.3 ± 2.1	15.7 ± 0.6	15.6 ± 0.4	16.1 ± 0.7

Table 7b. Hematological parameters (MCHC and RDW) of whole blood, final bags and loaded RBCs in four different buffers. Values are mean ± SD of 3 procedures.

	MCHC (g/dl)			RDW (%)		
	Time 0[h]	Time 30 min	Time 120 min	Time 0[h]	Time 30 min	Time 120 min
Whole blood[g]	34.6 ± 0.2	-	-	11.5 ± 0.2	-	-
Final bags	20.0 ± 1.2	-	-	23.1 ± 1.7	-	-
Saline	19.7 ± 0.7	19.7 ± 1.0	18.3 ± 0.1	23.8 ± 1.1	25.7 ± 1.4	28.5 ± 1.5
Phosphate buffer 10 mM	19.3 ± 0.9	18.1 ± 0.8	17.6 ± 0.7	23.2 ± 0.6	21.5 ± 2.7	24.5 ± 2.0
Phosphate buffer 20 mM	19.7 ± 0.7	18.0 ± 1.2	17.4 ± 0.6	23.1 ± 0.5	21.8 ± 1.0	21.2 ± 1.0
Phosphate buffer 30 mM	20.2 ± 0.9	18.5 ± 1.0	18.0 ± 0.7	22.4 ± 0.7	22.4 ± 2.1	21.7 ± 1.3

[g] Mean ± SD values of whole blood are the results of 3 analyses performed on the same initial blood.

[h] Time 0 hematological parameters of whole blood refer to values measured before the procedures; values of final bags refer to the end of the procedure, before splitting in the different buffers. After splitting, RBC indices were evaluated again and then followed over time.

At the end of the loading procedures, a mean volume (± SD) of 79 ± 0.3 ml loaded RBCs was obtained. Final bags were split in four conditions, i.e. three NaH_2PO_4 buffers at different final concentrations (10, 20 and 30 mM) and saline as control. After 30 min and 2 h incubation at room temperature, the percentage of free Hb and free HK activity in supernatants of centrifuged samples were as reported in the tables and graphs below.

Table 8. Mean ± SD (n = 3 procedures) of supernatant free Hb and free HK activity in loaded RBC samples after incubation in different buffers and high-speed centrifugation.

	Supernatant free Hb (%)[i]		Supernatant free HK (%)[j]	
	Time 30 min	**Time 120 min**	**Time 30 min**	**Time 120 min**
Saline (control)	26.1 ± 3.6	36.7 ± 4.3	37.3 ± 12.0	55.1 ± 12.5
Phosphate buffer 10 mM	15.6 ± 3.6	30.9 ± 4.9	27.8 ± 8.9	51.9 ± 16.3
Phosphate buffer 20 mM	8.5 ± 2.1	23.7 ± 4.7	12.1 ± 4.1	42.8 ± 12.6
Phosphate buffer 30 mM	8.1 ± 2.0	18.7 ± 3.7	8.8 ± 2.8	31.7 ± 11.6

[i] Percentage values were calculated assuming as 100% Hb the values resulting from CBC analysis of each one's respective sample after incubation and before centrifugation. [j] Percentage free HK was calculated assuming as 100% the value resulting from the total HK assay of final bags.

As expected, both supernatant free hemoglobin and free hexokinase activity increased during incubation in stressing conditions (RT incubation followed by centrifugation at 5000 *g*) in the presence of each of the tested buffers. The sample incubated in saline solution showed the highest amount of the two proteins, whose time-dependent increase, however, gradually diminished as NaH_2PO_4 concentration rose.

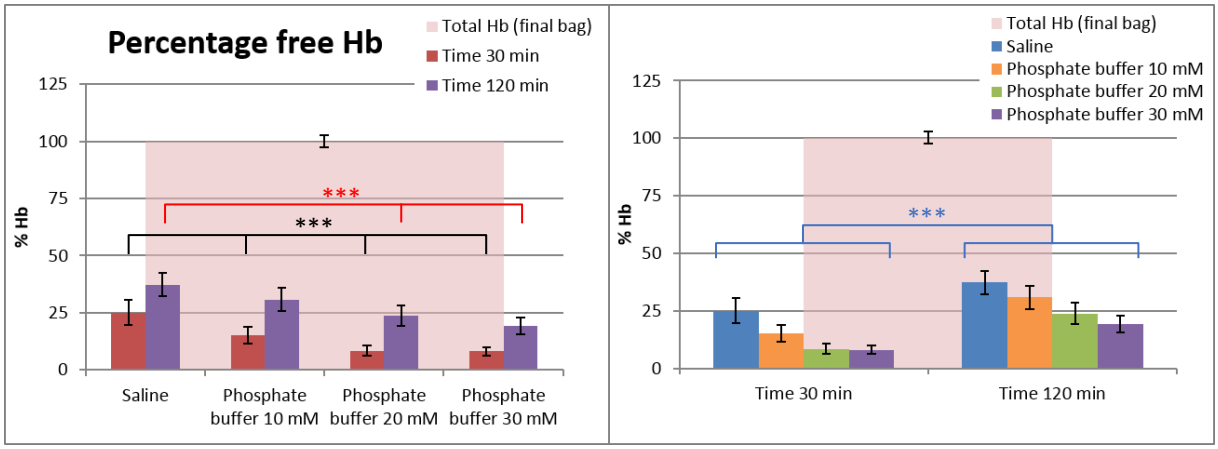


Figure 28. Graphical representation of supernatant free hemoglobin after stress test of loaded RBC samples. Values are mean ± SD of 3 procedures. The mean Hb value resulting from CBC analysis of final bags was considered as total Hb. Percentages were calculated referring to Hb values resulting from CBC analysis of each one's respective sample after incubation and before centrifugation. Data were grouped by tested condition (left) and by time of analysis (right). ***$p < 0.05$, Time 30 min, saline *vs* phosphate buffer 10 mM, *vs* phosphate buffer 20 mM and *vs* phosphate buffer 30 mM; ***$p < 0.05$, Time 120 min, saline *vs* phosphate buffer 20 mM and *vs* phosphate buffer 30 mM. ***$p < 0.05$, all conditions, Time 30 min *vs* Time 120 min.

In respect of free Hb (Figure 28), the effect of both time and buffer was very significant ($p < 0.01$ by two-way ANOVA); after 30 min incubation, saline was significantly less effective in preventing Hb leakage as compared to all other buffers. No difference was detected between phosphate buffers 20 mM and 30 mM. A similar significance rate could be observed after 2 h incubation, except for saline solution and phosphate buffer 10 mM, whose effect was not significantly different at this time point ($p < 0.05$, by two-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons).

When time points within each tested condition were compared to each other by the same statistical test, a significant variation was revealed between mean values at Time 30 min and Time 120 min ($p < 0.05$) for all conditions.

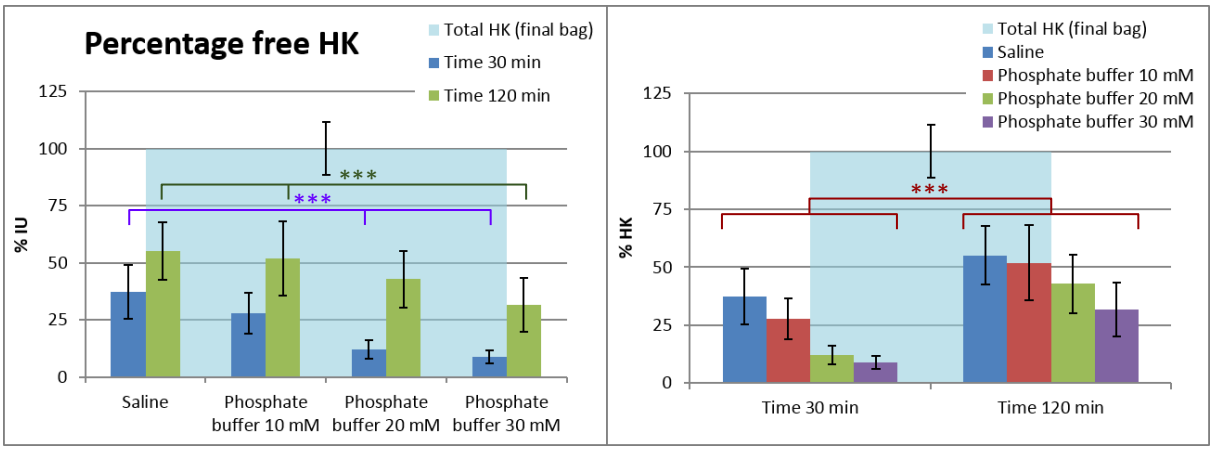


Figure 29. Graphical representation of supernatant free hexokinase activity after incubation of loaded RBC samples in different buffers in stress conditions. Values are mean ± SD of 3 procedures. The mean result of total HK assays in final bags was assumed as 100% HK. Data were grouped by tested condition (left) and by time of analysis (right). ***$p < 0.05$, Time 30 min, saline *vs* phosphate buffer 20 mM and *vs* phosphate buffer 30 mM; ***$p < 0.05$, Time 120 min, saline *vs* phosphate buffer 30 mM and phosphate buffer 10 mM *vs* phosphate buffer 30 mM. ***$p < 0.05$, all buffers, Time 120 min *vs* Time 30 min.

As regards free HK activity (Figure 29), the two-way ANOVA analysis revealed a decisive effect of the variable Time on HK in sample supernatants, whereas the tested buffers seemed to have a small influence on that ($p < 0.05$). If comparing the different conditions at the single time points, phosphate buffer 20 mM and 30 mM both had a significantly greater effect on free HK levels than saline solution at Time 30 min, whereas there was no difference among the three phosphate buffers. At Time 120 min, only phosphate buffer 30 mM proved to be better than both saline and phosphate buffer 10 mM, but no significant difference was detected compared to phosphate buffer 20 mM ($p < 0.05$, by two-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons).

The comparison between time points of the single conditions instead yielded a significant time dependent effect for all buffers ($p < 0.05$, by two-way ANOVA, followed by Tukey's *post hoc* test).

To make sure of actually evaluating membrane integrity, excluding that both Hb and HK increase was due to centrifugation-related RBC lysis, sample RBC counts before and after centrifugation were compared to each other. Data are reported in Figure 30.

The statistical analysis revealed no significant change in sample RBC numbers between conditions before and after centrifugation, nor differences were observed between buffers and control saline solution at each time point (by two-way ANOVA followed by Dunnett's test, $p > 0.05$ within each time point *vs* control saline, and $p > 0.05$ each condition after centrifugation *vs* before centrifugation).

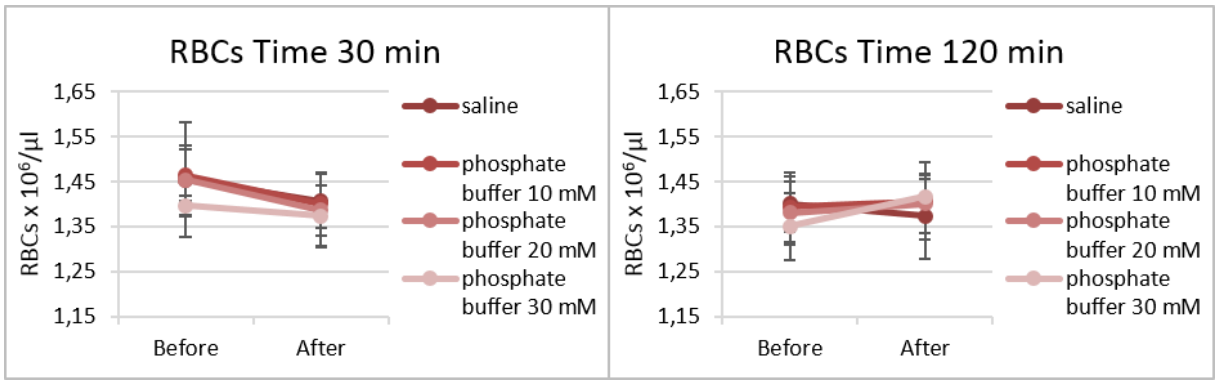


Figure 30. RBC counts before and after centrifugation at indicated incubation times. No significant change was detected following centrifugation in all the conditions analyzed, nor differences were revealed between buffers and control saline solution ($p > 0.05$, by two-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons).

Data emerging from the stability assay in different buffers suggest that the amount of both free hemoglobin and free hexokinase found in the supernatant fraction of samples increases over time in a time-dependent manner, but seems to be not contributed to by RBC lysis. The observed increase in Hb and HK should be therefore due only to membrane damages, and appears to be limited by the presence of rising concentration of NaH_2PO_4 in the final bags, with best results obtained with phosphate buffer 30 mM.

DISCUSSION

The genetic disease phenylketonuria (PKU) has been the first inborn error of metabolism to have been recognized as having a precise chemical etiology, i.e. hyperphenylalaninemia, due to a lack of activity of the hepatic enzyme phenylalanine hydroxylase (PAH, EC 1.14.16.1). Phisiologically, PAH metabolizes the amino acid L-Phe to synthesize other biologically important molecules (such as neurotransmitters) and preventing in the meanwhile its accumulation in body fluids. The severity of the clinical neurological manifestations characterizing untreated patients, the widespread diffusion of the disease itself (with its broad phenotypic variety), as well as the understanding of the etiologic agent have been altogether encouraging efforts in the field of therapy research and development since the first years after PKU discovery in the mid-1930s.

The golden standard of PKU treatments is still represented by a strict L-Phe-restricted diet, aimed at introducing only the amino acid amount necessary for normal development and functioning of the organism, according to each patient's age and specific needs. The numerous negative aspects of patient compliance to such dietary approach, both practical (poor palatability of foods, high economic cost, possible nutritional deficits) and social (psychological burden for patients and families in social contexts), and the life-long need for treatment have led to the development of alternative strategies to keep blood L-Phe near physiology, as much as possible independently of diet. Among them, a valid solution is cofactor supplementation in its pharmacological analog sapropterin dihydrochloride (Kuvan™), which has been extensively tested in clinical trials, reaching phase 4 in Europe (ClinicalTrials.gov ID NCT01082328). BH_4 supplementation proved to be effective, but only in patients with the milder forms of the disease, still maintaining a residual PAH activity. For all the other more severe forms of PKU, diet and diet-related treatments continue to be the only feasible choice able to reduce properly blood L-Phe.

In this general context, the strategy based on enzyme replacement/substitution therapy has emerged. The main purpose of this treatment is to provide a long-lasting enzymatic activity able to reduce the accumulation of toxic metabolites without adverse events. Despite its theoretical viability, this kind of approach presents some obstacles, first of all the host immune reaction against the foreign enzyme ultimately leading to its inactivation, especially upon multiple administrations. In the specific case of PKU, the non mammalian enzyme phenylalanine ammonia lyase (PAL, E.C. 4.3.1.24) has been selected due to its favorable structural and catalytic properties that make this enzyme easier to employ if compared to PAH. PAL oral [304] and subcutaneous administration [239] was tested in preclinical short-term studies, with positive results in terms of L-Phe reduction. However, longer-term L-Phe control is hampered by proteolysis and the host neutralizing antibody response, due to the far phylogenetic distance between PAL's organisms of origin and mammals. Several studies on PAL engineering to improve stability [255, 305] have ultimately led to a double mutant form of recombinant PAL from the cyanobacterium *Anabaena variabilis*, which currently represents the best performing

available variant [244, 255]. Additional PAL modifications by PEGylation have also been performed in order to further improve enzyme stability and to mask antigenic epitopes to the host immune system [253, 254]. Such strategy proved to be effective in preclinical studies on Pah-deficient mice [244, 305-307] and have led the company Biomarin Pharmaceutical Inc. (Novato, CA) to begin a clinical trial with the PEGylated form of recombinant AvPAL, administered subcutaneously [256; ClinicalTrial.gov ID NCT00925054]. The phase I clinical study in different cohorts of adult PKU patients treated with single increasing doses of rAvPAL-PEG demonstrated the efficacy of the enzyme in reducing blood L-Phe (at a dosage of 0.1 mg/kg body weight); however, even this single injection already induced the production of antibodies both against PEG and rAvPAL, causing some moderate hypersensitivity adverse events, even though the treatment was generally fairly well tolerated. The longer-term effectiveness of repeated treatment with the PEGylated enzyme is currently being investigated in phase 2 clinical trials (NCT01212744, NCT00925054, NCT01560286) but results have not been obtained yet. Phase 3 clinical trials are already starting or proceeding (NCT01889862 and NCT01819727, respectively). Although PEG has been used to modify several therapeutic molecules (mostly enzymes) thanks to its ability to attenuate the neutralizing immune response against the therapeutic agent [308], concerns about PEG metabolism and accumulation in the organism still remain [258, 309, 310], suggesting the usefulness of alternative strategies. The results of the phase I clinical study with rAvPAL-PEG confirm that, despite being convenient for enzyme pharmacokinetics, the employment of PEG as modifying agent elicits the production of specific antibodies against the PEG moiety itself, which can result in allergic manifestations or even in more severe forms of intolerance in already sensitized patients [256]. The production of anti-PEG antibodies reported by many Authors [256, 257, 310-314] could have in addition the effect of altering the PEG-conjugate biodistribution and bioavailability by acting on the complex clearance rate [315]. Administration through an opportune delivery system could therefore represent a valid and viable alternative to overcome such immunogenicity and bioavailability issues. Among the applicable methods, those exploiting red blood cells to deliver enzymes into circulation offer the best advantages [260, 261, 265-267, 274, 277]. In fact, clinical studies have demonstrated that RBCs are ideal carriers for enzymes in circulation, protecting them from premature inactivation both by plasma proteases and by neutralizing antibodies, particularly when repeated administrations are needed [287, 288, 290-292, 294]. Erythrocyte-mediated enzyme replacement therapy for PKU has been proposed for the first time in 1990 by Sprandel et al., in a short-term study demonstrating the effectiveness of this approach [296].

On these bases, therefore, our work consisted in the development of an RBC-mediated delivery system for the recombinant enzyme AvPAL, then administered to Pah-deficient mice to identify the most suitable dose and the long-term ability of repeated administrations in lowering and maintaining blood L-Phe levels as near as possible to physiologic “safe” values. Once preclinical investigations were

completed, we focused our attention on the development of the same strategy with human erythrocytes exploiting the existing EryDex technology, here applied to proteins.

In preliminary *in vitro* studies, we selected the best conditions to load rAvPAL in murine RBCs by means of a procedure of dialysis in hypotonic environment followed by isotonic resealing. The results showed that it is possible to load different amounts of enzyme by simultaneously varying both RBC hematocrit and enzyme concentration during the dialysis step. Previous studies conducted in our laboratory on RBC loading with proteins yielded different entrapment rates only when the enzyme was added in increasing amounts during dialysis [316]; therefore, this is the first evidence of the possibility to modulate the final protein content by acting also on Ht values of dialyzing RBCs.

The *in vitro* results indicate condition c in Table 2a (i.e. 40% Ht) as the best one to obtain the highest loaded amount of rAvPAL; however, the final RBC indices are not comparable to those of control native cells. This means that if these erythrocytes were injected into PKU mice, their life span in the bloodstream would be shorter and the therapeutic effect of the loaded enzyme not relevant in a long-term perspective. One of the primary objective was, indeed, to obtain final loaded erythrocytes maintaining as much as possible their starting characteristics, in order to guarantee a near normal mean half-life in circulation after reinfusion. Therefore, given the higher fragility of murine cells in comparison with human cells [303], we chose condition b (i.e. 50% Ht of dialyzing RBCs) as the best compromise between amount of loaded rAvPAL and corpuscular indices. Annexin V staining on final RBCs from the second verification experiment confirmed that RBC damage was limited to 4.9% of total loaded cells, implying that 95.1% have normal surface characteristics and would have a normal half-life *in vivo*. As expected from studies on L-Phe uptake kinetics by RBCs [317] and from rAvPAL K_m (0.060 ± 0.005) [243], murine rAvPAL-RBCs showed a pronounced ability to metabolize L-Phe, with amino acid reduction up to 80% the initial amount after 1 h incubation.

On the basis of these results, we performed the subsequent preclinical investigations using 50% Ht RBCs during dialysis. In the first *dose finding* study we aimed at evaluating the effectiveness of the strategy *in vivo* on a PKU murine model; therefore we set the experimental conditions at three scalar amounts of enzyme (0.25, 0.5 and 1 IU rAvPAL/mouse). The three doses of enzyme administered within erythrocytes proved to be able to dramatically decrease blood L-Phe levels in BTBR-Pah^{enu2} mice, peaking 24 h after treatment with no difference among doses. The lowest dose, however, showed a shorter duration of action, with blood L-Phe levels already starting to increase again from day 2 after treatment. In this mouse group, L-Phe reached back approximately 50% pre-treatment value after 8 days, in contrast with 21% and 25% of the other two doses. In fact, 0.5 and 1 IU/mouse doses seemed to be both supramaximal, in spite of a general statistically significant effect of the treatment in all the three groups until 8 days after infusion. When considering blood L-Phe on day 8 after treatment (i.e. the last day when all doses were statistically effective), both higher doses of 0.5 and 1 IU

rAvPAL/mouse proved to be statistically superior to the lowest one, but there was no difference between them; both yielded an L-Phe value around 200 µM, slight above the normal range of phisiological concentrations (50-110 µM, [34]). Indeed, rAvPAL-RBC efficacy seems to be correlated with RBC clearance from circulation. The half-life of loaded murine RBCs has been repeatedly reported to be in the range of 6 – 11 days [318-320], slightly reduced in comparison with native cells (range 12 – 14 days [320, 321]), due to a minimal loading-induced damage. However, when the total amount of administered enzyme overcomes the maximal amount needed for L-Phe metabolism, the amino acid reduction does not depend on RBC removal, since the remaining erythrocytes contain an amount of enzyme sufficiently high to ensure L-Phe metabolism; on the contrary, when the loading protocol yields a low rate of enzyme encapsulation per RBC unit, the number of circulating cells becomes limiting, with a metabolic efficacy decreasing proportionally to RBC clearance rate. In our *dose finding* study, we administered different amounts of loaded RBCs deriving from a single bulk loading procedure, and thus containing the same unitary amount of loaded enzyme. Therefore, we can suppose that the differences in treatment effects were due to the lower number of administered erythrocytes.

On the whole, these data demonstrate that blood L-Phe levels can effectively be modulated *in vivo* by injection of rAvPAL-RBCs. To perform the subsequent *repeated administration* studies we selected a minimum dose of 0.5 IU rAvPAL/mouse, given that there was no significantly different effect from the 1 IU/mouse dose.

Preliminary IgG evaluation showed a very slight induction of antibody production, apparently depending on the received dose. A high IgG production was reported only in two of the mice included in the experiment, which had already been treated with free rAvPAL in a previous preclinical study in our laboratory. In these cases, the second rAvPAL administration by RBCs induced a strong immune reaction, which however did not influence the treatment effect on L-Phe reduction, as suggested by the comparison between L-Phe levels of these mice and the mean values of their respective groups.

Moving on to the subsequent preclinical investigation, we wanted to assess the longer-term effect of this strategy in BTBR-Pah^{enu2} mice. The first step of the *repeated administration* study aimed at identifying the longer time lag between subsequent administrations of rAvPAL-RBCs allowing the maintenance of blood L-Phe levels in a safe range. Hence, we tested three injections of a dose of 0.67 ± 0.07 IU/mouse administered at 18-19 day intervals, regularly monitoring L-Phe over time. The results indicate the ability of multiple treatment to restore near physiologic L-Phe values. The effect was significant until 9-10 days after each administration, further confirming what observed as preliminary result from the *dose finding* study. Plasma antibody evaluation revealed a growing increase in IgG titers, which however exerted no influence on enzyme activity and treatment efficacy, thus confirming the validity of erythrocytes as protecting delivery system.

The removal of L-Phe excess from the circulation seemed also to restore L-Tyr metabolism, in particular the biosynthetic pathway of melanin. It is well understood that high L-Phe concentrations inhibit tyrosine hydroxylase, with consequent lack of neurotransmitter precursors and reduced activity of skin tyrosinase (E.C. 1.14.18.1), too [85, 322]. The decrease in blood L-Phe in BTBR-Pah^{enu2} mice following repeated treatment with rAvPAL-RBCs caused L-Tyr introduced with the diet to be metabolized again, with a reversal of mouse fur hypopigmentation observed at the end of the experiment, confirming the validity of the strategy notwithstanding the low number of repeated infusions.

The second step of the *repeated administration* study was carried out performing seven injections of the same dose of rAvPAL-RBCs every 9-10 days, as suggested by the first step. Treated mice experienced a reduction in blood L-Phe, whose levels kept on fluctuating between values of control healthy and PKU mice, never reaching back their respective pre-treatment level for the entire experimental period (70 days, Figure 23 of the Results section). As a whole, the amino acid levels of treated mice stayed significantly below those of control Pah-deficient mice receiving Hepes solution.

Once again as expected, antibody production was boosted by the repeated infusions, peaking at a similar titer as in Step 1. However, neutralizing IgGs had no effect on rAvPAL-RBC efficacy; the seven infusions were all able to act on L-Phe levels to the same extent, with no remarkable differences between the last administrations and the first ones.

The importance of overcoming the host immune response had already been highlighted by a previous preclinical study by Sarkissian et al. [244] on Pah-deficient mice treated with rAvPAL-PEG. One of the conclusions emerging from this remarkable work is the importance of the induction of immunologic tolerance against the therapeutic enzyme, when the latter is administered directly without carrier systems. The Authors demonstrated how the administration of gradually decreasing doses of rAvPAL-PEG (4 IU/mouse for 10 weeks, followed by another 6 week treatment with 2 IU/mouse) permitted to overcome the neutralizing effect of host antibodies, moreover providing further confirmation of the effectiveness of a therapeutic approach exploiting the PEGylated form of the enzyme. Nonetheless, a period of approximately 30 days after the first injection was necessary before blood L-Phe stabilization to sufficiently low values occurred. The strategy we applied in our work enabled us to obtain a better range of blood L-Phe levels in treated mice (100 – 900 μM), reaching amino acid concentrations closer to euphenylalaninemia than those observed after rAvPAL-PEG treatment (500 – 1200 μM). Most importantly, we achieved such effect with a lower dose of enzyme (mean 0.67 IU/mouse *vs* 1 IU/mouse, i.e. approximately half the lowest dose employed by Sarkissian and colleagues) with less frequency of administration (every 9-10 days *vs* weekly injections). The antibody response detected in the second step of the *repeated administration* study showed a decrease after the last infusion of rAvPAL-RBCs; we are inclined to interpret this phenomenon as a kind of immunologic tolerance arisen

consequently to the repeated injections of enzyme, similarly to what expressly carried out by Sarkissian et al [2008]. Further investigations would be necessary to verify such hypothesis.

Phenotypic evidence of treatment efficacy was noticed in Step 2 of the *repeated administration* study as well, with mice restoring fur pigmentation as in case of rAvPAL-PEG treatment. However, in our investigation, like for L-Phe levels, we managed to observe such effect after less frequent treatments with lower amounts of protein, further demonstrating RBC ability to protect the therapeutic enzyme since the first contact with the host immune system, while acting as circulating bioreactors.

Our approach indeed did not aim at reducing the antibody production; on the contrary, the immune response is effectively elicited due to antigen processing and presentation by macrophages, during the natural process of old/damaged RBC clearance from circulation. The major advantage concerns enzyme protection from the action of neutralizing IgGs independently of the titer, thanks to internalization inside the cell.

A similar approach has also been tested with recombinant PAH from *C. violaceum* encapsulated into erythrocytes and administered intravenously to BTBR-Pah^{enu2} mice [297]. Unfortunately, though improving enzyme pharmacokinetics, PAH-RBCs were not able to decrease blood L-Phe levels, probably because of a too low dose and/or specific activity of the administered enzyme. Another complication ensuing from the use of PAH as therapeutic enzyme is the continuous need for adequate BH_4 supplementation, to assure proper enzyme activity. Hence, the use of rAvPAL-RBCs has proved again to be a better choice of action.

Once verified the *in vivo* validity and viability of this enzyme substitution therapy approach, we moved on to the development and optimization of a protocol of loading of human erythrocytes, with the perspective of a future clinical application. To this purpose, we could exploit the technology owned by the company EryDel S.p.A. (Urbino, Italy), namely the Red Cell Drug Loading system, originally named EryDex system, consisting of a proprietary combination of an electromedical device, the Red Cell Loader® (RCL), and a disposable sterile kit, EryKit_01, projected to work together under the control of an opportune software (latest version 3.2.0) to obtain erythrocytes loaded with different therapeutic agents suitable for clinical use. The main example is provided by RBCs loaded with DEXA-21-P produced by means of RCL® and EryDex system which have been employed in several clinical studies for the treatment of many inflammatory diseases [281-284, 324-326], also obtaining the "Orphan Drug" designation by the European Medicines Agency (www.ema.europa.eu).

The successful development and optimization of a loading protocol performed by the EryDex system opportunely modified to fit to protein loading would therefore facilitate the transition of the enzyme replacement therapeutic approach from the lab to the clinics of PKU patients. Hence, we performed a series of *in vitro* experiments with human erythrocytes loaded with hexokinase, employed here as a model protein of rAvPAL, due to temporary unavailability of the latter. After a preliminary confirmation

of the optimal loading procedure applied to proteins, we also tested the possibility to further stabilize loaded RBC membranes in order to permit the long-term preservation of the final product, which, at the present status, must be reinfused immediately after the procedure, with no storage possibility. The *stress test* involving room temperature incubation followed by high-speed centrifugation aimed at verifying this hypothesis, by measuring the amount of both supernatant free Hb and HK originating from the leakage across RBC membranes. The type of stabilizing solutions employed in these tests have been chosen by EryDel Company, independently of the nature and metabolic needs of the loaded protein.

In the first experiment, the effect of both different RBC-HK contact times and addition of buffer solutions to the final product was analyzed. The results deriving from the three procedures conducted with RBC-HK contact times set at 3, 5 and 10 minutes could only orient our decisions, since only one procedure per condition could be carried out and therefore no statistical analysis was possible. We could conclude that the current standard procedure involving a 10-minute loading step allowed a better RBC recovery with comparable protein entrapment rate and acceptable RBC corpuscular indices. In fact, the shorter contact times tested here seemed to be both not sufficiently able, or at least no more than the standard protocol, to produce RBCs with parameters nearer to the range of untreated whole blood. We consequently selected the reference standard protocol for the following experiments.

The whole EryDex system has been projected to yield a final product meeting international criteria for transfusion products, whose main requirement are reported in a dedicated manual [327]. In particular, an amount of free supernatant proteins lower than 0.5 g/unit is required for final product reinfusion. EryDel's *in line* measurements on waste saline solution during the last washing step of the procedure have widely demonstrated the absence of free supernatant Hb, which is thought to be the major (if not unique) contributor to the total protein content of the aqueous phase. In our experiments, the determination of supernatant free Hb and HK was employed to evaluate the alterations suffered by RBC membranes following the *stress test*, assuming that these proteins could leak out the cells through a damaged membrane. The first tests with 20 mM NaH_2PO_4 or $NaHCO_3$, two compounds naturally found in blood, suggested a better resistance of RBC membranes when incubated with phosphate buffer. The increasing amounts of free Hb and HK were reduced in fact in this condition in comparison with both bicarbonate buffer and saline solution, the latter included as negative control. Moreover, this positive result was detected in all three loading protocols, suggesting that phosphate buffer effect is independent of any possible additional stress experienced by RBCs during a longer permanence in hypotonic environment.

The second experiment was conducted with the purpose of assessing whether different final concentrations of phosphate buffer had the same effect on RBC stabilization upon stress test. The

loading protocol was set at a fixed 10-minutes RBC-HK contact time for all the analyzed procedures and the final products were incubated in the presence of 10, 20 or 30 mM phosphate buffer, considering saline solution as negative control. RBC mean cellular hemoglobin (MCH) remained constant over time in samples analyzed before centrifugation, suggesting no detectable loss of hemoglobin during incubation at room temperature, independently of the buffer. Following centrifugation, an increase in both supernatant free Hb and HK was observed in all conditions. Phosphate buffer addition proved to be effective in limiting both parameters in comparison with control saline solution, and such effect was more evident as final buffer concentration rose. No contribution to free proteins was provided by RBC lysis, as shown by comparisons of CBC analyses performed on samples before and after centrifugation, which excluded significant decreases in RBC number.

A similar positive effect of membrane stabilization as the one emerged from our experiments had already been observed in previous EryDel's tests, when loaded RBCs were incubated with plasma obtained from the initial whole blood. This might be due to the restoration of a native physiologic environment, optimal for RBC maintenance in good conditions. If we consider that plasma naturally contains NaH_2PO_4, we can hypothesize a possible explanation for the similar behavior; however, the exact molecular mechanism underlying the stabilizing action exerted by phosphate buffer on RBC membranes has still to be clarified. One possible explanation might be provided by the importance of phosphates for ATP production. Previously it has been demonstrated that the preservation of the biconcave shape as well as the pronounced flexibility typical of RBC membrane is strictly ATP-dependent [328, 329]. *In vitro* experiments clearly demonstrated how erythrocytes deprived of their ATP supplies displayed limited membrane mobility, as a consequence of the lack of ATP-mediated interaction between the membrane lipid layer and cytoskeleton, normally accounting for cell deformability [329]. Such phenomenon might have had a role in RBC resistance to our stress test, in the sense that RBCs incubated with growing concentrations of phosphate buffer could benefit from a larger amount of phosphates to regenerate their ATP supplies. ATP regeneration, in turn, might have allowed keeping a proper cell membrane elasticity, thus enabling erythrocytes to better resist to the strong gravitational force generated during high-speed centrifugation, with limited membrane damages and protein leakage.

All these are hypothesis that might deserve further investigations. Indeed, to our knowledge these experiments are the first attempts to verify and understand the phenomenon of membrane stability, and have been performed to identify the best conditions capable of long-term stabilization of RBCs submitted to the loading procedure.

CONCLUSION

AND

FUTURE PERSPECTIVES

In our investigations, we have demonstrated that murine RBCs loaded with recombinant AvPAL are effectively able to act as bioreactors to decrease blood L-Phe both *in vitro* and *in vivo* in BTBR-Pah^{enu2} mice, the most widely used animal model of human PKU.

We were able to select the most suitable loading parameters yielding the best entrapment of rAvPAL while keeping optimal RBC functioning, and to identify the minimum dose of enzyme capable of maintaining *in vivo* blood amino acid levels near the physiologic condition. We succeeded in defining a proper schedule of repeated administrations, which proved to be effective in the long-term control of L-Phe. We managed to obtain protein-loaded human erythrocytes meeting international requirements for blood components [327] by employing the EryDex system, with additional preliminary data orienting future decisions to further improve final product characteristics.

Our future objective is now to verify whether the early adoption of such therapeutic approach could positively affect the neurological outcome in adult age. To this purpose, we are planning a new experiment involving newborn BTBR-Pah^{enu2} mice, which will be treated early and repeatedly with rAvPAL-RBCs and will be finally evaluated in their neurocognitive performance.

In conclusion, we demonstrated the great potential of rAvPAL-RBCs as enzyme replacement therapy for the treatment of phenylketonuria, thus opening new perspectives for the development of enzyme replacement therapies for other disorders involving enzyme deficiencies. In fact, many other inborn errors of metabolism, which share with PKU a similar pathophysiological mechanism, characterized by a progressive blood accumulation of (neuro)toxic compounds [330], could therefore benefit from the long-term advantages of the therapeutic approach here described, which are better understood if considering that such patients are usually bound to life-long treatment. The availability of a treatment possibility like the one proposed here would therefore mean for all these patients and their families a great improvement in everyday quality of life.

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