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Mitochondrial pharma-Q-genomics: Targeting the OXPHOS cytochrome *b*

Aurora Gómez-Durán[1], David Pacheu-Grau[1], Manuel J. López-Pérez[1], Julio Montoya[1] and Eduardo Ruiz-Pesini[1,2,*], eduruiz@unizar.es

Genetic variation in human cytochrome b generates structurally different coenzyme Q binding pockets, affects the coupling efficiency of the oxidative phosphorylation system and susceptibility to different medical conditions. As modification of coupling efficiency has already been shown to have therapeutic interest, these structural differences might be used to develop new drugs and allow for personalized medicine, giving rise to a new field: mitochondrial pharmacogenomics.

Human mitochondrial DNA (mtDNA) encodes 24 RNAs (2 rRNAs and 22 tRNAs) and 13 polypeptides of the oxidative phosphorylation (OXPHOS) system. One of these polypeptides, cytochrome *b* of the electron transport chain (ETC) complex III (CIII), is central to OXPHOS function. Cytochrome *b* contains two coenzyme Q binding sites: the outer Qo and the inner Qi. At Qo, two ubiquinols are oxidized to ubiquinones and their electrons are sent to two different pathways. Two electrons are sent downstream where ultimately reduce oxygen to water, two other are directed towards Qi, where a ubiquinone is reduced to ubiquinol. Concurrently, cytochrome *b* pumps protons to the intermembrane space, which contributes to the electrochemical gradient used for ATP production.

The importance of the Q binding sites is demonstrated by the fact that Qo inhibitors, such as strobilurins, are widely used as agricultural fungicides [1]. Strobilurins are natural fungicides produced by various basidiomycetes. These organisms developed means to protect their own mitochondrial respiratory chains. In the case of the strobilurin A-producing basidiomycete, *Mycena galopoda*, natural resistance is achieved by a G143A mutation of the target site [2]. Thus, differences in Qo sites between crops and their pathogenic fungi have allowed the development and use of Qo inhibitors in agriculture. Other Qo inhibitors exert notable inhibitory action on the respiratory processes of *Plasmodium falciparum*, the parasite that causes severe malaria. Qo inhibitors such as myxothiazol and stigmatellin have been available for many years but are highly toxic to humans and not suitable for therapeutic use. However, the *P. falciparum* Qo site has unusual structural features which may help drive drug selectivity [3]. A new compound, atovaquone, is active not only for the malaria parasite, but also for microorganisms that cause pneumonia (*Pneumocystis jirovecii*) and toxoplasmosis (*Toxoplasma gondii*). Again, interspecies differences in the drug target have allowed the development of therapeutic compounds active against the parasites with few adverse effects on humans.

Several mutations resulting in resistance to atovaquone have been reported in *P. falciparum* isolated from patients after treatment failure. For example, Y268S/C mutations remove an aromatic residue important for stabilizing an interaction between the drug and residues within the Qo site [4]. Additionally, several mutations that cause resistance to Qo inhibitors have been described in plant pathogen fungi. The alanine in the cytochrome *b* 143 position of *Blumeria graminis*, *Sphaeroteca fuliginea* or *Plasmopara viticola* inhibits drug binding through simple steric hindrance [1,5,6]. Hence, intraspecies differences in the drug target allow distinct behaviors to a particular drug.

Usually, mutations causing resistance affect sites important for the cytochrome *b* function. Therefore, many of mutations are also associated with loss of fitness [7]. For example, the yeast Y279C mutation (Y268C in *Plasmodium*) affects ubiquinol binding and decreases cytochrome bc1 activity to 50% of the control [8]. This mutation (Y278C in humans) has also been found in a patient suffering from a multisystem disorder [9]. It is important to realize that fitness costs for mutations causing resistance might vary with environmental conditions and energy demands.

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If differences in parasite's Q sites can affect biological fitness and drug response, then important questions arise. Is there genetic variation in the Q sites of human cytochrome *b*? Does this variation have a phenotype? What are the molecular mechanisms? Are these mechanisms susceptible to modification? Can this variation be used for a personalized medicine?

Human mitochondrial DNA genetic variation at coenzyme Q binding sites of cytochrome *b*

Because of its particular location and genetic features, mtDNA accumulates mutations at a high rate [10]. Because nearly all mtDNA sequence is coding DNA, most mutations will affect a gene. When these mutations occur in the germinal line and survive in populations, new mtDNA genetic backgrounds are originated. Because mtDNA is exclusively transmitted by maternal lineage, new genotypes will be directly related to their progenitors and will be very similar to other descendents of this progenitor mtDNA. Groups of phylogenetically related mtDNA genotypes are known as mtDNA haplogroups.

The cytochrome *b* gene is 1141 base pairs long, approximately 7% of the whole mtDNA, and many mtDNA mutations will hit it. In fact, an analysis of more than 3,500 human mtDNA sequences has shown that 124 (32.6%) of the 380 amino acids of cytochrome *b* are polymorphic and 52 (28.3%) of the 184 amino acids that constitute the Q sites (Qi, 15-40 and 190-239 [11]; Qo, 120-182 and 250-294 [12]) are also variable. Many of these polymorphisms in the Q sites have been found in one or a small number of individuals, but several define very important haplogroups. For example, the A229T mutation in Qi characterizes African L0a and Asian M30 haplogroups, A193T labels Asian haplogroup G1 and H16R, F18L and L236I define European haplogroups U5a, Uk/J1c and JT, respectively. In the Qo site, A122T characterizes African haplogroup L2bc, G251S defines Asian haplogroup G1 and T158A and D171N label European haplogroups U5a1 and J2, respectively. The overall frequency of JT (including J1c, J2 and T), Uk and U5a (including U5a1) haplogroups in Europe is higher than 25% [13,14], meaning that one fourth of the European population harbors polymorphisms in the Q sites of cytochrome *b* (Fig. 1).

Phenotypic differences due to genetic variation of cytochrome *b* coenzyme Q binding sites

Using a phylogenetic tree that included more than 3,500 human mtDNA sequences and aligning 276 mammal species, we have found that human cytochrome *b* Q sites show less variation than the rest of the protein. Additionally, amino acids in these sites are more highly

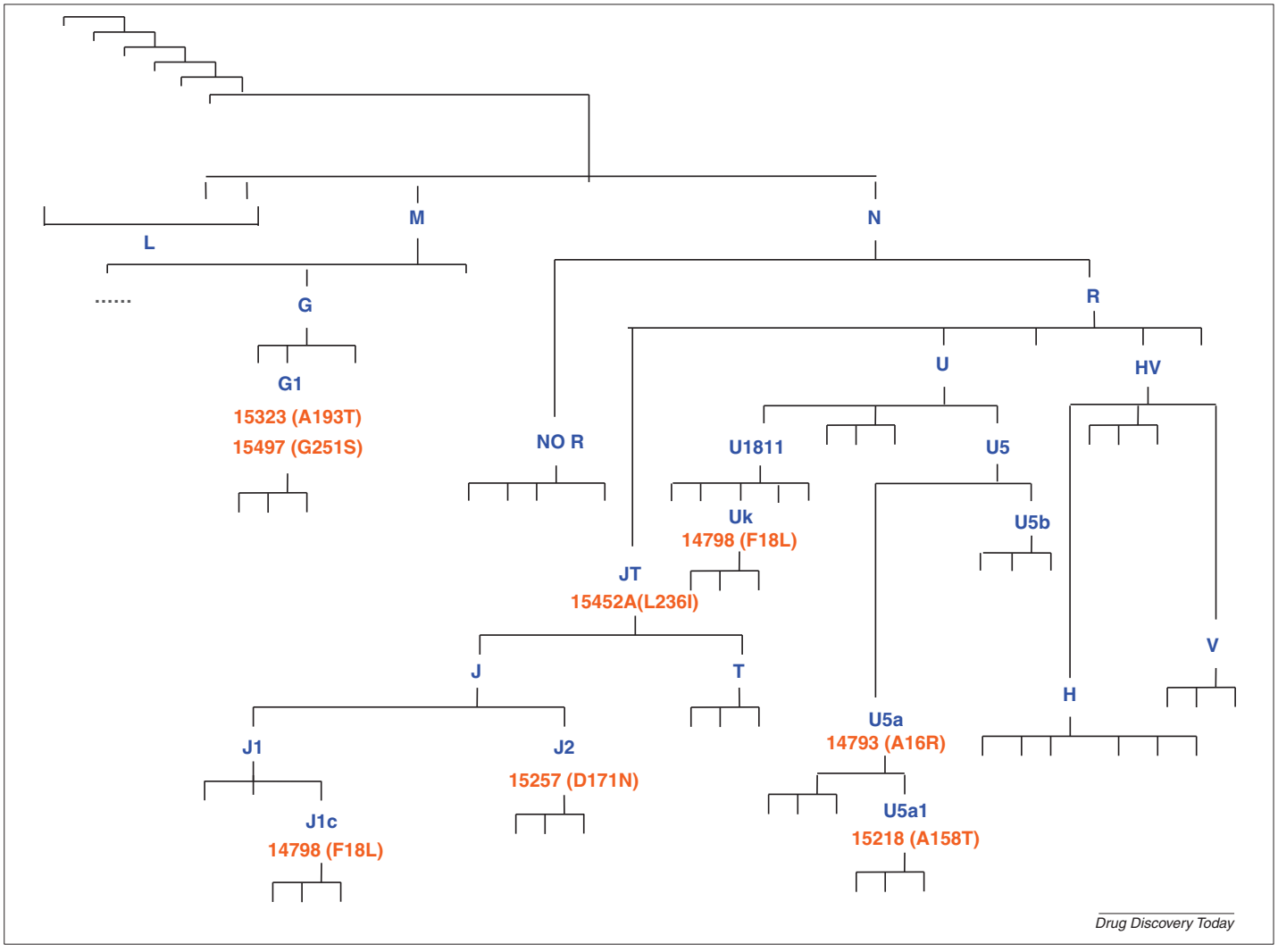

FIGURE 1

mtDNA phylogenetic tree. Important mtDNA haplogroups defined by cytochrome *b* Q site mutations are represented.

conserved than those found outside of them. This suggests that negative selection is stronger at these sites. Because selection implies phenotypic differences, it is possible that Q site mutations have an effect on human phenotypes. The importance of Q sites is evident after the observation that seven out of the nine pathologic missense mutations in cytochrome *b* are located in these sites (Qi – G34S, S35P; Qo – S151P, G166E, G251D, Y278C and G290D) [15] (Fig. 2). However, these mutations are very pathologic and will be quickly removed, causing their population frequencies to be very low.

Alternatively, population analysis has shown that cytochrome *b* and its Q sites are particularly variable in temperate zones of the world [16,17] (Fig. 2) and that positive selection may have played a role in shaping this variation [13,18]. Thus, distinct environmental conditions, such as climate, could differentially affect the biological fitness and select for particular mtDNA genotypes. In this sense, it has been shown that mtDNA lineages with cytochrome *b* mutations, like U5a, were substantially enriched in northern Europe [13]. MtDNA haplogroups adapted to different environmental conditions would show distinct OXPHOS capacity and dissimilar susceptibility to particular phenotypes. Supporting this suggestion, epidemiologic studies have shown that mtDNA haplogroups J and Uk are susceptibility factors for Leber's hereditary optic neuropathy (LHON) and resistance factors against aging and Parkinson's disease. Additionally, J and U5a haplogroups were elevated among HIV-1 infected people who display accelerated progression to AIDS and death and the Uk haplogroup is correlated with a significant increase in the risk of developing breast cancer [19]. Moreover, the G251S mutation, defining mtDNA haplogroup G1, has been associated with obesity in Japanese population [20].

Unfortunately, complete linkage disequilibrium in the mtDNA prevents assignment of a particular phenotype to a specific polymorphism. However, molecular analyses allow refinement of these associations. Thus, it has been shown that biochemical and molecular-genetic differences between mtDNA haplogroups H and Uk are probably due to the F18L substitution in the cytochrome *b* Qi site [19]. By contrast, site-directed mutagenesis of the aspartic acid at position 187 to asparagine in *Rhodobacer sphaeroides* (D171N in humans) alters the photosynthetic apparatus and influences cellular physiology [21].

OXPHOS coupling efficiency

As we have previously commented, cytochrome *b* contributes to generation of an electrochemical gradient. In addition to ATP production, this gradient is used for many other purposes, such as importing proteins and substrates into the mitochondria, apoptosis, thermogenesis, maintenance of cytosolic calcium levels and production of reactive oxygen species (ROS).

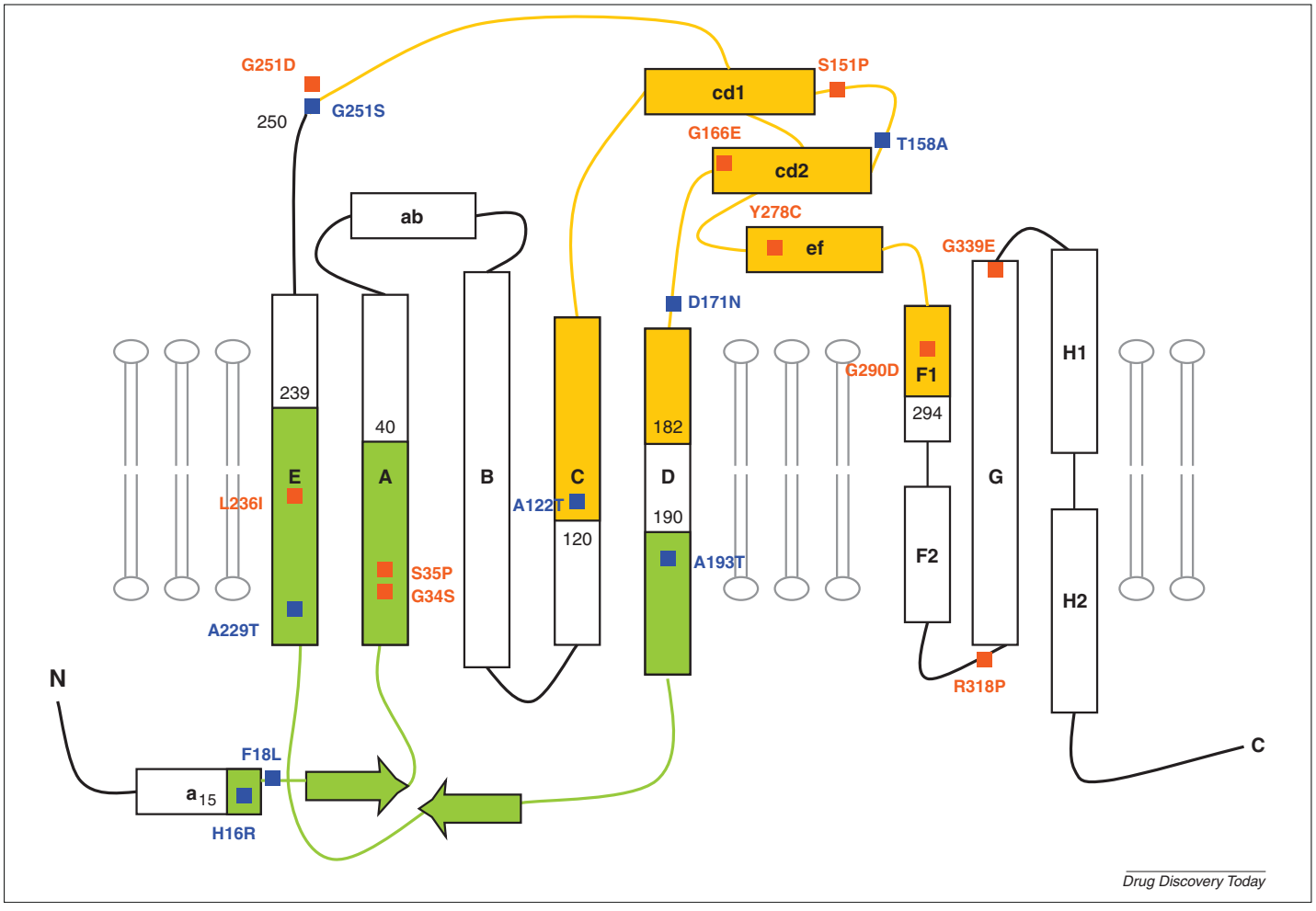

FIGURE 2

Genetic variation of cytochrome *b* Q sites. Pathologic mutations (red) and population polymorphisms (blue) affecting cytochrome *b* Q sites are indicated. Qi and Qo are represented in green and yellow colors, respectively.

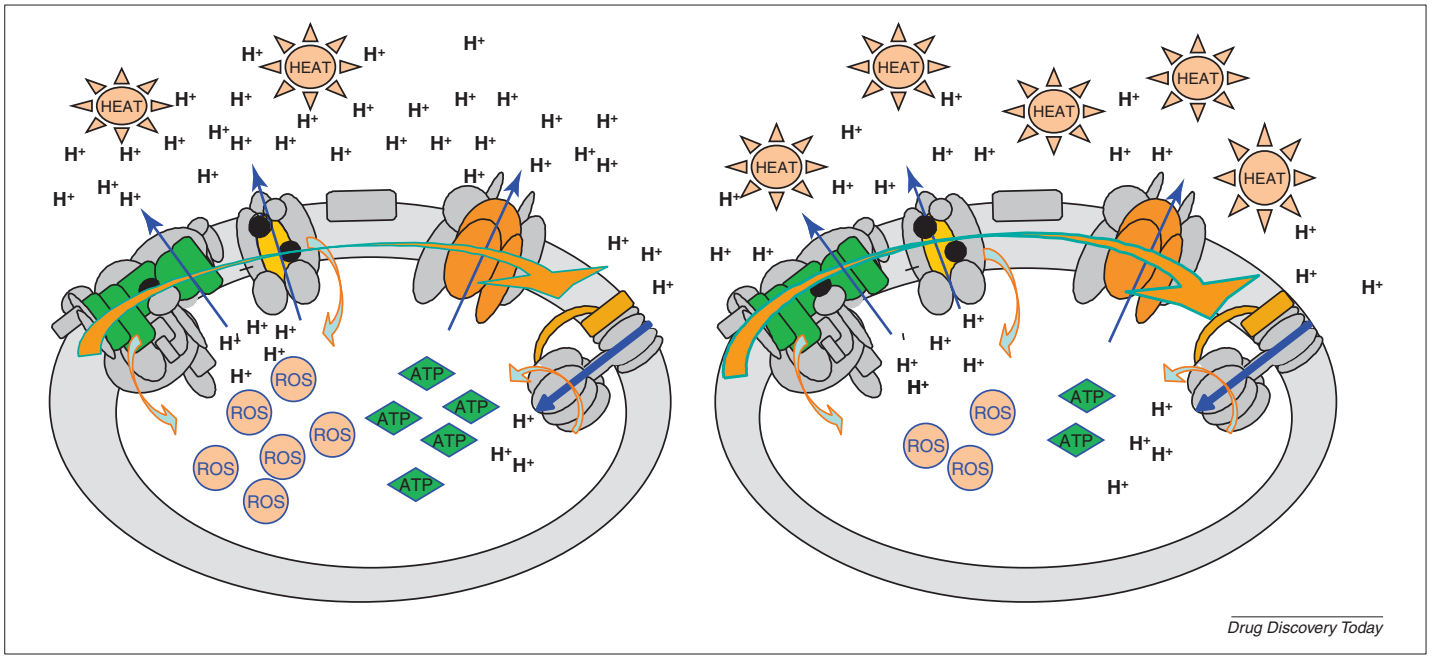

FIGURE 3

OXPHOS coupling efficiency. Highly coupled (left) and uncoupled (right) OXPHOS systems.

These compounds (ATP, calcium and ROS), can act as second messengers and trigger intracellular retrograde responses that affect cell homeostasis. There is a balance between all of these functions and, thus, a tight coupling between transported electrons and pumped protons in CIII with returned protons to the matrix through ATP synthase (CV) will produce ATP efficiently, and, therefore, less substrate will be consumed (Fig. 3).

However, it is possible that cytochrome *b* mutations affect coupling efficiency, either by decreasing the number of protons pumped per transported electron (electron slipping, decoupling) or by favoring the return of intermembrane protons to the matrix independent of the CV proton channel (proton leak, uncoupling) [22]. In such cases, the proton gradient would be diminished, and these mitochondria would generate less ATP per calorie consumed and require higher substrate consumption. At the same time, a lower electrochemical gradient would facilitate electron flow and heat production, ETC intermediates would be less reduced and fewer ROS would be produced (Fig. 3). Hence, individuals harboring cytochrome *b* uncoupling mutations would be more prone to clinical problems resulting from energy insufficiency, such as LHON. However, they would also produce more heat, making them better adapted to living in cold climates. Moreover, their ETC would generate fewer ROS and they would be protected against disorders that are dependent on oxidative stress, such as aging and age-linked disorders [13,18,23]. Similarly, it has also been shown that the Qo site is necessary to increase cytosolic ROS in hypoxic conditions, which are required to stabilize hypoxia-inducible factor 1α (HIF-1α). These mitochondrial ROS could serve as therapeutic targets for many HIF-dependent pathological processes, including cancer [24].

Thus, cytochrome *b* Q site mutations may affect OXPHOS coupling efficiency, which has already been the target of therapeutic interventions.

Targeting OXPHOS coupling efficiency

2,4-*p*-Dinitrophenol (DNP) is a hydrophobic weak acid with delocalized negative charge. In mitochondria, the DNP anion is protonated in the intermembrane space, moves into the matrix where it is deprotonated, thus regenerating the DNP anion, and expelled into the intermembrane space. In this way, DNP dissipates the mitochondrial proton gradient and increases the ETC rate and substrate consumption. Thus, more NADH is reoxidized, and NAD^+ appears to be a neuroprotective agent through various regulatory pathways, such as those involving the histone deacetylase sirtuin 1 (SIRT1) and poly (ADP-ribose) polymerase 1 (PARP1) [25]. Therefore, neurological conditions might benefit from treatment with DNP. Indeed, rats treated with DNP showed significant protection against brain damage caused by striatal injection of the N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid, which models the excitotoxic brain injury in neurodegenerative conditions. Additionally, DNP also reduced the infarct volume in a model of focal ischemia-reperfusion injury in rat brains and increased white matter sparing in a rat contused spinal cord model [26].

By increasing the electron flow rate in the ETC, red-ox intermediates would remain less reduced and fewer ROS would be produced. Therefore, treatment with DNP would be beneficial for those phenotypes dependent on oxidative damage. Accordingly, it has been shown that DNP leads to decreased ROS production and an increase in replicative life span of *Saccharomyces cerevisiae* [27]. The addition of DNP to the nutritional mixture of *Drosophila melanogaster* larvae significantly increased the average life span of the flies [28]. Moreover, treatment of mice with DNP decreased ROS levels and oxidative damage, and enhanced longevity [29].

In the early 1930 s, DNP was widely used in humans as a weight-loss drug [30]. Although it was very effective, the therapeutic index was razor thin and many people suffered irreversible harm, such as vision loss or even death [31].

Previous observations indicate that the chemical modification of OXPHOS coupling efficiency may have very important therapeutic consequences. However, as previously noted, the therapeutic index of DNP is very low and safer chemical uncouplers should be sought out. One method of finding these safer compounds

would be through the use of drugs with very specific targets.

Conclusion: coenzyme Q binding sites as pharmacologic targets in human therapy

In *S. cerevisiae*, an isoleucine to phenylalanine mutation at position 17 of cytochrome *b* causes diuron-resistance. Interestingly, mammals appear to be naturally resistant to diuron [32] as 80%, including humans, contain phenylalanine at this position (position 18 in humans). However, individuals from mtDNA haplogroups J1c and Uk have a F18L mutation. Might these individuals be more susceptible to diuron? Is it possible to develop drugs that, acting on these sites, decrease the OXPHOS coupling efficiency and have therapeutic interest? Could variation in Q sites be used for personalized medicine? We have a convenient model to pursue this aim: the transmitochondrial cell lines or cybrids. These lines have identical nuclear genetic background and are grown in the same environmental conditions, with mtDNA genotype being their only difference [33]. Therefore, it is now possible to test large chemical compound libraries on cybrid collections that include mtDNA haplogroups defined by important polymorphisms in cytochrome *b* Q sites.

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Aurora Gómez-Durán
David Pacheu-Grau
Manuel J. López-Pérez
Julio Montoya
Departamento de Bioquímica, Biología Molecular y Celular, Centro de Investigaciones, Biomédicas En Red de Enfermedades Raras (CIBERER), Instituto Aragonés de Ciencias de la Salud (I+CS), Spain

Eduardo Ruiz-Pesini
Departamento de Bioquímica, Biología Molecular y Celular, Centro de Investigaciones, Biomédicas En Red de Enfermedades Raras (CIBERER), Instituto Aragonés de Ciencias de la Salud (I+CS), Spain

Fundación ARAID, Universidad de Zaragoza, 50013 Zaragoza, Spain