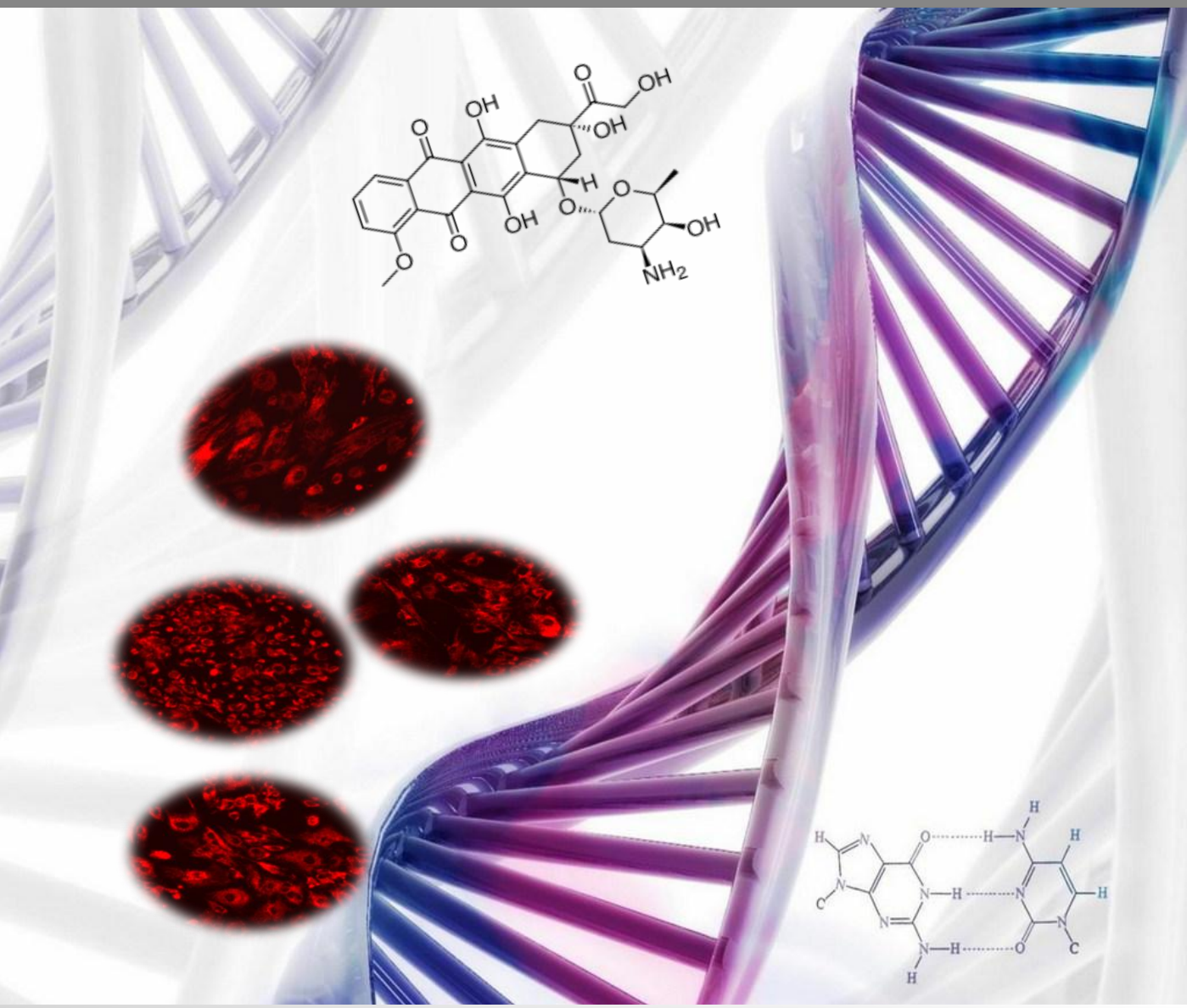
Idiosyncrasy of drug-induced mitochondrial liabilities: From mitochondrial DNA single nucleotide polymorphisms to mitochondrial sirtuins



Cláudia Sofia Viriato Alves Pereira

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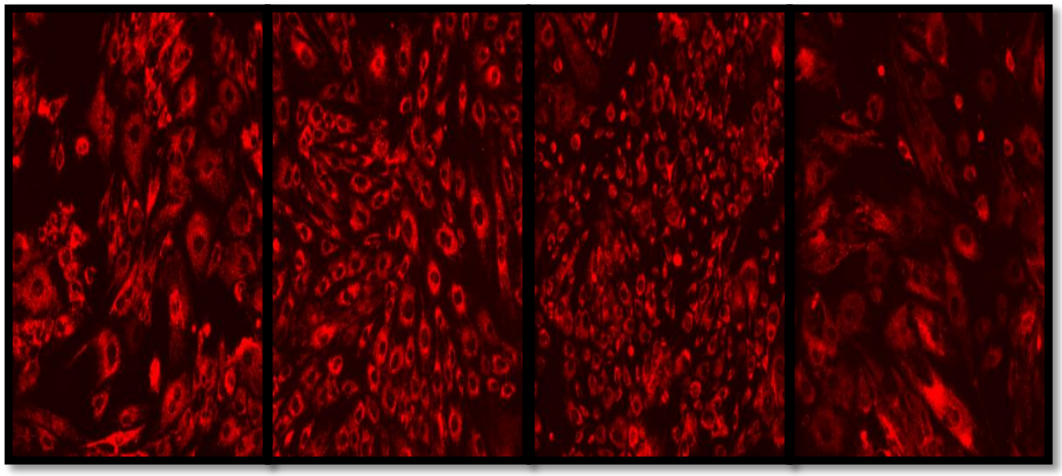

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Idiosyncrasy of drug-induced mitochondrial liabilities: From mitochondrial DNA single nucleotide polymorphisms to mitochondrial sirtuins

FCTUC FACULDADE DE CIÊNCIAS
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"(…) Nobody said it was easy
No one ever said it would be this hard
I was just guessing, at numbers and figures
pulling your puzzles apart.
Questions of science; science and progress
do not speak as loud as my heart"

The Scientist, Coldplay

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List of Abbreviations

mtΔΨ - Mitochondrial transmembrane electric potential

AceCS$_2$ - Acetyl-CoA synthetase 2

ADP - Adenosine diphosphate

ADR(s) - Adverse drug reaction(s)

AIDS - Acquired immunodeficiency syndrome

AIF - Apoptosis inducing factor

AKT - Protein Kinase B

ALT - Alanine transaminase

AMP - Adenosine monophosphate

AMPK - 5' adenosine monophosphate-activated protein kinase

ANT - Adenine nucleotide translocator

APAP - Acetyl-para-aminophenol (acetominophen)

APC(s) - Antigen-presenting cell(s)

ATP - Adenosine triphosphate

AZT - Azidothymidine (zidovudine)

B$_2$ (TFB$_2$M) - Transcription factor B2

BAX - Bcl-2–associated X protein

BN - Brown Norway

CAD - Caspase-activated DNase

CCCP - Carbonyl cyanide *m*-chlorophenyl hydrazone

CoA - Coenzyme A

CoQ - Coenzyme Q10 or ubiquinone

$CoQH_2$ - Ubiquinol

CPS - Carbomoyl phosphate synthase 1

CR - Calorie restriction

CREB - cAMP response element-binding protein

CYP - Cytochrome P450 superfamily

D-loop - Displacement loop

DCLF - Diclofenac

DCM - Dilated cardiomyopathy

DEAF - Maternally inherited deafness or aminoglycoside-induced deafness

DILI - Drug-induced liver injury

DMDF - Diabetes mellitus + deafness

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DOX - Doxorubicin

ER/PR - Estrogen receptor /Progesterone receptor

ERR - Estrogen related receptors

ESOC - Encephalomyopathy

ETC - Electron transfer chain

FADD - Fas-Associated protein with Death Domain

FAD - Flavin adenine dinucleotide

FAOOH - Fatty acid hydroperoxides

FBSN - Familial bilateral striatal necrosis

FCCP - Carbonylcyanide-4-trifluoromethoxyphenylhydrazone

FDA- Food and Drug Administration

FICP - Fatal infantile cardiomyopathy plus

FOXO1 - Forkhead box protein O1

GDH - Glutamate dehydrogenase

GPx - Glutathione peroxidase

GSH - Reduced glutathione

GSSG - Glutathione disulfide

GST - Glutathione-S-transferases

GTP - Guanosine-5'-triphosphate

HFD - High fat diet

HIV - Human immunodeficiency virus

HK II - Hexokinase II

HLA - Human leukocyte antigen

hSIRT3 - Human sirtuin 3

hSIRT3-H248y-HA - Human sirtuin 3 deacetylase mutant

HSP - Heat shock proteins

IDR(s) - Idiosyncratic drug response(s)

IGF - Insulin-like growth factors

IMS - Intermembrane space

JNK(s) - c-Jun N-terminal kinase(s)

KSS - Kearns–Sayre's syndrome

LCAD - Long chain acyl coenzyme A dehydrogenase

LHON - Leber hereditary optic neuropathy

LPS - Lipopolysaccaride

MAPK - Mitogen-activated protein kinase

MDA - 3,4-methylenedioxyamphetamine

MDR1 - Multidrug resistance protein 1

MEFs - Mouse embryonic fibroblasts

MELAS - Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

MHC - Major histocompatibility complex

MICM - Maternally inherited cardiomyopathy

MM - Mitochondrial myopathy

MMC - Maternal myopathy and cardiomyopathy

MNGIE - Mitochondrial neurogastrointestinal encephalopathy

MnSOD - Manganese superoxide dismutase

MPTP - Mitochondrial permeability transition pore

mRNA - Messenger ribonucleic acid

MRP- Multidrug-resistance-associated protein

MTERF - Mitochondrial transcription termination factor

NAD^+- Nicotinamide adenine dinucleotide

$NADP^+$- Nicotinamide adenine dinucleotide phosphate

NAPQI - N-acetyl-p-benzoquinone imine

NARP - Neurogenic muscle weakness-ataxia retinitis pigmentosa

NAT - N-actyltransferases

NF-kB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NRF - Nuclear respiratory factors

NRTI(s) - Nucleoside reverse transcriptase inhibitor(s)

NSAIDs - Nonsteroidal anti-inflammatory drugs

OCR - Oxygen consumption rate

OPA 1 - Optic atrophy 1 dynamin-like protein

OSCC - Oral squamous cell carcinoma

OTC - Ornithine transcarbomoylase

OXPHOS - Oxidative phosphorylation

PARP - Poly (ADP ribose) polymerase

PCR - Polymerase chain reaction

PDH - Pyruvate dehydrogenase

PEM - Progressive encephalomyopathy

PEO - Progressive external ophthalmoplegia

PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI - Pharmacological interaction

PLOOH - Phospholipid hydroperoxides

POLG - DNA polymerase subunit gamma encoded gene

PPAR - Peroxisome proliferator-activated receptor

PXR - Peroxiredoxin

RNA - Ribonucleic acid

ROS - Reactive oxygen species

rRNA - Ribosomal ribonucleic acid

tRNA - Transfer ribonucleic acid

SDH - Succinate dehydrogenase

Smac/DIABLO- Second mitochondria-derived activator of caspases

SNP(s) - Single nucleotide polymorphism(s)

SOD - Superoxide dismutase

SUMO - Small ubiquitin-like modifier

TCA - Tricarboxylic Acid

TFAM - Mitochondrial transcription factor A

FMN - Flavin mononucleotide

TMRM - Tetramethylrhodamine methylester

TNF - Tumor necrosis factor

mTOR - Mammalian target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1 (FRAP1)

TRITC - Isothiocyanate derivative

UCP_2 - Mitochondrial uncoupling protein 2

VDAC - Voltage-dependent anion channel

VEGF - Vascular endothelial growth factor

VPA - Valproic acid

WT - Wild type

"Among other things, you'll find that you're not the first person who was ever confused and frightened and even sickened by human behavior. You're by no means alone on that score, you'll be excited and stimulated to know. Many, many men have been just as troubled morally and spiritually as you are right now. Happily, some of them kept records of their troubles. You'll learn from them — if you want to. Just as someday, if you have something to offer, someone will learn something from you. It's a beautiful reciprocal arrangement. And it isn't education. It's history. It's poetry."

(From The Catcher in the Rye, J.D. Salinger, 1951)

Summary

Mitochondria are not only cell furnaces but are also the main targets for many toxic compounds. Mitochondrial DNA (mtDNA) variations, including single nucleotide polymorphisms (SNPs) have been proposed to be involved in idiosyncratic drug reactions (IDRs). Although a rare phenomenon, IDRs are a serious and sometimes fatal complication. Therefore, IDRs are a major challenge for pharmaceutical companies which see their costly drugs being withdrawn from the market. However, current *in vitro* and *in vivo* models lack the genetic diversity observed in the human population and are not suitable to evaluate mitochondrial idiosyncratic drug-induced toxicity.

The objective of this thesis was to identify novel intrinsic aspects of host-dependent mitochondrial physiology that may contribute to IDRs. These two intrinsic properties are mitochondrial DNA (mtDNA) SNPs and the content/expression of mitochondrial sirtuins. The first specific aim was to identify whether different cell strains with distinct mtDNA SNPs have different mitochondrial bioenergetic profiles and resistance to drug-induced toxicity. An *in vitro* system composed of four strains of mouse embryonic fibroblasts (MEFs) with mtDNA polymorphisms was used for this specific aim. We sequenced mtDNA from embryonic fibroblasts isolated from four mouse strains, C57BL/6J, MOLF/EiJ, CZECHII/EiJ and PERA/EiJ, with the latter two being sequenced for the first time. The bioenergetic profile of the four strains of MEFs was investigated at both passages 3 and 10, in order to investigate cell culture aging as well. Our results showed clear differences among the four strains of MEFs at both passages, with CZECHII/EiJ having a lower mitochondrial fitness when compared to C57BL/6J, followed by MOLF/EiJ and PERA/EiJ. The second objective in this specific aim was to test seven drugs known to impair mitochondrial function in our *in vitro* model for their effect on cellular ATP content in both glucose- and glucose-

free, galactose-containing media. Our results demonstrated strain-dependent differences in the response to some of the tested drugs. We propose that this model is a useful starting point to study compounds that may cause mitochondrial off-target toxicity in early stages of drug development, thus decreasing the number of experimental animals used.

For the second specific aim, we investigated the role of mitochondrial sirtuins on drug-induced toxicity. Mammalian sirtuins are a family of seven protein deacetylases involved in many different cellular functions including calorie restriction, life span and metabolic stress. Sirtuin 3 (SIRT3) is localized in the mitochondrial matrix, where it regulates the acetylation levels of metabolic enzymes and antioxidant protection. In order to demonstrate the role of SIRT3 against drug-induced toxicity, we used a golden-standard agent to induce cardiotoxicity. Doxorubicin (DOX) is a widely used anti-cancer agent known for its dose-dependent and cumulative cardiotoxicity. The second specific aim in this thesis aimed to understand if DOX-induced toxicity is regulated by SIRT3 expression levels. We used a cardiomyoblast cell line, H9c2, to study SIRT3 under different expression levels. Interestingly, H9c2 cells have minimal levels of detectable SIRT3 mRNA, hence we used a transfection system to over-express this sirtuin. Cell mass, intracellular ATP levels, cell death and caspase 3/7 activities were evaluated in untreated and DOX-treated human SIRT3 (hSIRT3)-overexpressing cells, hSIRT3-H248Y-HA (mutant enzymatic form of hSIRT3) overexpressing cells and pcDNA control H9c2 cells. The results showed that hSIRT3 overexpression protected H9c2 cells from DOX-induced cell death and caspase-like activity, when compared with control cells, after 24h of DOX treatment. Surprisingly, hSIRT3-H248Y-HA overexpression also protected cells against DOX-induced cell death and caspase activity, but it decreased the ATP levels when compared to the control cells treated with DOX.

The role of mitochondria in IDRs is here further explored. We come to the conclusion that in fact it is likely that both aspects here investigated (mtDNA SNPs and SIRT3 content) may impact mitochondrial response to drugs. Since both can be host-dependent, we anticipate them to be a component, at least to a certain degree, of IDRs.

Sumário

As mitocôndrias não são somente as fornalhas das células, como também os alvos principais de vários compostos tóxicos. As variações no DNA mitocondrial (mtDNA), incluindo as alterações singulares de nucleotídeos (SNPs) têm sido propostas como estando envolvidas nas reações idiossincráticas a fármacos (IDRs). Embora sendo um fenómeno raro, as IDRs são complicações sérias e por vezes, até fatais. Por isso, as IDRs são o maior desafio das empresas farmacêuticas que se deparam com fármacos de elevado valor económico a serem retirados do mercado. Os modelos *in vitro* e *in vivo* que existem atualmente, não são adequados para avaliar a toxicidade idiossincrática mitocondrial induzida por fármacos e não têm em conta a diversidade genética observada na população humana.

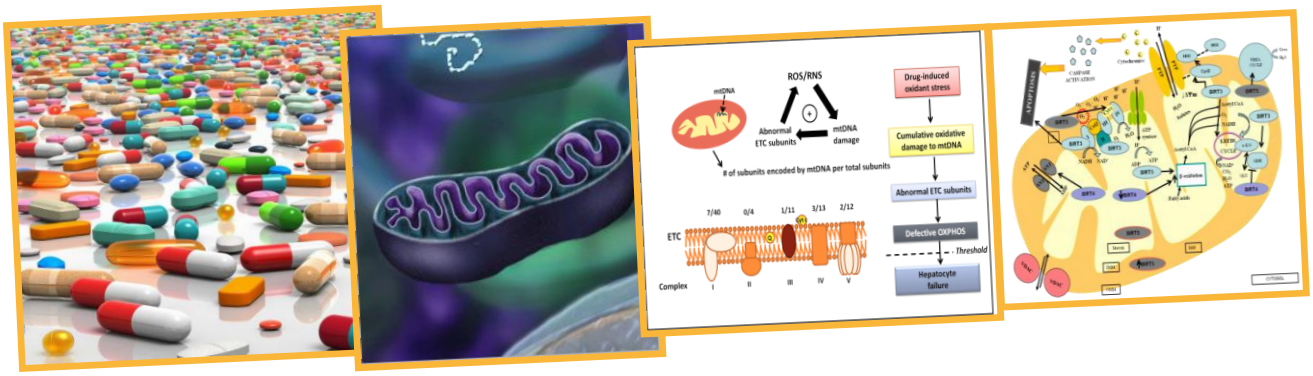
O objetivo desta tese foi identificar novos aspetos intrínsecos, dependentes do hospedeiro, relativos à fisiologia mitocondrial, que possam contribuir para as IDRs. Estas duas propriedades intrínsecas são os SNPs no mtDNA mitocondrial e a quantidade/expressão das sirtuínas mitocondriais. O primeiro objetivo específico foi identificar se diferentes linhagens celulares com diferentes SNPs no mtDNA tinham diferenças na bioenergética mitocondrial e na resistência à toxicidade induzida por fármacos. Um sistema *in vitro* composto por quatro linhagens celulares de fibroblastos embrionários de ratinho (MEFs), que continham SNPs no mtDNA foram usados com este objetivo específico. Sequenciámos o mtDNA dos fibroblastos embrionários isolados de quatro estirpes diferentes de ratinho, C57BL/6J, MOLF/EiJ, CZECHII/EiJ e PERA/EiJ, sendo os dois últimos sequenciados pela primeira vez. O perfil bioenergético das quatro linhagens celulares de MEFs foi investigado para as passagens 3 e 10, de forma a compreender também o efeito do envelhecimento celular em cultura. Os nossos resultados demonstraram

diferenças evidentes entre as quatro linhagens celulares de MEFs em ambas as passagens, tendo as CZECHII/EiJ um menor desempenho a nível mitocondrial quando comparadas com as C57BL/6J, seguidas das MOLF/EiJ e PERA/EiJ. O segundo objetivo específico foi testar no nosso modelo *in vitro* sete fármacos conhecidos por danificarem a função mitocondrial, através dos seus efeitos no conteúdo celular de ATP, em culturas que continham meios com glucose e sem glucose, mas contendo galactose. Os nossos resultados demonstraram diferenças dependentes de cada linhagem celular na resposta a vários fármacos. Propomos que este modelo seja um ponto de partida para a avaliação de compostos que causam disfunção mitocondrial não direcionada, em fases precoces do desenvolvimento de fármacos, diminuindo desta forma, o número de animais usados nas experiências.

No segundo objetivo específico, investigámos o papel das sirtuínas mitocondriais na toxicidade induzida por fármacos. As sirtuínas dos mamíferos são uma família de sete proteínas desacetilases, envolvidas em diferentes funções celulares, nomeadamente na restrição calórica, longevidade e stresse metabólico. A sirtuína 3 (SIRT3) está localizada na matriz mitocondrial, onde regula os níveis de acetilação de enzimas metabólicas e a proteção antioxidante. De forma a demonstrar o papel da SIRT3 contra a toxicidade induzida por fármacos, usámos um composto chave de cardiotoxicidade. A doxorubicina (DOX) é um composto vastamente usado como agente anti-cancerígeno, conhecido pela sua cardiotoxicidade cumulativa e dependente da dose. O segundo objetivo específico desta tese foi entender se a toxicidade induzida pela DOX é regulada pelos níveis de expressão da SIRT3. Usámos uma linha celular de cardiomioblastos, as H9c2, para estudar a SIRT3 sob diferentes níveis de expressão. Curiosamente, as células H9c2 têm níveis de mRNA muito reduzidos de SIRT3, sendo que usámos um sistema de transfecção para

sobrexpressar esta sirtuína. A massa celular, o conteúdo celular de ATP, a morte celular e a atividade de caspases 3/7 foram avaliadas em células tratadas com DOX e não tratadas que sobrexpressavam SIRT3 humana (hSIRT3); em células que sobrexpressavam a hSIRT3-H248Y-HA (forma enzimaticamente inactiva da hSIRT3) e em células H9c2 controlo pcDNA. Surpreendentemente, a sobrexpressão da hSIRT3-H248Y-HA também protegeu as células da morte celular e da atividade de caspases induzida pela DOX, mas diminui os níveis de ATP quando comparados com os das células controlo tratadas com DOX.

O papel da mitocôndria nas IDRs foi estudado nesta tese. Chegámos à conclusão de que de é de facto muito provável que ambos os aspetos aqui investigados (os SNPs no mtDNA e o conteúdo de SIRT3) possam ter um impacto na resposta das mitocôndrias a fármacos. Uma vez que ambos são fatores dependentes do hospedeiro, antecipamos que possam ser uma componente, pelo menos em parte, das IDRs.



1- General Introduction

Section 1

Idiosyncratic drug-reactions: the past and the future

"Adverse drug reactions are a major complication of modern drug therapy; they are a considerable cause of patient morbidity and account for a significant number of patient deaths."
(Park et al., 1992)

1.1 Idiosyncratic drug toxicity: an overview

Idiosyncratic drug toxicity is a serious problem for the pharmaceutical industry since it is the most frequent reason for drug withdrawal from the market by the Food and Drug Administration (FDA) (Jefferys *et al.*, 1998). Adverse drug reactions (ADRs) can include different complications such as anaphylasis, blood dyscrasias, hepatotoxicity and severe skin reactions (Park *et al.*, 2000). Idiosyncratic drug reactions (IDRs) are expected to occur in less than 1:10 000 patients (Lee, 2003). Even occuring in a minority of patients, the consequences are most of the times fatal. The most common class of therapeutics involved are anti-infective, anticonvulsants, antipsychotics, antidepressants and non-steroidal and anti-inflammatory drugs (NSAIDs) (Park *et al.*, 2000). From a pharmaceutical and clinical point of view, it is crucial to prevent IDRs, however the scarcity of *in vivo* and *in vitro* models to evaluate idiosyncratic reactions and the lack of clear mechanistic pathways, makes its prediction generally impossible. Therefore, IDRs are in the majority of the times later recognized during phase IV, the post-licensing stage of drug development.

Most IDRs appear to be caused by immunological mechanisms, although other mechanisms may be present. For instance, it is possible that genetic polymorphisms present in mitochondrial DNA (mtDNA) can result in risk factors for IDRs, and it is also quite possible that mitochondrial damage can act as an initiation sign for induction of the onset of an immune response (Uetrecht, 2008).

Clearly, the basis for IDRs is far from being understood. There is growing evidence that ADRs are more diverse and complex than previously recognized and thus, it is crucial to continue to carry on basic research in this field to create new and more realistic models to evaluate drug toxicity and in particular, occurring in small populations of patients.

In this section the mechanisms of idiosyncratic toxicity and its main characteristics will be discussed.

1.1.1 The unclear definition of idiosyncrasy

The term "idiosyncratic" refers in general terms to specific characteristics, particular to one individual. However, there are different definitions for IDR, including an extreme sensitivity to low drug doses (Peters, 2005) or a gradual toxic response rather than all or nothing (Boelsterli and Lim, 2007). Another definition states that an IDR is an adverse reaction that does not occur in most people within the range of clinically used dosages. Besides, ADRs that involve the known pharmacological effects of the drug are often excluded (Park *et al.*, 1998). For instance, the rare cardiovascular events that have been attributed to cyclooxygenase-2-specific agents presumably involve the inhibition of cyclooxygenases (Uetrecht, 2007). Therefore, those reactions would not be called idiosyncratic because they involve a known pharmacological effect of the drug, even though they are patient-specific.

When allergologists use the term IDR, they fail to include ADRs that are immune-mediated but, instead, the reactions that involve the pharmacological effects of the drug are actually considered. There are other terms that can be very often used with varying degrees of overlapping with IDRs, including type B reactions (which do not show simple dose-response relationship), hypersensitivity, and allergic reactions (Pirmohamed *et al.*, 1998). Additionally, IDR can be defined as toxic responses determined by individual susceptibility (i.e. host factors) that increase the *penetrance* and *expressivity* of the intrinsic toxicity of a drug (or drug metabolite)(Boelsterli, 2003).

There is no general agreement on the use of the term IDR; thus, it is unlikely that the nomenclature of IDRs will be standardized; in other words, it can be used to describe different types of reactions and can actually be used to mean different things. Despite the different definitions IDR will be consider along this thesis as an adverse reaction that does not occur in most patients and does not involve the known pharmacological effects of a drug (Uetrecht, 2008).

1.1.2 Main characteristics of idiosyncratic drug responses

It has been described that a delay of one week or more between the initiation of drug treatment and the onset of the adverse reaction from the first exposure exists (Uetrecht, 2007). However, in exceptional cases, IDRs may occur within days of the first dose of the drug. The interval of time that takes to ADRs to occur is dependent not only of the different type of IDR but also of the different drug used. For instance, maculopapular rashes usually occur after one or two weeks of treatment with drugs such as metamizole (Torres *et al.*, 2009) while IDR-induced hepatitis occurs

commonly after one to two months of therapy (Lawrenson *et al.*, 2000). Some drugs such as troglitazone have toxicity delays that are often longer and it can actually occur only after a year or even more since the starting of the treatment (Graham *et al.*, 2003). Also, drug-induced lupus can have a delay of more than a year (Borchers *et al.*, 2007). In contrast to the first exposure, the delay in the onset of symptoms in a patient who previously suffered from IDR is usually very short and can actually occur in minutes. On the other hand, there are cases where the onset of symptoms after a second-hit can also be delayed (Guest *et al.*, 1998) or even may not happen (Warkentin and Kelton, 2001).

Idiosyncratic reactions are usually referred to be dose-independent. It is true that for most patients, a relationship between the occurrence of IDR and drug dose does not exist, but it is self-evident that every biological effect has a dose-response relationship and that a dosage without having associated IDR will exist. When a patient is allergic to penicillin it is usually possible to administer a small dose (about one ten thousandth the normal therapeutic dose) followed by slowly increasing doses until the therapeutic success is achieved (Uetrecht, 2007). IDRs occur rarely for drugs given at a dose of 10 mg per day or less. In contrast, drugs such as procainamide (Knowles *et al.*, 2003) and felbamate (Dieckhaus *et al.*, 2002), where the dose is often one gram per day or more, are more expected to cause IDRs.

Most IDR effects can also be caused by other co-existing agents or be idiopathic, i.e., with an unknown cause. For example, a large number of IDR-unrelated liver failure appears to exist (Gow *et al.*, 2004), and although the histology can provide clues to the etiology, hepatic IDRs cannot be differentiated from idiopathic liver failure or viral hepatitis. Drug-induced hepatic failure has an inflammatory component that is usually less pronounced than that with viral hepatitis but it is impossible to know

whether the inflammation is the cause or the consequence of hepatic damage. Some hepatic IDRs are associated with eosinophilia (Uetrecht, 1999), which is considered evidence of an immune-mediated reaction. Most drug-induced rashes are also presumed to be immune-mediated. Idiosyncratic drug-induced liver injury (DILI) will be discussed in more detail later at section 3.

1.1.3 Genetic associations with idiosyncratic drug reactions

Establishing a link between a specific genotype and the risk to a specific IDR has been an open question. A small number of associations with genetically polymorphic metabolic enzymes and the risk of IDR have been found. For instance, the risk of drug-induced toxicity with isoniazid and sulfonamide antibiotics is greater in patients that are slow acetylators (Huang *et al.*, 2002; O'Neil *et al.*, 2002). However, the relative risk is very small and since 50% of North Americans are slow acetylators, this could not be the reason behind this IDR. Other studies are focused on the association between polymorphisms in drug metabolic enzymes, although none of them has been conclusive (Leeder, 1998). It is possible that such associations exist but results suggest that differences in drug metabolism are not the major risk factor for IDRs.

If IDRs are immune-mediated, then specific human leukocyte antigen (HLA) genotypes could be linked to IDRs. Studies were also negative or showed that those associations were weak (Pirmohamed and Park, 2001). In contrast to older studies, strong associations have been recently found between HLA-B*5701 and hypersensitivity reactions to *abacavir* in Australia, where the odds ratio is 960 (Mallal *et al.*, 2002). In contrast, this may not be a risk factor in black populations (Hughes *et al.*, 2004). Other very strong association was found between HLA-B*1502 and carbamazepine-induced

Stevens-Johnson syndrome, with an odds ratio of 895 (100% sensitity, 97% sensitivity) in Han Chinese, among other examples (Phillips, 2006).

There are also other risk factors for the development of IDRs such as sex, age, weight and disease state. Some studies showed that women were an increased risk regarding some drugs such as halothane-induced hepatitis and clozapine-induced agranulocytosis (Pollard and Coombs, 1980; Alvir *et al.*, 1993). The risk of liver toxicity increases with aging as well for drugs such as isoniazid and acetaminophen. It is also often assumed that patients with preexisting liver disease have also a higher risk of idiosyncratic DILI (Russo and Watkins, 2004). Infectious diseases such as mononucleosis, HIV infections, and possibly herpes virus, were also associated with higher risk of IDRs (Pullen *et al.*, 1967; Rabaud *et al.*, 2001; Kano *et al.*, 2004).

1.1.4 Proposed mechanisms for idiosyncratic drug reactions

There is not a definitive mechanistical understanding for IDRs and it is very likely that there are many different mechanisms involved. However, there is a general consensus that many can be immune-mediated. In addition, there is a large amount of evidence to suggest that chemically-reactive metabolites are responsible for many IDRs and this is a strong component in several hypotheses. Although it is not known whether reactive metabolite screenings have significant predictive value for the risk of IDRs, the formation of a large amount of reactive metabolite is considered a significant liability for a drug candidate.

1.1.4.1 The proposed hypothesis

In 1935, it was reported that it was not possible to induce an immune response with small molecules unless they were chemically reactive and bound to proteins; this led to the *Hapten Hypothesis* (Figure 1.1.1)

(Landsteiner and Jacobs, 1936). Haptens are molecules of low molecular weight (LMW) of less than 1000 daltons (Chipinda *et al.*, 2011) that bind irreversibly to a carrier molecule (protein) to induce an immune response. This hypothesis was first used to explain both humoral and cellular immune responses to LMW chemicals in guinea pigs observed by Landsteiner (Landsteiner, 1936). Briefly, the drug-modified protein is bound by antigen-presenting cells (APCs) processed and further presented by major histocompatibility complex (MHC) to helper T cells. The recogniziton of drug modified proteins by T cells receptors is referred to as signal 1 (see Figure 1.1.1).

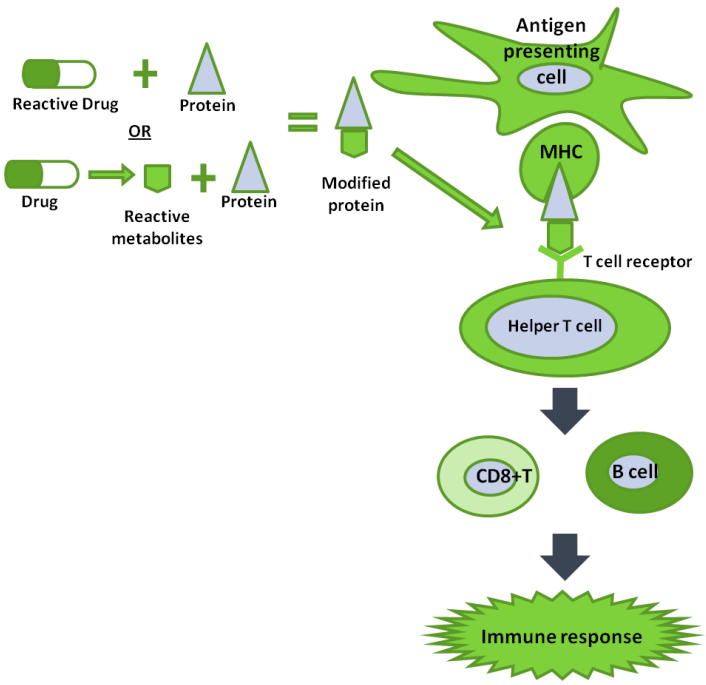


Figure 1.1.1 - The hapten hypothesis. The drug or reactive metabolites bind to proteins which will be modified and seen as foreigner by the immune-system activating the T cells. First, the modified protein is taken by the antigen presenting cells (APCs) processed and drug-modified peptides are then presented in the context of MHC-M to helper ($CD4^+$) T cells. The recognition of the processed antigen by the T cell receptor (TRC) is the signal 1 which will lead to an immune response *(Adapted from Uetrecht, 2007).*

For over 50 years, immunologists thought that the immune system functions by making the distinction between self and nonself. In 1994, Matzinger (Matzinger, 1994) challenged the Hapten Hypothesis with a new hypothesis. This danger hypothesis is based on the principle that the signals which control an immuneresponse are endogenous and not exogenous, being raised by stress factors or tissue injury (Fig. 1.1.2). Therefore, it was argued that, in general, foreign proteins do not generate a significant immune response in the absence of an adjuvant which could stimulate the antigen-presenting cells (APCs). In addition to signal 1 (referred previously), co-stimulation of T cells by activated APCs is required for an immune response, and this is the so-called signal 2. Without signal 2, the response is immune tolerance. In this hypothesis, danger signals from stressed cells stimulate APCs and upregulate the co-stimulatory molecules. If we extend this hypothesis to IDRs, it may be that some reactive metabolites can act as danger signals rather than haptens, or both effects may be important to the onset of IDR. The danger hypothesis does not address the issue of signal 1, which could be provided by a drug, by the drug-modified peptide or by an autoantigen (see Figure 1.1.2).

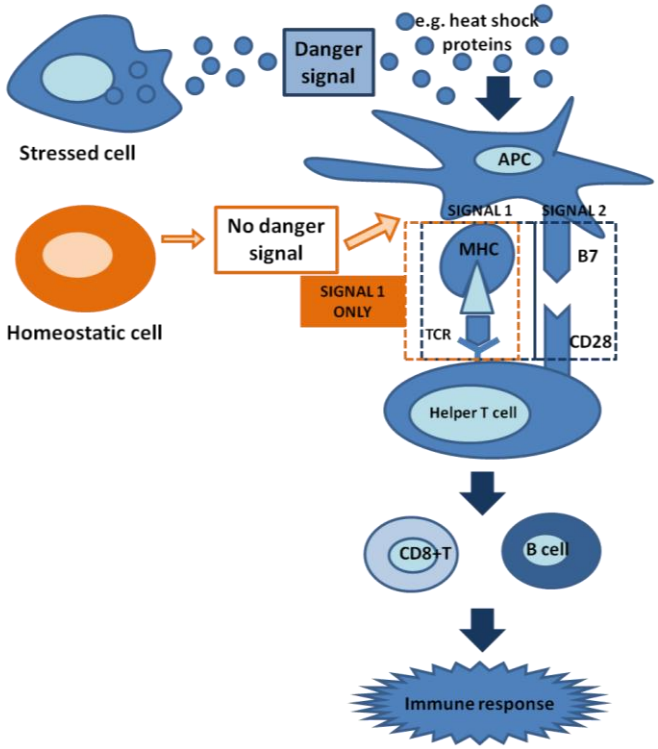


Figure 1.1.2 - The danger hypothesis. Cells which undergo stressful events produce danger signals such as heat shock proteins (HSP) which activate APCs, inducing up-regulation of costimulatory molecules as B7. The signal 2 refers to the costimulation of T cells by interaction between APCs and Tcells, for instance, between B7 and CD28. When signal 2 is absent, the response is tolerance. This hypothesis does not explain the issue of signal 1 *(Adapted from Uetrecht, 2007).*

Another hypothesis is the pharmacological interaction (PI) hypothesis which consists in drug binding to the MHC-TCR complex reversibly leading to signal 1 and an immune response to the parent drug, in some cases leading to an IDR (Figure 1.1.3) (Pichler, 2002). This hypothesis, similarly to the hapten hypothesis does not address the signal 2 issue.

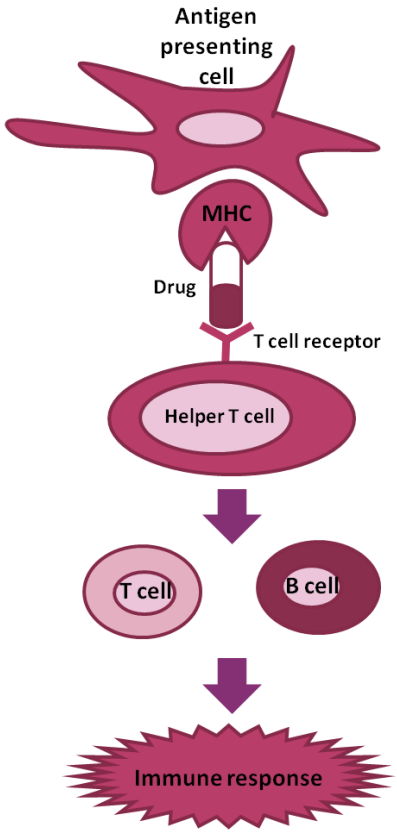


Figure 1.1.3 - Pharmacological interaction (PI) hypothesis refers to drugs that bind directly to the MHC-TCR complex and lead to signal 1 and the onset of an immune response. In the opposite to danger hypothesis, this one does not address the issue of signal 2 *(Adapted from Uetrecht, 2007).*

Some IDRs, in particular the ones that involve the liver, are referred to as *metabolic idiosyncrasy,* once they lack the inflammatory symptoms such as fever and rash, or lack of immediate onset or rechallenge (Zimmerman, 1976). One example is troglitazone-induced hepatotoxicity which is typical of metabolic idiosyncrasy, although recently it was also related with autoantibodies (Murphy *et al.*, 2000; Maniratanachote *et al.*, 2005). Various studies have suggested that troglitazone-induced liver failure was mediated by oxidative stress, mitochondrial damage, inhibition of bile salt transport, and apoptosis (Julie *et al.*, 2008); however, none of the studies are truly

convincing, nor do explain the idiosyncratic nature of the reaction. Another example is valproate-induced hepatotoxicity, which conveys mitochondrial markers of dysfunction such as steatosis and mitochondrial toxicity which apparently does play an important role in some IDRs (Baillie, 1992). Although it does not explain the idiosyncratic nature of these reactions, mitochondrial diseases appear to be a risk factor for valproate-induced hepatotoxicity.

Another hypothesis, especially for the mechanisms of hepatotoxicity, is the *Inflammagen Hypothesis* which was formulated by Roth. It is suggested that idiosyncratic liver toxicity results from coincident exposure to a drug and to some *inflammagen* such as lipopolysaccaride (LPS) (Roth *et al.*, 2003; Wheeler *et al.*, 2003). Nonetheless, it is unlikely that the previous mechanism can explain most of the idiosyncratic drug responses. Drug-induced liver injury and IDRs will be debated in detail in Section 3.

1.1.5 The scarcity of animal models of idiosyncratic drug reactions

"The uselfuness of an animal model depends on how closely it resembles the disease or condition to which is compared" (Scarplelli et al, 1997)

Unfortunately, idiosyncratic reactions have the same nature in animals and in humans. In other words, these reactions occur in a very small number of individuals and they are not visible in the majority of the animal population. Many attempts have been made to find good animal models either by manipulating their immune system and/or metabolism, but overall, those attempts ended up failing or involving other mechanisms of toxicity rather of the idiosyncratic type. Manifestation of the same drug response can be different in different individuals, and it is difficult to predict the diversity of

reactions seen in the human population by using an animal model. A solution would be by using animals with different genetic backgrounds, for instance, by studying strains of mice, with different polymorphisms in their genetic code. Besides, the animals that have been used for safety prediction of new compounds are healthy. In general, these animals are quite resistant to the drug effects so the right model should be a susceptible strain or transgenic animal. Anyway, a possible way of overcoming the inherent difficulties would be by using an animal model which could mimic a disease that is likely to occur in the population of patients who took the specific drug in the first place.

In spite of all challenges, an animal model can be used to study the mechanisms behind adverse reactions and must be similar to that of the adverse reactions that occur in humans, but the problem is that until now, we are not certain of the mechanisms involved in human adverse reactions as well. Some examples of previously reported models will be described below, which perhaps can shed some light on the mechanisms behind drug-induced idiosyncratic adverse reactions.

1.1.5.1 Halothane-induced liver injury

Halothane causes severe idiosyncratic liver toxicity in some patients (Moult and Sherlock, 1975). Studies reported that halothane does not lead to rat induced-liver toxicity, however, for instance, if the animals were first treated with Phenobarbital and then exposed to Halothane under hypoxic conditions, this would led to mild liver toxicity (McLain *et al.*, 1979). Antibodies against trifluoroacetylated (TFA)-protein were associated with an idiosyncratic response in humans (Vergani *et al.*, 1980). Eosinophilia and autoantibodies were found to be part of halothane-induced hepatotoxicity symptoms which suggests immune sensitization (Walton *et al.*, 1976). This

would make us think that halothane-induced toxicity in humans is an immune-mediated mechanism. Other studies reported that guinea pigs exposed for 1 hour to halothane suffered from hepatic necrosis, although no increase in transaminases was observed (Hughes and Lang, 1972). By increasing the exposure to 4h consistent hepatic injury was observed in guinea pigs, although the damage was reversible (Lunam *et al.*, 1985). Later, halothane was administered in combination with buthionine sulfoximine to deplete glutathione, with a high mortality rate achieved (Lind *et al.*, 1994). Unfortunately, this observation is more likely to be a cytotoxic effect than an immune-mediated reaction and does not represent a good model. To conclude, the model here described may not mimic what happens in some patients but it is still a reasonable model for early steps in the immune response to the oxidative metabolite of halothane and immune tolerance may be what limits the degree of toxicity.

1.1.5.2 Penicillamine-induced autoimmunity in brown Norway rats

D-penicillamine therapy has been used in the treatment of rheumatoid arthritis, cystinuria, and Wilson's disease (Emery, 1989). In humans, treatment with D-penincillamine is associated with high incidence of autoimmune pathologies such as lupus-like syndrome and myasthenia gravis. In a similar way, D-penicillamine also causes autoimmune syndromes in Brown Norway (BN) rats (Donker *et al.*, 1984; Tournade *et al.*, 1990). The rat model of D-penicillamine-induced lupus was described for the first time in 1984 (Donker *et al.*, 1984) and the symptoms included rash, antinuclear antibodies, hepatic necrosis, arthritis and weigh losse. D-penicillamine administration after 3-4 weeks of treatment showed that rats eventually died from what appeared to be disseminated intravascular coagulation. Furthermore, circulating antinuclear antibodies were found in sera of BN rats as immune complexes. Three to eight weeks after D-penicillamine

administration in a dosage of 20 to 50 mg per day, 73% of Brown Norway (BN) rats became ill while no adverse effects were observed in Lewis (LEW) and Sprague-Dawley (SD) rats in the same treatment. Like IDRs in humans, there is a delay between the starting of the drug and the onset of the autoimmune syndrome, and it is considered idiosyncratic because it only occurs in BN rats (although only occurs in 50-80% of treated animals). This model provides a similar mechanism and a reasonable tool to study the mechanism of drug-induced lupus in humans.

1.1.5.3 Nevirapine-induced skin rash

Nevirapine is a non-nucleoside reverse transcriptase inhibitor used in the treatment of human immunodeficiency virus (HIV) infections. This drug causes a high incidence of skin rash and also liver toxicity (Shenton *et al.*, 2003). Sprague-Dawley rats were administered with 150mg/kg/day to investigate *in vivo* covalent binding of nevirapine to liver proteins (Shenton *et al.*, 2003). Two out of 4 Sprague-Dawley rats administered with nevirapine developed erythema at 4 and 6 weeks, respectively. In addition, excessive scratching was observed around the nose/mouth area as well as loss of body weight, although liver toxicity was not observed in this model (Shenton *et al.*, 2004). The incidence of skin rash in patients is superior to the incidence of liver toxicity, so it was suggested that this model could represent the population who develops skin rash only. Although there is still no available model for nevirapine-induced hepatotoxicity, the skin rash in humans and in rats suggests a common mechanistic pathway. Studies in this toxicity model showed a definite role for the immune system in the development of the skin rash. The rash is strain and sex dependent, with 0% incidence in all male animals treated and a 20-100% incidence in female Sprague-Dawley and BN rats, respectively (Shenton *et al.*, 2003).

1.1.6 Pharmacogenetics and IDRs: future perspectives

The term pharmacogenetics was first used in the late 50's, and is defined as the study of genetic factors which affect drug response. Pharmacogenomics refers to the whole-genome application of pharmacogenetics which has been concerned with single-gene effects (Daly, 2010). The term pharmacogenomics can also be related with the development of new drugs using genomic information. The genetic basis for inter-individual variation in drug response has been broadly studied and it is now recognized that all human genes have extensive genetic polymorphisms and this results in functionally significant alterations. The main question is how this knowledge can shed some light on IDRs? How can genetic knowledge of human polymorphisms contribute to create better safety platforms/models to test drug safety and avoid drug withdrawal from the market? How can this knowledge help us to understand the mysterious mechanisms of IDRs? With this in mind, it is important to focus on the most common class of genetic variation in humans, the presence of *Single nucleotide polymorphisms* (SNPs) in the human genome (Meyer, 2000).

SNPs are single-base differences in the DNA sequence observed between individuals, which occur naturally throughout the human genome in a frequency > 1% (Sachidanandam *et al.*, 2001). On the other hand, a single base alteration that occurs in < 1% of the population is called mutation. SNPs are normally classified as coding SNPs (in the translated region of the gene), noncoding SNPs (outside the translated region such as promoter region) and random SNPs. The coding SNPs can be additionally classified as synonymous (when single base substitutions do not cause a change in the resultant amino acid) and nonsynonymous (when single base substitutions cause a change in the resultant amino acid) (Sauna and Kimchi-Sarfaty, 2011). SNPs may occur at any position in the gene structure, and based on

location SNPs can be classified as intronic, exonic or occurring in promoter regions. A complete list of all SNPs can be found at the National Center for Biotechnology Information website. At least 60,000 SNPs are within coding regions (coding SNPs) and are, therefore, more likely to be functionally active. This high density SNP map provides an opportunity to perform SNP profiling to identify genetic factors predisposing for further IDRs. Some examples which can actually interfere in drug metabolism and predispose to ADRs are cytochrome P450 enzymes, namely polymorphisms occurring in CYP2D6 (Cytochrome P450 2D6), MDR1 (multidrug resistance protein 1), KCNE2 (altered potassium channels) and HLAs (human leukocyte antigen) genes, among many others (Pirmohamed and Park, 2001). The availability of cost-effective SNPs genotyping technologies may allow a search for pharmacogenetically active genes which can predispose an individual to an idiosyncratic reaction, which can lead the way to personalized medicine. If one could predict the patients who are likely to have an IDR to a specific drug, "risky" drugs could be used safely.

Furthermore, maping SNPs can also be made in animals. One of the goals of the Mouse Phenome project (http://phenome.jax.org/db/q?rtn=docs/aboutmpd) is to map SNPs. The Mouse Phenome database begun in 2000 and has been funded by grants from the National Institutes of Health and other sources, and is headquartered at The Jackson Laboratory. Actually, different strains of laboratory mice are being used to collect phenotypic and genotypic characteristics of these animals, which are then made accessible online (Bogue *et al.*, 2007). Therefore, the future for animal studies will probably pass through the usage of different mice strains, which can actually mimic the genetic diversity seen in the human population and therefore, can represent a better framework to predict drug response and the

predisposition to IDRs. A few toxicogenomics studies and other interesting models will be presented later in Section 3 of this thesis.

To conclude this section, it is also important to note that besides genetic variations, host and environmental factors such as disease, alcohol consumption, smoking habits and even diet may also be significant sources of variability and may interact with the genetic factors increasing or decreasing the risk of ADRs.

Summary points of Section 1:

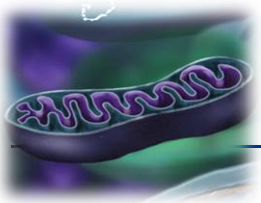
- Although a rare occurrence, idiosyncratic drug reactions are a major problem in drug development, representing a major cause for drug withdrawal.

- It is not clear what makes drug reactions idiosyncratic but genetics may play an important part and polymorphisms might predispose to drug-induced toxicity.

- The mechanistic pathways of IDRs are far from being understood but there is evidence that most IDRs might be immune-mediated, although there are probably many exceptions.

- Animal models are scarce and very few have been validated to the study of IDRs, although representing an important tool for mechanistic studies.

- In the future, the SNPs mapping should provide crucial information to the creation of personalized medicine, increasing the possibility to predict IDRs among the human population.



Section 2

Mitochondria: more than just cells furnaces

"Complex structures can only be maintained by the continual flux of energy. Life, therefore, is the interaction between structure, energy, and information, with information required for both structure and energetic." (Wallace, 2010a)

1.2 The origins of Mitochondria: when two entities collide

Once upon a time, around 1-2 billion years ago, an ancient bacterium found a shelter inside of a host cell. Inside the host, the bacterium found enough food and protection against the hostile environment of the outside world. Soon the host cell and the bacteria became inseparable entities. This extraordinary collision of two entities gave rise to a synergically superior eukaryote and it is described by the Endosymbiotic Theory of *Margulis* (1975) (Margulis, 1975). What happened to the bacterium then? It is believed that it became integrated into the host cell and evolved into an organelle which we now call mitochondrion.

This story that has been told has intrigued and fascinated many scientists all over the world and mitochondria became a famous organelle because of its unique and extraordinary characteristics and importance in a living eukariotic cell.

Mitochondria are one of the most fascinating organelles in cells and will be explored in detail in the following pages. This section will focus entirely on mitochondrial bioenergetics and genetics which will be the basis for Section 3, where the role of mitochondria in drug-induced toxicity and IDRs will be discussed in detail.

1.2.1 Mitochondrial structure and organization

When mitochondria were first identified in eukaryotic cells in 1898 by Carl Benda, these organelles were described as a collection of free-floating individual vesicles existing in hundreds of copies, remaining freely in the cytosol (Palade, 1952; Palade, 1953). At the time, imaging techniques revealed that mitochondria are composed by an outer membrane (OM), by an inner membrane (IM) with convolutions (cristae), by an intermembrane space (IMS) and by an inner space, the matrix. More recently, with the help of new and more refined technologies, a new look into mitochondria was possible (Frey and Mannella, 2000; Frey *et al.*, 2002). The organelle morphology can range from wide network of long tubules to a collection of small and individual vesicles (See figure 1.2.1.). What is defined as a mitochondrion is actually a sectional view of a single mitochondrial tubule. Furthermore, the combination of several microscopy techniques, allowed to better analyze mitochondrial organization (Yaffe, 1999). The association of three-dimensional light and electron microscopy allowed for the study of concomitant morphology changes in mitochondria. For instance, real-time transitions in the morphology of the mitochondrial network from a tubular reticulum or a collection of small circular fragments was observed (Benard and Rossignol, 2008).

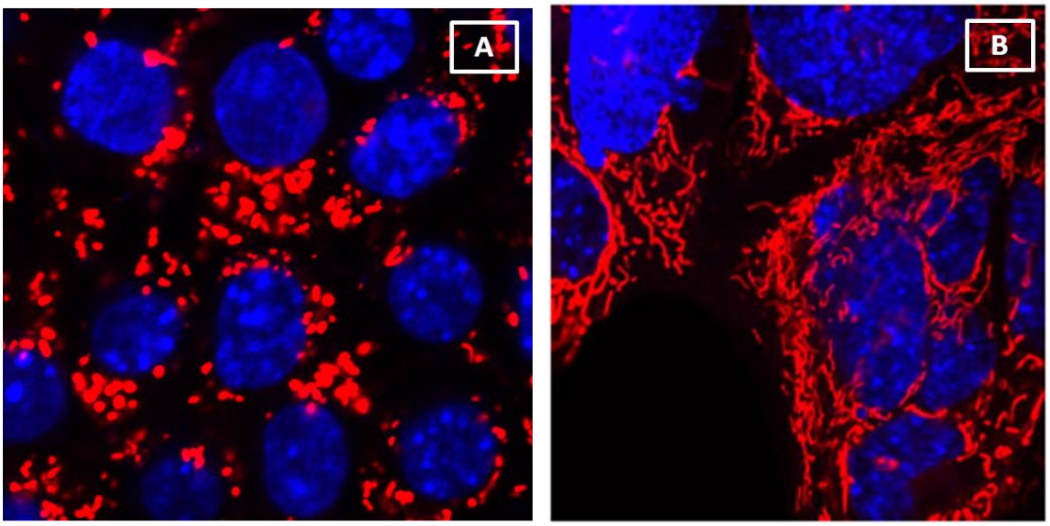


Figure 1.2.1 - Mitochondrial morphology in embryonic P19 carcinoma cell line in the undifferentiated state (**A**, small round mitochondria) and after retinoic-acid-directed differentiation (**B**, longer mitochondrial tubules). The red labeling originates from the fluorescent probe tetramethylrhondamine methylester (TMRM, 100 nM), which accumulates in polarized mitochondria. The blue labeling originates from Hoechst 33342 (1 microgram/mL) fluorescence after binding to nuclear DNA. Images were obtained by confocal microscopy in an Eclipse C1 confocal device (Nikon, Melville, USA). Total magnification (600x). Unpublished images from Dr. Paulo J. Oliveira (used after oral permission).

1.2.2 Mitochondrial biogenesis regulation

Mitochondria are dynamic organelles which are continuously being generated and eliminated. Mitochondrial biogenesis is a highly controlled and regulated event which involves the coordination of two distinct genomes, as we will see later. Many transcription factors such as the nuclear respiratory factors (NRF) control respiratory gene expression in mammals. NFR-1 was the first factor to be identified, with later NRF-2 being also discovered to have the same characteristics (Evans and Scarpulla, 1989; Evans and Scarpulla, 1990). Both factors function as positive regulators of mitochondrial-related gene transcription, and are vital for the

generation of new mitochondrial components, as observed by the fact that inactivation of NRF induces early embryonic lethality (Huo and Scarpulla, 2001). Both NFR-1 and NFR-2 are known to regulate the transcription of many genes involved in OXPHOS, such as complex I, II, III, IV and ATP synthase subunits, as well as genes involved in the machinery of mtDNA transcription and replication and genes involved in protein import to mitochondria (Evans and Scarpulla, 1990; Kelly and Scarpulla, 2004). Several nuclear receptors are also related with regulation of mitochondrial biogenesis and metabolism, such as peroxisome proliferator-associated receptors (PPARα, PPARβ and PPARγ) and ERRα, since they regulate and coordinate the expression of genes involved in fatty acid oxidation and the respiratory chain (Gulick *et al.*, 1994; Giguere, 2008).

Importantly, the regulation of the transcription factors and nuclear receptors is regulated by a family of co-activators, the PGC-1 family (PGC-1α, PGC-1β and PRC). PGC-1α was first identified as a crucial factor in adaptative thermogenesis (Puigserver *et al.*, 1998) and PGC-1β and PRC-1 found to be homologous to PGC-1α (Lin *et al.*, 2002a). This family of activators plays a central role in the transcriptional regulation of mitochondrial biogenesis and metabolism through binding to NRF-1 and -2, PPARs and ERRs, and therefore is considered "master regulators" of the mitochondrial biogenesis program. Indeed, overexpression of PGC-1α or PGC-1β in cultured cells and transgenic mice resulted in increased mitochondrial content (Wu *et al.*, 1999; Lehman *et al.*, 2000). Furthermore, the overexpression of either PGC-1α or PGC-1β in the skeletal muscle induces its conversion to oxidative type muscle fibers (Lin *et al.*, 2002b; Arany *et al.*, 2007). In addition, a role for oxidative stress in the regulation of mitochondrial biogenesis has been demonstrated (Piantadosi and Suliman, 2012). The cell energy supply was shown to be protected from conditions

that damage mitochondria by an inducible transcriptional program regulating mitochondrial biogenesis that operates in large part through redox signals involving the nitric oxide synthase and heme oxygenase-1/CO systems. The redox events lead to the elimination of defective mitochondria (Piantadosi and Suliman, 2012). Caloric restriction also plays a role in mitochondrial biogenesis and longevity in several animal models; through regulation of the sirtuin pathway, namely sirtuin 1 (SIRT1) (Haigis and Guarente, 2006). Sirtuin 1 activation by resveratrol or its analogs increase mitochondrial energy output and biogenesis in mice through the deacetylation and activation of PGC-1α (Lagouge *et al.*, 2006). This mechanism will be described later at Section 4. Importantly, PGC-1α is regulated in response to metabolic changes and nutrient availability (Housley *et al.*, 2009). Indeed, Sirtuin 1 (SIRT1) and AMP-activated kinase (AMPK) were found to regulate PGC-1α activity. SIRT1 and AMPK are two important metabolic sensors as both are regulated by the NAD^+/NADH and ATP/AMP ratios, respectively. In conditions of fasting, SIRT1 was reported to deacetylate and activate PGC-1α (Rodgers *et al.*, 2005; Gerhart-Hines *et al.*, 2007) (Figure 1.2.2). More recently, AMPK was also found to regulate PGC-1α activity since AMPK phosphorylates PGC-1α and primes it for deacetylation (Canto and Auwerx, 2009). As such, AMPK and SIRT1 are able to integrate external cues and adapt mitochondrial metabolism by regulating PGC-1α activity and thereby the biogenesis program.

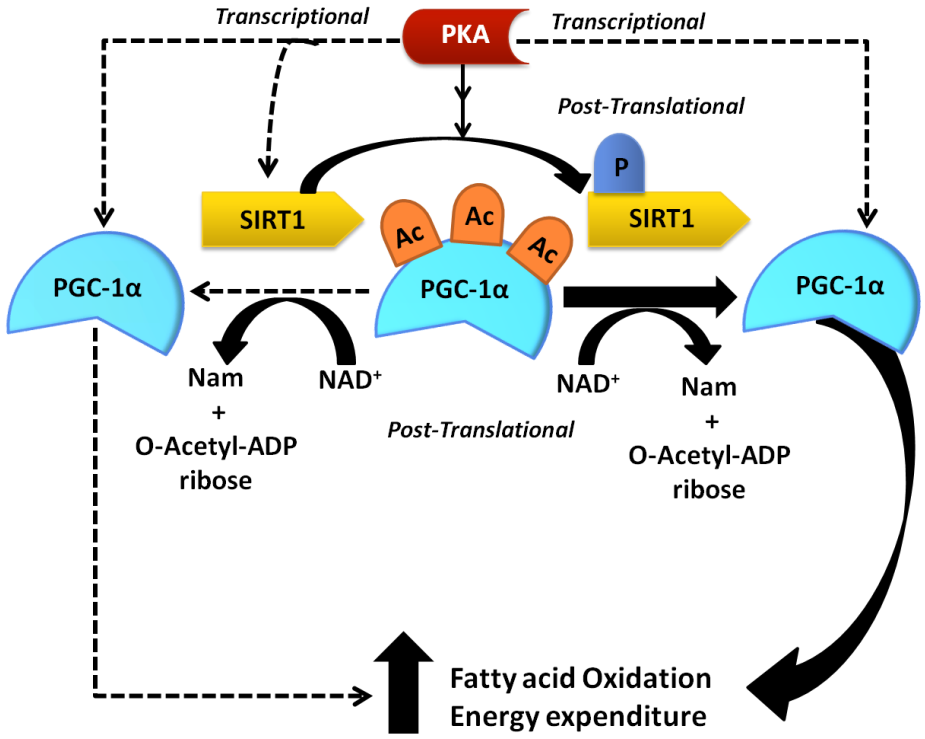


Figure 1.2.2 - SIRT1 serine 434 phosphorylation after stimulation of cAMP/PKA pathways which enhances the efficiency of NAD^+ co-substrate utilization and increases overall catalytic capacity will also induce PGC-1α deacetylation and increased fatty acid oxidation and energy expenditure. Adrenergic agonism also induces transcription in both SIRT1 and PGC-1α (Adapted from Gerhart-Hines *et al.*, 2007).

1.2.3 The basics of mitochondrial bioenergetics

Mitochondrial bioenergetics gained attention in the late 40's and 50's with the works of Lehninger, Racker, Chance, Boyer, Ernst, and Slater, among others. The "chemiosmotic hypothesis", formulated in the 60s by Mitchell, prevailed (Madeira, 2012).

Mitochondria are the main arena for the production of the majority of energy cells need for their normal activity. Energy production is achieved by

electron transfer in the respiratory chain through a process called oxidative phosphorylation (OXPHOS) (Chance and Williams, 1956; Mitchell, 1961). Aerobic tissues rely on OXPHOS for ATP production and the overall process is highly controlled and involves integration and balance of the oxidation of fatty acids, pyruvate, ketoacids and a range of other intermediary metabolites and regulatory ions such as calcium.

1.2.3.1 Mitochondrial energetics: OXPHOS and ATP production

"Oxidative phosphorylation is the culmination of energy yielding metabolism in aerobic organisms. All oxidative steps in the degradation of carbohydrates, fats, and amino acids converge at this final stage of cellular respiration, in which the energy of oxidation drives the synthesis of ATP" (in Lehninger et al., 2005)

During glycolysis, glucose is converted to pyruvate in the cytosol, reducing cytosolic NAD^+ to NADH. Pyruvate is channeled to enter mitochondria via pyruvate dehydrogenase (PDH) yielding acetyl CoA, NADH + H^+, and CO_2. Acetyl CoA enters the Krebs cycle or Tricarboxylic Acid (TCA) cycle generating NADH + H^+ (Rich, 2003). Fatty acids are oxidized in mitochondria via β-oxidation to generate Acetyl-CoA, NADH + H^+ and reduced FAD co-factor. Two electrons are then transferred from NADH to the OXPHOS complex NADH dehydrogenase (Complex I) or from $FADH_2$-containing enzymes as the electron transfer factor dehydrogenase or succinate dehydrogenase (SDH, complex II) to reduce ubiquinone (coenzyme Q10, CoQ) to ubiquinol ($CoQH_2$). The electrons are then transferred to complex III (bc_1 complex), cytochrome c, complex IV (cytochrome c oxidase, COX), and finally to oxygen, generating H_2O. In fact, during glycolysis, only 2 molecules of ATP are formed while during OXPHOS, ATP production yield is up to 38 molecules of ATP. In fact, OXPHOS is the peak of energy metabolic yield.

Overall, the respiratory chain involves five enzymatic complexes, the NADH-ubiquinone oxidoreductase (complex I); the succinate-ubiquinone oxidoreductase (complex II); the ubiquinol-ferrocytochrome c oxidoreductase or cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and finally, complex V or F1Fo ATP synthase. Other crucial components are two electron carriers, the lipid soluble coenzyme Q (CoQ) and the soluble protein cytochrome c (cyt c). The energy that is then released as electrons flow down the redox potential through the ETC is used to pump protons across the mitochondrial inner membrane space by complexes I, III and IV creating a proton electrochemical gradient ($\Delta p=\Delta\Psi+\Delta\mu^{H+}$), which is acidic and positive in the intermembrane space and negative and alkaline in the matrix side. The potential energy (also called driving force) stored in the protonmotive gradient (Δp) is used to import proteins and calcium to the mitochondrial matrix, to generate heat and to synthesize ATP. The maximal protonmotive force across the inner membrane is around 180-220 mV (Mitchell and Moyle, 1967; Mitchell, 2011). The energy to convert ADP+Pi to ATP comes from the flow of protons through the ATP synthase (complex V) back into the matrix. The matrix ATP is also exchanged by cytosolic ADP through the ANT (adenine nucleotide translocator) located in the inner mitochondrial membrane. The production of ATP is dependent on the efficiency by which the protons are pumped out of the matrix by ETC complexes and by the efficiency by which proton flux through complex V can yield ATP. This means that the electron transfer is coupled to OXPHOS via proton gradient, but this machinery is actually prone to proton leakage, leading to variations in the coupling efficiency of the respiratory chain. Furthermore, alterations in the coupling efficiency can influence the generation of reactive oxygen species (ROS), leading to apoptosis (Orrenius *et al.*, 2003). Finally, each of the ETC complexes incorporates multiple electron carriers. Complex I and II utilize

flavins and iron-sulfur (Fe-S) centers. The Krebs cycle enzyme aconitase also utilizes a Fe-S group, susceptible to oxidative stress. Complex III encompasses a Fe-S center plus cytochromes b and c_1-Complex IV encompasses two Cu centers plus cytochromes a and a_3 (figure 1.2.3).

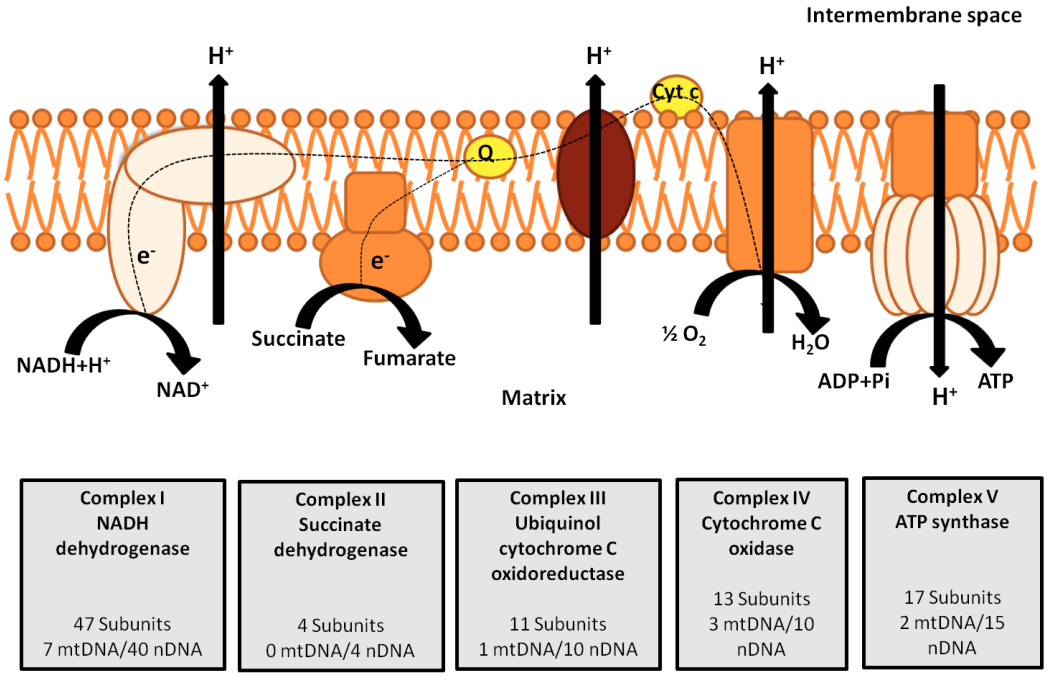


Figure 1.2.3 - Mitochondrial respiratory chain. Four enzyme complexes (complexes I - IV) and two intermediary substrates (coenzyme Q and cytochrome c) compose the respiratory chain in mammals, and also the complex V. The NADH+H+ and succinate produced by the intermediate metabolism are oxidized further by the mitochondrial respiratory chain to establish an electrochemical gradient of protons, which is finally used by the F1F0-ATP synthase (complex V) to produce ATP, the only form of energy used by the cell (Adapted from Bellance *et al.*, 2009).Legend: mt- mitochondrial, n- nuclear, H^+-proton, NAD^+-Nicotinamide adenine dinucleotide, NADH- Nicotinamide adenine dinucleotide (reduced form), e^-- electron, cyt c- cytochrome c, ADP- adenosine diphosphate, ATP- adenosine triphosphate, Q- ubiquinone.

1.2.4 Reactive oxygen species production by OXPHOS

The mitochondrial electron transport chain (ETC) is one main source of superoxide anion production in a living cell (Esposito *et al.*, 1999; Miwa *et al.*, 2003; Aquilano *et al.*, 2006; Hamanaka and Chandel, 2010). During OXPHOS, a small percentage of electrons are trapped by oxygen, which then becomes superoxide anion ($O_2^{\cdot-}$) (Indo *et al.*, 2007). Damage in the electron transport chain or in the inner mitochondrial membrane can increase superoxide anion production. Although some controversy still exists, it appears that two of the electron transport chain complexes are responsible for most of ROS production, namely complex I, the NADH-ubiquinone oxidoreductase (Jezek and Hlavata, 2005; Marzetti *et al.*, 2011) and complex III, the ubiquinol-cytochrome *c* oxidoreductase (Viola and Hool, 2010; Yen *et al.*, 2011). Superoxide production can be increased when the electron transport chain decreases its flux due to the formation of a high membrane potential under non-phosphorylating and maximum coupled conditions (Inoue *et al.*, 2003). It has also been demonstrated that superoxide anion can escape from the intermembrane space through porin, a voltage-dependent anion channel (VDAC) located in the mitochondrial outer membrane (Jezek and Hlavata, 2005). Besides oxidation of proteins, nucleic acids and membranes, one important consequence of oxidative stress is the induction of the mitochondrial permeability transition pore (MPTP). Induction of the MPTP, once irreversible, can lead to mitochondrial depolarization, loss of calcium control, mitochondrial swelling, secondary oxidative stress and inhibition of ATP synthesis, besides leading to cell death (Azzolin *et al.*, 2010). Membrane alterations can lead to the release of cytochrome c once the mitochondrial outer membrane is disrupted. Thus, MPTP induction can trigger cell death, either through apoptosis, if enough ATP is present to maintain caspase activity or through

necrosis after complete bioenergetic failure of the cell. Despite extensive studies, the composition and mechanism of the MPTP are still under debate (Azzolin *et al.*, 2010). An emerging hypothesis highlights the importance of the ATP synthase for the MPTP induction (Chinopoulos and Adam-Vizi, 2012). See figure 1.2.4 for a schematic representation of mitochondrial ROS production.

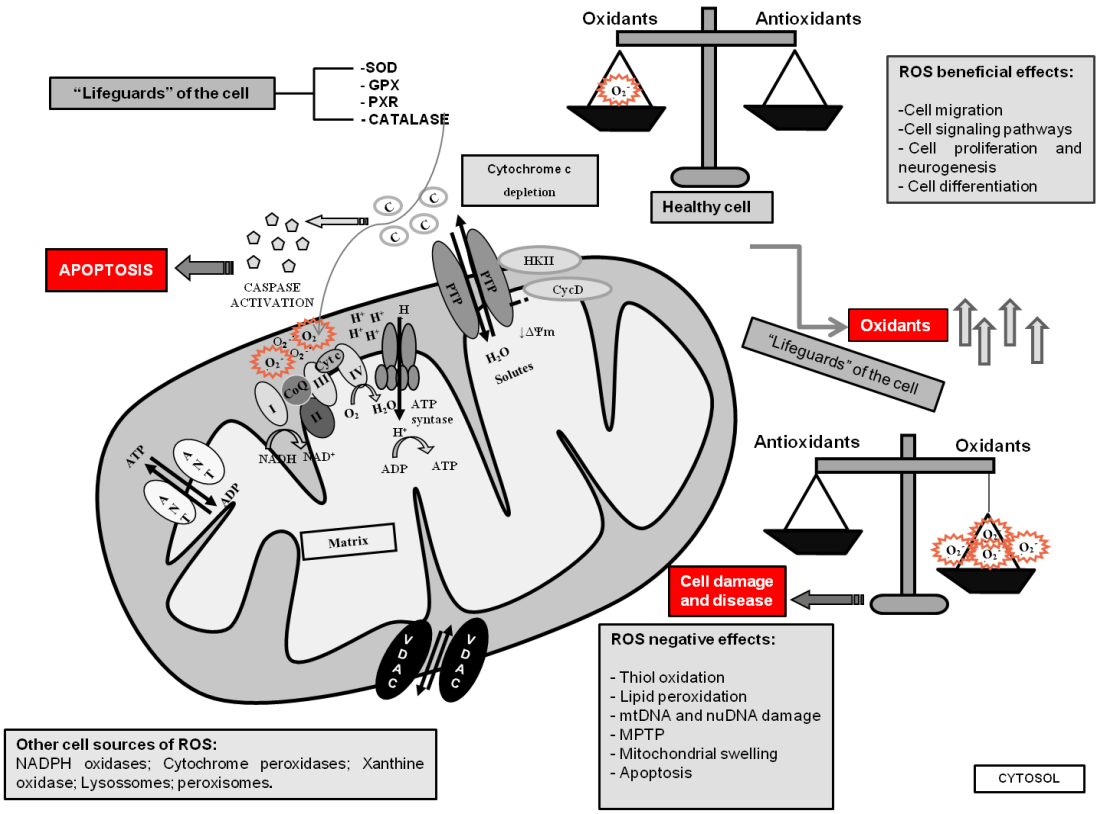


Figure 1.2.4 - Mitochondria: the main source of ROS production of the cell. Superoxide anion is mainly produced at mitochondrial complex I and III. The excessive ROS accumulation can lead to an imbalance between the antioxidant mechanisms (enzymes such as SOD, catalase, GPx, PXR) and ROS being produced in the cell. When ROS accumulate, mitochondrial and cell damage might occur. Increased ROS can damage mtDNA and nuclear DNA, and can cause thiol oxidation and lipid peroxidation, which can ultimately lead to cytochrome *c* release from the mitochondria, MPTP opening and apoptosis. Legend: SOD, superoxide dismutase; GPx, glutathione peroxidase; PXR, peroxiredoxin; VDAC, voltage dependent anion channel; MPTP, mitochondrial permeability transition pore; HKII, hexokinase II; CycD, cyclophilin D; ANT, adenine nucleotide translocator; Cytc, cytochrome c, CoQ, coenzyme Q.

1.2.4.1 Reactive oxygen species formation and the benefits for the cell

Reactive oxygen species are not only toxic cellular metabolic products that are frequently associated with many diseases but can also be critical intermediates of cellular signaling pathways. For example, an oxidative burst is necessary for insulin/IGF-1 pathway modulation (Mahadev *et al.*, 2001) and vascular endothelial growth factor (VEGF) signaling (Colavitti *et al.*, 2002). A recent review from *Hamanaka et al* (Hamanaka and Chandel, 2010) underlines a new insight into modulation of local oxidative stress and its advantages to the cell, which should also be taken into consideration when studying the effects of antioxidants. It has now become clear that ROS can function as second messengers in signal transduction (Hansen *et al.*, 2006) and that low levels of ROS are required for cellular processes such as proliferation and differentiation, pointing a new direction for the modulation of ROS production as a means to achieve a beneficial therapeutic outcome.

1.2.5 The antioxidant guardians of cells

Cellular antioxidant defenses have evolved to provide an important network system for prevention, interception and repair of oxidative damage. Antioxidant defenses consist of nonenzymatic scavengers and quenchers, known as antioxidants, as well as enzymatic systems including superoxide dismutases and hydroxyperoxidases, such as glutathione peroxidase, catalase and other hemoprotein peroxidases (Koch *et al.*, 2004; Koehler *et al.*, 2006).

Superoxide dismutase (SOD) exists as three different isoforms : SOD1 is a Cu/Zn SOD located in the cytoplasm and in the intermembrane space (1993; Achilli *et al.*, 2005; Abba *et al.*, 2009), SOD2 is a manganese superoxide dismutase that is exclusively located in the mitochondrial matrix (MnSOD)(Asimakis *et al.*, 2002) and SOD3 is a Cu/Zn SOD that has an

extracellular localization (Antonyuk *et al.*, 2009; Jung *et al.*, 2011). Mice lacking mitochondrial MnSOD (SOD2) cannot survive more than a few days after birth suggesting that control of a tight level of mitochondrial superoxide anion is critical for cell survival (Andreassen *et al.*, 2000; Araujo *et al.*, 2011). Superoxide dismutase accelerates the elimination of superoxide by increasing the rate constant for spontaneous dismutation. Acting like a scavenger of superoxide, SOD catalyzes its dismutation to H_2O_2 in the matrix. Superoxide anion that is released to the intermembrane space is partly controlled by the intermembrane space Cu-Zn SOD.

Glutathione (GSH) is the most abundant intracellular non-protein thiol which reduces cysteine disulfide bonds formed within cytoplasmic proteins by serving as an electron donor. Glutathione-based systems, including glutathione-S transferase and the thioredoxin system, constitute the major redox buffer in the cytosol (Aniya and Imaizumi, 2011). Since the concentration of the two glutathione forms (GSSG/GSH) is so much higher than that of any other system, the GSH/GSSG pool dominates the principal redox buffer of the cell (Meredith and Reed, 1982; Hansen *et al.*, 2006). Mitochondrial matrix glutathione represents 10-15% of the total glutathione in the liver as well as in renal proximal tubules. Since the enzymes for GSH synthesis are not present in the matrix, GSH has to be imported to mitochondria from the cytosol. The fact that mitochondrial glutathione (10mM) content is higher than in cytosol (7mM) suggests its importance in the cellular GSH content (Meredith and Reed, 1983). However, in mitochondria, glutathione is found mainly in its reduced form and its pool accounts for 10-15% of the total cellular amount (Mari *et al.*, 2009).

Glutathione peroxidase (GPx) exists in two forms in mitochondria: GPX1 and phospholipid-hydroperoxide GPX (PHGPx) (Blokhina *et al.*, 2003; Al

Hadithy *et al.*, 2010). The phospholipid hydroperoxide glutathione peroxidase is inducible under various stress conditions. This enzyme catalyses the regeneration of phospholipid hydroperoxides using the reducing power of GSH, being present in the cytosol and in the inner mitochondrial membrane of animal cells, while GPX1 occurs mainly in the mitochondrial matrix. Both enzymes use reduced glutathione (GSH) in order to reduce hydrogen peroxide into water, generating glutathione disulfide (GSSG) in the process, which can form protein-mixed disulfides and thus inhibit protein function (de Haan *et al.*, 2004; Fernandes *et al.*, 2007). The enzyme glutathione reductase can regenerate GSSG to GSH by using NADPH as a co-factor.

Another system capable of degrading H_2O_2 involves proteins of the thioredoxin family. These proteins work jointly with thioredoxin-dependent peroxidase reductases (peroxiredoxins) and a family of selenium-independent glutathione-S-transferases (GST). GSTs catalyze GSH-dependent reduction of both phospholipid hydroperoxides (PLOOH) and fatty acid hydroperoxides (FAOOH) and their respective aldehydes. Thioredoxin, which is especially concentrated in the endoplasmic reticulum, is a thiol-specific antioxidant that reduces disulfide bridges of proteins under oxidative stress conditions. Thioredoxin produces mostly intra-molecular disulfides and has different substrate preferences (Arner and Holmgren, 2000; Inarrea *et al.*, 2007). NADPH is a source of reducing equivalents for both thioredoxin and glutathione systems. NADPH-dependent thioredoxin reductase activity reduces 12kDa proteins of the thioredoxin family together with GSH or peroxiredoxins, maintaining protein thiols in the reduced state (Jezek and Hlavata, 2005). Peroxiredoxin (PXR) also converts H_2O_2 to H_2O using thioredoxin as a reducing agent.

There are two forms of PXR that are found in the mitochondrial matrix, PXRIII and PXR V (Sanchez-Font *et al.*, 2003; Cha *et al.*, 2009).

Although the activity of catalase, which converts H_2O_2 to water, is restricted in most tissues to peroxisomes, the enzyme represents 0.025% of total heart mitochondrial protein and it is important for detoxifying mitochondrial-derived H_2O_2. Hence, catalase represents a key antioxidant defense mechanism for myocardial tissue (Schriner *et al.*, 2000; Chan *et al.*, 2006). Catalase is also present inside liver mitochondria where it takes part in the oxidative stress defense system (See figure 1.2.4). It should also be emphasized that if liver and heart mitochondria catalase share similar detoxification functions, enzyme activity may control the amount of hydrogen peroxide available to produce hydroxyl radical and trigger the MPTP. Also, it is known that electron chain complexes and Krebs cycle enzymes in heart mitochondria can be inactivated by hydrogen peroxide. Target enzymes include succinate dehydrogenase, alpha-ketoglutarate dehydrogenase and aconitase (Salvi *et al.*, 2007). Due to its high Km, catalase functions at high H_2O_2 concentrations (e.g. in skeletal muscle, red blood cells), on the other hand at low H_2O_2 concentrations the enzyme exhibits a nonspecific peroxidase activity. In the liver, catalase removes endogenous H_2O_2 formed in the peroxisomes and it has been shown to protect hepatocytes more efficiently from H_2O_2 formed by peroxisomal oxidases than that formed by the outer mitochondrial membrane-bound monoamine oxidase (Siraki *et al.*, 2002). The most economical way of removing H_2O_2 is through catalase activity, since no reducing equivalents are consumed in the process.

1.2.6 The origin of mitochondrial DNA (mtDNA)

The mitochondrial energy production system originates from two distinct genomes, the nuclear and mitochondrial DNA (Wallace, 2010a). This feature creates a demand for elaborated regulatory processes to coordinate gene expression in response to cellular demands for ATP synthesis (Falkenberg *et al.*, 2007). As previously described, mitochondria are related to protobacteria and the eukaryotic cell originated by possibly fusion events between ancient cells (Yang *et al.*, 1985). Phylogenetic comparisons showed that there is co-segregation between the presence of mitochondrial DNA (mtDNA) and a functional respiratory chain (Wallace, 2007a). Traffic of mtDNA to the nucleus was described, with genes for many respiratory chain subunits being transferred to the nucleus, during evolution (Wallace, 2007a). There are 13 genes of the electron transfer chain (ETC) encoded by mtDNA. The reason for the localization of these genes in mtDNA is thought to be originated from the hydrophobicity of the gene products, which may prevent mitochondrial import if the gene is relocated to the nucleus. An alternative hypothesis is that mtDNA has been kept for regulatory purposes and that the biogenesis of the oxidative phosphorylation requires an inter-play between the respiratory chain subunits and the mtDNA (Allen, 2003). About 200-250 nucleus-encoded proteins are needed to express mtDNA-encoded proteins, but the reason why this costly arrangement has been maintained during evolution is still uncertain (Rotig and Munnich, 2003).

1.2.6.1 The mitochondrial genome and its features

Human mtDNA is a 16,569-bp double stranded and circular genome. Each mammalian cell contains hundreds of mitochondria and thousands of mtDNA copies. Each molecule contains 37 genes, encoding large and small

RNA (12S rRNA and 16S rRNA), 22 transfer rRNAs (tRNA) and 13 key respiratory chain (RC) subunits. The structural proteins encoded by mtDNA include seven subunits from Complex I (ND1-ND6 and ND4L), one subunit from Complex III (cytochrome b), three subunits from Complex IV (COXI- III), and two subunits from Complex V (ATPase 6 and 8) (Figure 1.2.5) (Anderson *et al.*, 1981).

There are three particular and important features of mtDNA: a) maternal inheritance, b) heteroplasmy and c) mitotic segregation. The inheritance of mtDNA is almost exclusively maternal, although some rare exceptions have been reported (Schwartz and Vissing, 2003). The clinical importance of this phenomenon is that pathogenic mutation in an affected mother is transmitted to all her offspring, but only her daughters will transmit the mutation to their progeny. A disease that has sex expression in both genders but that is not transmitted by the father is highly suggestive of a mtDNA deficiency (Wallace *et al.*, 2010; Park and Larsson, 2011).

Under normal conditions, all copies of mtDNA in a cell are identical, which is called homoplasmy. However, when mutations occur in mtDNA, normal (wild-type) and mutant mtDNA co-exist within the same cell, which is called heteroplasmy (Wallace and Fan, 2010). Since there are multiple mitochondria within a cell, there are hundreds of copies of mtDNA and the percentage of mutant mtDNA can vary between 0 and 100%.

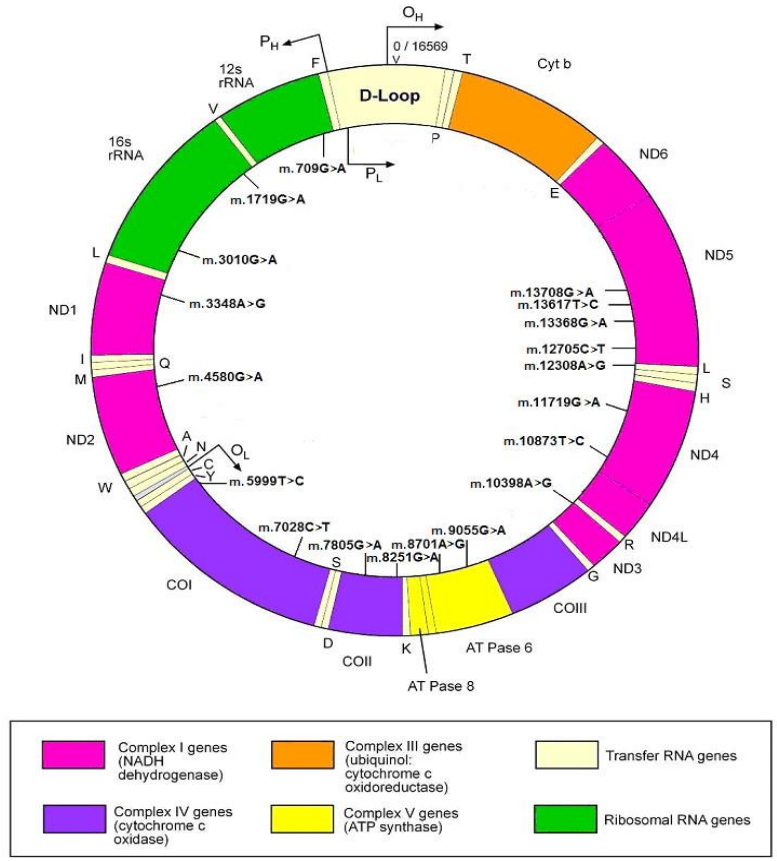


Figure 1.2.5 - Mammalian mtDNA is a circular and double-stranded genome composed by the heavy (PH) strand and the light (PL) strand. The non-coding region (D-loop) contains a triplex structure with a nascent H strand and the promoters for the transcription of both strands (HSP and LSP) as well as the origin of replication of the leading strand (O_H). The origin of replication of the lagging strand (O_L) is located in a cluster of tRNA genes (Taken from Rosa *et al.*, 2008, with permission).

In contrast, cells usually contain two normal alleles in the nucleus or one abnormal allele (50% mutant, 50% heterozygote), or two abnormal alleles (100% mutant, homozygote, or compound heterozygote) (Figure 1.2.6). A minimum number of mutant mitochondrial genomes are required for the expression of a mitochondrial dysfunction or disease, and this is the so called "threshold effect" (DiMauro and Moraes, 1993). This effect is only relative since the percentage of mutated genomes that is necessary to impair

mitochondria varies according to the metabolic requirements of the cell or tissue at any given time, the particular mtDNA mutation involved, and the nuclear genetic background. In general, the higher amount of mtDNA mutations, the more severe the disease state is, although occasional exceptions exist (Davis and Sue, 2011). Furthermore, mutations in the mtDNA have been shown to cause common age-related disease phenotypes including blindness, deafness, cardiovascular disease, neurological disease, renal and endocrine dysfunction (Wallace and Fan, 2010). It is generally assumed that age-associated mtDNA mutations are caused by accumulated damage during aging but recent studies support the hypothesis that somatic mtDNA mutations created during embryogenesis may contribute to the creation of aging phenotypes in adult life (Larsson, 2010).

Mitochondria replicate and divide at any time during the cell cycle and the proportion of mutant mtDNA passed onto the daughter cell at cell division may not be identical to that of the parental cell (Bogenhagen and Clayton, 1977). Besides, in response to metabolic demands of the whole cell, wild-type and mutant mtDNA may replicate at varying degrees during cell cycle. Therefore, the percentage of mutant mtDNA may vary quickly between the parent and the daughter cells. This phenomenon of mitotic segregation of heteroplasmic mtDNA mutations in somatic tissues and the principles for maternal transmission of mtDNA are the cornerstones in mammalian mitochondrial genetics (Wallace, 2010b). Unfortunately, these phenomena are not clearly understood at the cell biological level despite their fundamental importance.

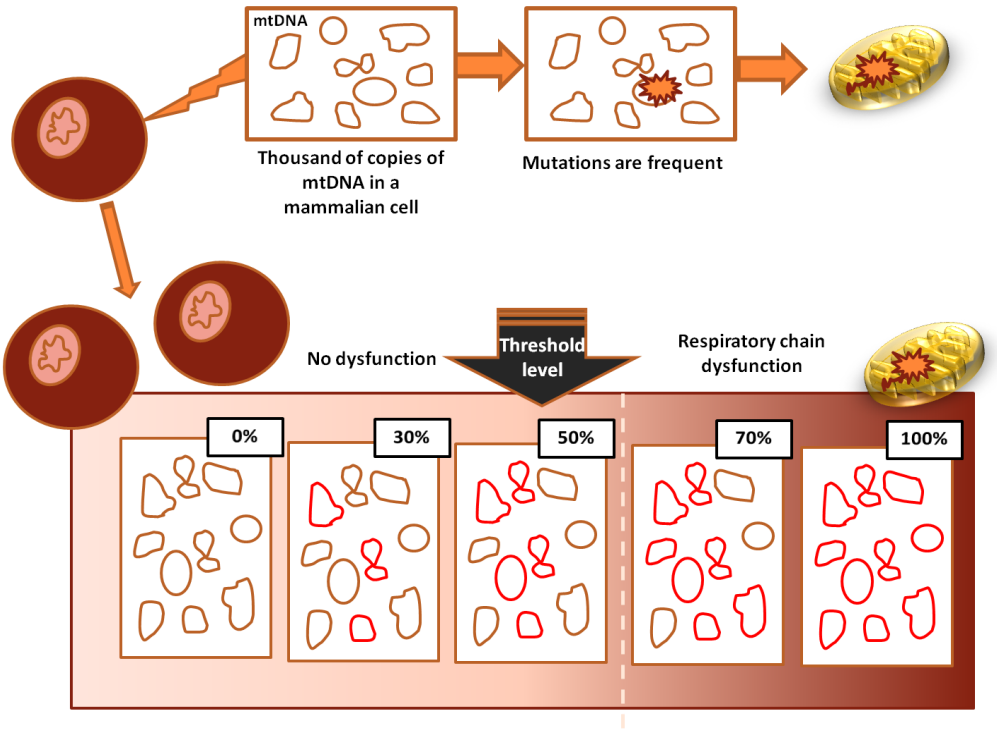


Figure 1.2.6 - Clonal expansion of mutated mtDNA copies in a cell. The mechanism of mitotic segregation is still not clearly understood but can lead to the loss of somatic mtDNA mutations in some cells and clonal expansion in some others. Respiration chain dysfunction can occur when the heteroplasmic mtDNA mutation is above a certain threshold level, the so-called threshold effect (Adapted from Larsson, 2010).

1.2.6.2 Maintenance of mtDNA: an overview

Mitochondria have independent replication, transcription and translation systems. The mitochondrial genome replicate in two phases: the replication starts at the heavy-strand (H) replication origin and continues clockwise around the mtDNA molecule. When the light-strand (L) replication origin is exposed as a single strand, the second strand is replicated in the opposite direction, starting from the light-strand replication origin (Wallace, 1982). The two strands have different base compositions, which lead to different

densities in alkaline cesium chloride gradients (Falkenberg *et al.*, 2007). The mtDNA is densely packed with genes and contains only one noncoding region, the long displacement loop (D loop). The D loop contains the promoters for L and H transcription and the origin of replication of the H strand (O_H). This DNA replication is sometimes abortive and stops at the end of the D loop, thus producing a triple-stranded D-loop structure containing a nascent H strand. Mitochondrial DNA (mtDNA) replication is conducted by the heterotrimeric DNA polymerase γ (Pol γ) and continues throughout the lifespan in both proliferating and post-mitotic cells (Kaguni, 2004). The first model described for mammalian replication also referred to as the *asymmetric replication model* states that the initiation of leading strand replication occurs at O_H and is dependent on an RNA primer formed by transcription from the long strand promoter (LSP) (Clayton, 1991). This model predicts that the lagging strand also undergoes continuous DNA synthesis without the need for repeated priming and thus without formation of Okazaki fragments. More recently, a new model has been proposed in which mtDNA replication in mammals begins from multiple origins and proceeds via a coupled leading strand and lagging-strand synthesis (Yang *et al.*, 2002). The two mtDNA strands are transcribed from specific promoters into polycistronic RNA, which will be further processed into rRNA, tRNA and mRNA. It is important to note that both models predict the involvement of a limited number of enzymes, and further biochemical work should help to clarify this situation.

Transcription of the circular mtDNA molecule produces 13 mRNAs, all encoding oxidative phosphorylation components, and 2 rRNAs and 22 tRNAs needed for mitochondrial translation of these mRNAs. Transcription is not only necessary for mtDNA gene expression but also for the production of RNA primers necessary for the initiation of mtDNA

replication at O_H (Falkenberg *et al.*, 2007). The transcription machinery consists of the RNA polymerase (POLRMT), mitochondrial transcription factor B2 (TFB2M) and mitochondrial transcription factor A (TFAM) (Falkenberg *et al.*, 2002). The latter is absolutely essential for the initiation of transcription and there is not even abortive transcription in its absence. Besides, TFAM has a role in packing mtDNA (Falkenberg *et al.*, 2002). Transcription is regulated by mitochondrial transcription terminating and initiating factors, such as MTERF1, which binds downstream of the rRNA genes and has been proposed to play a role in termination of both H and L strand transcription (Fernandez-Silva *et al.*, 1997).

Until recently, it was assumed that mammalian mitochondria were devoid of mtDNA repair mechanisms. The existence of short-patch base excision repair systems to remove damaged bases in mtDNA refuted the previous line of thoughts (Bogenhagen, 1999). Recently, the existence of a mitochondrial long-patch base excision repair was also reported (Szczesny *et al.*, 2008). However, other repair systems such as mismatch repair, homologous recombination, and nonhomologous and joining, are either absent or remain to be discovered in mammalian mitochondria (Bogenhagen, 1999; de Souza-Pinto *et al.*, 2008). In addition, mitochondrial DNA is packaged in DNA-protein aggregates referred to as nucleoids (Garrido *et al.*, 2003). The presence of TFAM in nucleoids has been reported and other proteins can also be putative components. Future studies need to be performed in order to clarify the term nucleoid and its components.

The mitochondrial proteome is dynamic, both the polypeptide composition and the relative abundance of a given polypeptide may vary in mitochondrial proteomes of different tissues as well as in the same tissue

over time (Rotig and Munnich, 2003). The mitochondrial mRNA are translated into the mitochondrial matrix with nuclear encoded machinery but following a specific genetic code, different from the nuclear code. Mitochondrial translation requires ribosomal proteins and tRNA synthetases. In total, approximately 100 different proteins and tRNA synthetases are involved (Rotig and Munnich, 2003).

Mitochondrial genes have no introns and intergenic sequences are absent or limited to a few bases. Some genes overlap and in some instances, terminations codons are not encoded, but are generated post-transcriptionally by polyadenylation (Davis and Sue, 2011).

1.2.7 Mitochondrial fusion and fission

Mitochondrial network is dynamic and undergoes continuous fusion and fission to maintain their function (Detmer and Chan, 2007; Hoppins *et al.*, 2007). Mitochondrial fission and fusion require GTP hydrolysis and are both crucial for normal mitochondrial function, as well as for the redistribution of lipids and proteins along the cell (Nakada *et al.*, 2001). Fission is a necessary event for the correct redistribution of mitochondrial DNA during cell division and also for the transport of mitochondria to daughter cells during mitosis and meiosis (Hales, 2004). On the other hand, fusion is a mechanism by which neighboring mitochondrial membranes fuse. It is accepted that this event occurs as a mean of recovering the activities of damaged/depolarized membranes, which ensures the proper mixing of metabolites and mtDNA (Twig *et al.*, 2008). Another perspective might be that fission allows for genetic complementation between two mitochondria, which promotes ATP synthesis in oxygen-deprived regions of a cell through electrical transmission of mitochondrial membrane potential across the mitochondrial network. Actually, the energy from GTP

hydrolysis is crucial to facilitate the transmission of calcium signals and membrane potential across distances in the cell (Szabadkai *et al.*, 2004). Loss of fission results in a large network of fused mitochondria, whereas fusion disruption results in small and dividing mitochondria. Fusion is regulated by the proteins Mnf1, Mnf2, and Opa1 (Chen and Chan, 2005), while fission is regulated by the proteins Drp1 and Fis1 (Otsuga *et al.*, 1998; Mozdy *et al.*, 2000). These proteins and others were found in both cardiac and non-cardiomyocyte cells and are regulated by post-translational modifications, mediated by ubiquitin and SUMO (small ubiquitin-like modifier) ligases (Zungu *et al.*, 2011).

1.2.8 Mitochondrial DNA mutations and disease

Mutations in the mtDNA can induce alterations of mitochondrial function. A database of reported mtDNA mutations is available online (www.mitopa.org). Pathogenic alterations in the mtDNA can be classified into point mutations, large scale rearrangements (duplications, single deletions, multiple deletions) as well as mtDNA depletion (Davis and Sue, 2011). In most cases, mtDNA mutations are heteroplasmic, since both normal and mutant mtDNA is present. Point mutations include amino acid substitutions and protein synthesis mutations (tRNA and rRNA). An example of a point mutation is for instance the A3243G mutation in the $tRNA^{Leu}$ that is responsible for Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome (Goto *et al.*, 1990; Goto, 1995). This syndrome is characterized by a childhood onset of intermittent hemicranial headaches, vomiting, proximal limb weakness, recurrent neurologic deficits, stroke-like episodes, lactic acidosis and occasionally ragged red fibers (MERRF) in muscle biopsies. Another example can be the missense mutation A8344G in the mitochondrial $tRNA^{Lys}$ gene which accounts for myoclonic epilepsy with ragged red fibers

(Shoffner *et al.*, 1990). This disease is characterized by encephalomyopathy with myoclonus, ataxia, hearing loss, muscle weakness, and generalized seizures. The Leber's hereditary optic neuropathy and neurogenic muscle weakness-ataxia retinitis pigmentosa (NARP) or Leigh syndrome are known to be induced by mutations in mitochondrial genes encoding structural proteins (Tatuch *et al.*, 1992). Leber's syndrome is associated with 18 missense mutations in mtDNA, which can act individually or in association with each other to cause the disease. Complex I deficiency is also a frequent cause of mitochondrial disorders, representing more than 30% of the cases (Wallace *et al.*, 1988a; Triepels *et al.*, 2001). The symptoms are truncal hypotonia, prenatal and postnatal growth retardation, encephalopathy, and liver failure which account for the main clinical characteristics of this dysfunction. Most of the patients identified with a Complex I disorder presented Leigh or Leigh-like syndrome (Sarzi *et al.*, 2007), but cardiomyopathy was also reported. Complex III deficiency is, in contrast to complex I, a relatively rare cause of respiratory enzyme dysfunction and the clinical symptoms are very heterogeneous (Mourmans *et al.*, 1997). Cytochrome c oxidase (COX) deficiency is one of the most frequent causes of mitochondrial disorders in childhood and it is also clinically heterogeneous, including Leigh syndrome myopathies and liver failure, among others (DiMauro *et al.*, 2012). Mitochondrial DNA deletion syndromes have been found in patients with autosomal dominant external ophthalmoplegia (PEO) from mutations in TWINKLE, ANT1, POLG, POLG2 and OPA1. These patients showed muscle weakness and ophthalmoplegia among other symptoms. Finally, mtDNA depletion syndromes involve at least 9 genes such as TYMP, POLG, DGUOK, TK2, SUCLA2, MPV17, SUCLG1, RRM2B and C10orf2. Mutations in these genes may cause depletion of the mtDNA in different tissues, specially the muscle and liver (Wallace *et al.*, 2010).

It is important to notice that only 10-15% of all cases presented a mtDNA mutation, at least among the pediatric patients (Koenig, 2008). Therefore, in most cases, nuclear genes defects are presumably responsible for the respiratory chain deficiency. For instance, mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome and POLG-related disease are known to be caused by nDNA encoded genes (Wallace *et al.*, 2010). Very briefly, the MNGIE syndrome is an autosomal recessive disease caused by mutations in the *TP* gene (Nishino *et al.*, 1999). The reduction of TP activity results in reduced/abnormal mtDNA synthesis, causing multiple deletions, mtDNA depletion and point mutations. Mutations in the DNA polymerase subunit gamma (POLG) gene can be inherited as autosomal recessive or dominant traits. This disorder is associated with ophthalmoplegia, epilepsy, Parkinsonism, and children might present hepatocerebral disease associated with mtDNA depletion, among other complications (Davis and Sue, 2011). See figure 1.2.7.

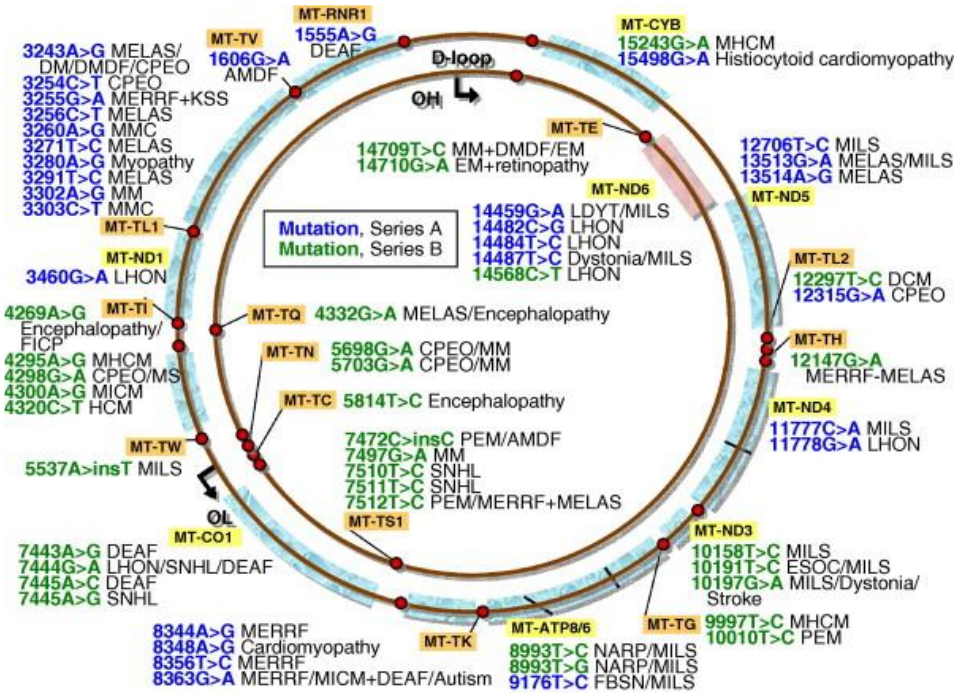


Figure 1.2.7 - The mtDNA map of the 61 mtDNA pathological point mutations found through the extensive suspension array-based detection system. Figure legend: DCM, dilated cardiomyopathy; DEAF, maternally inherited deafness or aminoglycoside-induced deafness; DM, diabetes mellitus; DMDF, diabetes mellitus + deafness; EM, encephalomyopathy; ESOC, epilepsy, strokes, optic atrophy, and cognitive decline; FBSN, familial bilateral striatal necrosis; FICP, fatal infantile cardiomyopathy plus, a MELAS-associated cardiomyopathy; KSS, Kearns–Sayre's syndrome; LHON, Leber hereditary optic neuropathy; MHCM, maternally inherited hypertrophic cardiomyopathy; MICM maternally inherited cardiomyopathy; MM, mitochondrial myopathy; MMC, maternal myopathy and cardiomyopathy; MS, multiple sclerosis; PEM, progressive encephalomyopathy. Abbreviations and information about mutations are annotated in the MITOMAP database (Taken from Nishigaki *et al.*, 2010, and used here after obtaining permission).

1.2.9 Age-related diseases and mitochondrial reactive oxygen species

Mitochondrial dysfunction and ROS have been implicated in age-related diseases and certainly in the aging process. More than 50 years ago, *Harman* postulated the "free radical theory" of aging (Harman, 1992; Harman, 2003). The theory is based on the principle that gradual accumulation of damage within intracellular macromolecules during aging, could have been

caused by the harmful effects of free radical production during aerobic metabolism. Later, Harman renamed his initial proposal the "mitochondrial theory of aging" (Raffaello and Rizzuto, 2011). Studies have shown a direct link between mtDNA mutations and mammalian aging (Seo *et al.*, 2010).

In support of the idea that mitochondria play an important role in the aging process, mice with a proofreading-deficient version of PolgA, which accumulate mtDNA mutations that are associated with impaired respiratory-chain function, have a shorter life span and display age-related phenotypes at an early age (Trifunovic *et al.*, 2004). Surprisingly, these changes were not accompanied by increased levels of oxidative stress, which has also been confirmed in humans; therefore, these reports have been questioning the role of oxidative stress as a cause of normal aging. As such, and even though many investigators agree on the relationship between mitochondria dysfunction, aging and ROS production, the topic is still under debate (Jang and Remmen, 2009). However, it is clear that the decline of the integrity of mitochondria as a function of age is implicated in aging and age-related diseases, such as diabetes, neurodegeneration, cancer, atherosclerosis/vascular aging, heart failure and myocardial infarction (Park and Larsson, 2011) . Interestingly, and further supporting the key role of mitochondria in the aging process, caloric restriction (the only known intervention that extends lifespan), through the modulation of IGF-1, TOR, sirtuins, and AMP kinase signaling pathways, has been demonstrated to increase mitochondrial mass and improve mitochondrial oxidative stress (Wallace and Fan, 2010).

Recent findings shed further light on the strong linkage between aging and metabolism, opening new and unexplored scenarios in the field of drug development. Some compounds including resveratrol and SRT1720, have been describe to activate SIRT1 and evidence suggests that this activation

can extend lifespan, at least in part due to their ability to increase mitochondrial biogenesis (Lagouge *et al.*, 2006; Pearson *et al.*, 2008; Baur, 2010; Zarse *et al.*, 2010). Recently, two papers from Dipak Das lab were rectracted from the Free Radical Biology & Medicine journal. Both dealt with the possible protective role of resveratrol in cardioprotection (Mukherjee *et al.*, 2009) as well as with the importance of resveratrol in redox regulation of survival signals (Das *et al.*, 2008). Thus, this recent and unfortunate news created a shadow in the protective role of resveratrol.

Metformin, a biguanide drug has been found to extend lifespan of nondiabetic mice and also *C. elegans* (Onken and Driscoll, 2010). Insulin-like signaling in *C.elegans* activates the transcription factor SNK-1 which is known to participate in the defense against ROS by mobilizing the phase 2 detoxification response and is referred to as the longevity-promoting factor (Tullet *et al.*, 2008). Furthermore, the target of rapamycin (TOR) pathway is also a major nutrient-sensing pathway that, when genetically downregulated, increases life span in evolutionarily diverse organisms, including mammals (Bjedov et al, 2010).

1.2.10 Mitochondria and calcium homeostasis

Alterations in cytosolic calcium concentration provide signals to control important cellular events such as muscle contraction, neurotransmitter release, alterations in gene transcription and even cell death, among other events (Berridge *et al.*, 2000; Bootman *et al.*, 2001). The capacity of isolated mitochondria to accumulate calcium in the matrix depending on the proton gradient was first described in 1962 (Vasington and Murphy, 1962). Calcium should be accumulated inside the mitochondrial matrix with a 1 million fold concentration than in cytosol, according to the Nernst equation (Giacomello et al, 2007). However, all the experimental measurements of

intramitochondrial calcium concentrations refuted this theory. The paradox was solved when it was discovered that calcium accumulation by mitochondria was dependent not only of an electrogenic uniporter or mitochondrial calcium uniporter but also on antiporters (Na^+/Ca^{2+} or H^+/Ca^{2+}) responsible for calcium export from mitochondria. These organelles thus act as localized cytosolic calcium buffering organelles, modulating several events of calcium feedback inhibition and activation. The mitochondrial efflux mechanisms assisted by the matrix calcium buffering activity, maintain a constant mitochondrial calcium concentration of 700-1000 nmol of Ca^{2+}/mg of protein before bioenergetic breakdown occurs (Rizzuto *et al.*, 1998). Mitochondrial calcium accumulation also occurs through a "rapid uptake mode" (RaM) mechanism which acts on a millisecond timescale, as well as through a mitochondrial isoform of the ryadonine receptor which has been described in some excitable cells (Altschafl *et al.*, 2007).

1.2.11 Mitochondria and cell death: a short overview

Cell death can occur through necrosis due to the activation of the MPTP, which compromises ATP production, since the mitochondrial inner membrane becomes freely permeable to protons leading to OXPHOS uncoupling. The ATP synthase can revert its activity and hydrolyze ATP in order to maintain the mitochondrial membrane potential ($mt\Delta\Psi$), resulting into a further decrease in intracellular ATP (McMillin and Pauly, 1988). Cell swelling occurs during necrosis due to alterations in the cytoskeleton and inhibition of the Na^+/K^+ pump causing loss of the selective permeability of the membrane. Apoptosis involves the activation of a group of cysteine proteases called "caspases" (cysteine-dependent aspartate-specific proteases), which form a complex cascade of responses. There are initiator caspases (2,8,9 and 10) and the effector caspases (3,6 and 7), and also,

inflammatory caspases (1,4,5) (Cohen, 1991). Two partly interdependent routes lead to apoptosis, the extrinsic and intrinsic pathways (Hengartner, 2000). Mitochondria play a leading role in the intrinsic pathway which is generally characterized by intracellular apoptotic stimuli (Figure 12.8). Several "death signals" such as ROS, DNA damage and excessive mitochondrial calcium influx, among others, promote the release of cytochrome c and other apoptotic factors such as Smac/DIABLO, and the apoptosis-inducing factor (AIF) from the intermembrane space, by two basic mechanisms: a) through the opening of MPTP, leading to mitochondrial swelling and outer membrane rupture or b) formation of pores in the outer membrane by pro-apoptotic proteins (BAX and BAK), including BAX (Halestrap *et al.*, 2000). Figure 1.2.8. presents more details on the role of mitochondria on cell death.

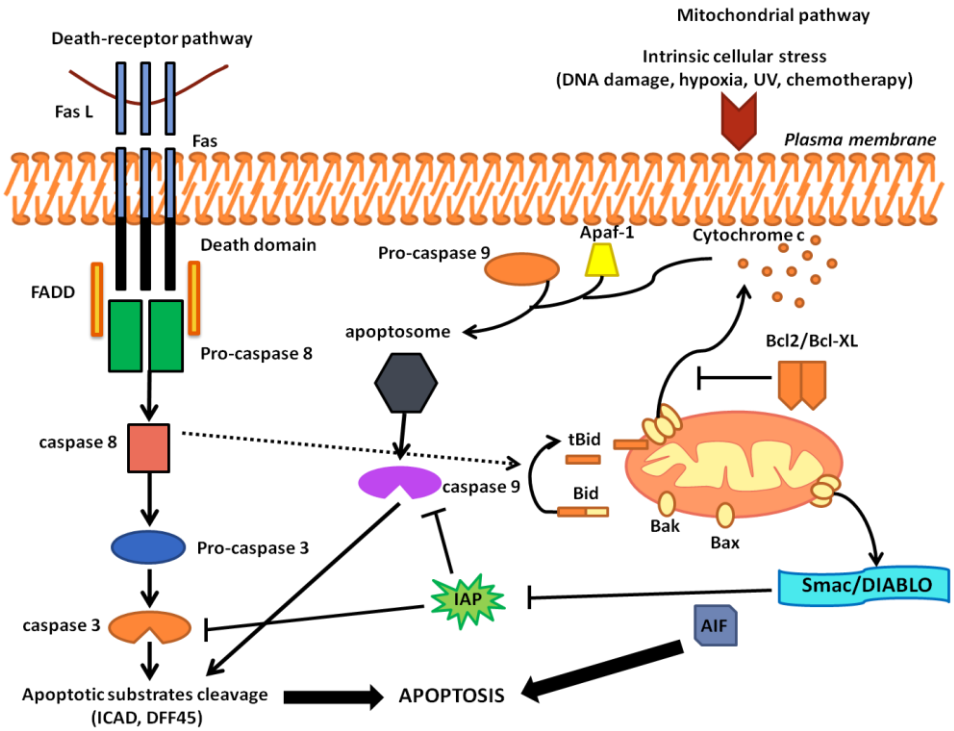


Figure 1.2.8 - Mitochondrial apoptotic pathway. Death can be induced by the binding of ligands (such as FasL) to specific receptors (such as FAS) located at the cell surface. FAS contains a cytoplasmic death domain where FADD (Fas-associated death domain) can bind in the presence of FasL, and recruit Pro-caspase 8 for subsequent activation to caspase 8, inducing downstream caspase 3 activation. Caspase 3 cleaves I-CAD, the inhibitor of CAD (Caspase-activated DNase), which is released to enter the nucleus and cleaves DNA. In addition, caspase 8 cleaves Bid protein, resulting in truncated Bid (tBid) that, upon dimerisation of Bax or Bad, causes the release of cytochrome c from mitochondria. The mechanisms by which Bax leads to mitochondrial membrane permeabilisation and subsequent release of pro-apoptotic factors are still unclear. Bax can interact with the MPTP, or form channels by self-oligomerization. This leads to the mitochondrial release of cytochrome c and Smac/Diablo (Smac: second mitochondrial- derived activator of caspase; Diablo: direct IAP-binding protein with low pI), AIF (apoptosis inducing factor) and various procaspases. Bcl2 inhibits the release of cytochrome c and AIF in the cytoplasm and prevents the opening of the MPTP. In the cytosol, cytochrome c binds to Apaf-1 (apoptosis- protease- activating factor). Both proteins form the apoptosome, which converts procaspase 9 in caspase 9. This results in activation of downstream effector caspases. Smac/DIABLO binds to IAP (Inhibitors of apoptosis) and prevent them from inhibition of the caspase 9 and caspase 3 activation. The AIF has an indirect role in chromosome degradation as it activates endonuclease G, a DNase that moves from the mitochondria to the nucleus during apoptosis. Interestingly to note, mtDNA is not fragmented during apoptosis (Adapted from Bellance *et al.*, 2009).

Summary points of Section 2:

➢ The oxidative phosphorylation system involves two genomes, the nuclear and mitochondrial DNA, which are in a constant cross-talk regarding regulation of mitochondrial function.

➢ The mtDNA is a small circular genome and it is very susceptible to oxidative damage. The mitochondrial genome has three main features: maternal inheritance, heteroplasmy and mitotic segregation.

➢ mtDNA mutations lead to mitochondrial dysfunction which consequently is the cause for many syndromes. However, nDNA mutations also affect mitochondrial function and lead to mitochondrial associated-pathologies.

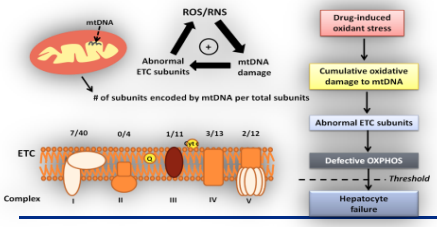
➢ Mitochondria are no longer seen as a single entity. These organelles are a dynamic network of long tubules composed by an inner membrane, outer membrane, cristae, intermembrane space and matrix.

➢ Mitochondrial biogenesis is highly regulated by both genomes and recently, caloric restriction, sirtuins and PGC-1α have been described as important players in this process.

➢ Mitochondria are in constant motion and the network undergoes fusion and fission which are crucial events to maintain mitochondrial functional homeostasis.

➢ Mitochondrial respiratory chain leads the most effective ATP yield of the cell. Complexes I to IV are coupled to complex V to produce ATP. The ATP generation results from a proton gradient formed between the matrix and the intermembrane space.

➢ Mitochondria are not only key players in metabolism but also have a role in ROS production, antioxidant defenses, calcium homeostasis and cell death.



Section 3

Mitochondria: from a toxicological tool to a likely player in drug-induced idiosyncratic reactions

1.3 Mitochondria as a tool in toxicology and pharmacology

Mitochondria are unquestionably key players in many different and crucial events for life. The finding that certain compounds can damage mitochondrial function has increased the attention of pharmaceutical companies which aimed to design and develop drugs that can specifically target mitochondria for therapeutic purposes. Since mitochondrial dysfunction is increasingly implicated in drug-induced toxicity, mitochondrial fractions are now used to reduce the late stage attrition of drug candidates and allow the design of safer drugs (Dykens *et al.*, 2007; Labbe *et al.*, 2008).

Mitochondria can actually be used as bio-sensors for the improvement of drug safety in many different target organs such as the liver and the heart, which can be achieved with methods and models available to investigate drug-induced mitochondrial dysfunction. Furthermore, the underlying genetic or acquired mitochondrial abnormalities are a major determinant of susceptibility for a number of drugs that target mitochondria and also cause liver injury. The knowledge that mitochondrial damage implies a gradually accumulating and initially silent mitochondrial injury in heteroplasmic cells which reaches a critical threshold and triggers liver injury, is consistent with the symptoms of idiosyncratic drug-induced liver injury (DILI). Thus, it has been hypothesized that mitochondria can be involved in the mechanisms of idiosyncratic DILI. Nevertheless, this is still an hypothesis since the lack of

experimental evidence, in part due to a lack of experimental models, is still difficult to surpass.

This section of the present thesis will focus on mitochondrial drug-induced toxicity in different organs and, especially in DILI which originates from mitochondrial abnormalities. Furthermore, the present experimental tools to study the involvement of mitochondria in idiosyncratic DILI will be debated.

1.3.1 Mitochondria as a target of drug-induced organ toxicity

The use of mitochondria for testing drug safety provides a source for screening many compounds in many different tissues. Mitochondrial toxicity is frequently evaluated in the heart, liver, kidney, and also, in the brain and testis. Many studies demonstrated that mitochondria act as primary or secondary targets of drug-induced toxicity, which can be useful to understand the mechanisms of toxicity of drugs and open new perspectives to its application (Figure 1.3.1). Molecular and mitochondrial effects of some drugs, including FDA "Black box" warnings for mitochondrial toxicity, namely cardiovascular, hepatotoxicity and renal toxicity, will be discussed next.

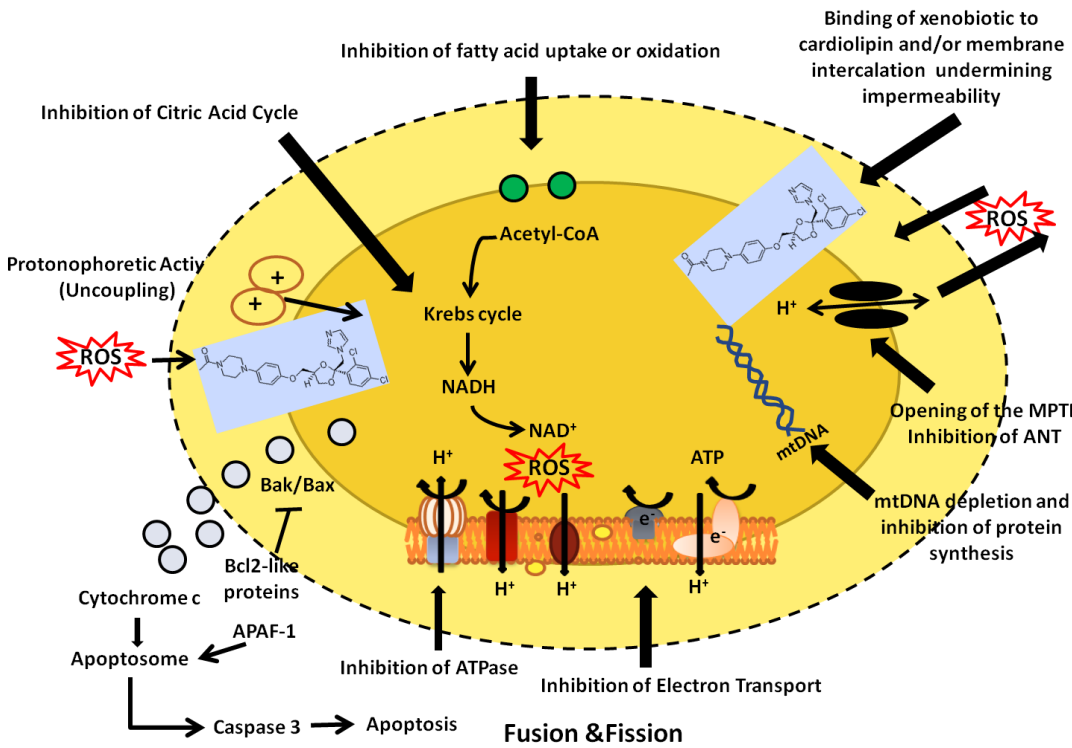


Figure 1.3.1 - Possible drug targets in mitochondria. In addition to the inhibition of electron transport and its uncoupling from the membrane electric potential, mitochondria can be inhibited in many different ways. Inhibition of mitochondrial transporters or intermediate metabolism that fuel OXPHOS, as well as, direct effects of drugs on cardiolipin can alter mitochondrial function. Mitochondrial toxicity frequently accelerates the autoxidation of electron transport components that yields oxygen-centered free radicals. By contrast, drugs that impair DNA replication or protein synthesis will decrease mitochondrial and hence bioenergetic capacity over a longer time period. In addition, many drugs can precipitate irreversible mitochondrial collapse via formation of the MPTP leading to release of pro-apoptotic factors such as cytochrome *c*. Drugs that alter the normal equilibrium between pro-apoptotic and anti-apoptotic proteins, such as Bak/Bax and Bcl-2, among many others, can also induce mitochondrial failure. Abbreviations: APAF-1, apoptotic protease activating factor 1; ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore; ANT, adenine nucleotide translocator; ATP, adenosine triphosphate (Adapted from Marroquin *et al.*, 2007).

1.3.1.2 Mitochondrial drug-induced cardiovascular toxicity

Mitochondrial dysfunction due to cardiotoxicity is very often associated with the side effects of several drugs. For example, zidovudine (AZT) is the most well know nucleoside reverse transcriptase inhibitor (NRTI), prescribed for acquired immune deficiency syndrome (AIDS) therapy (Lewis *et al.*, 2006). Zidovudine is characterized as a mitochondrial poison and induces the formation of superoxide anion causing oxidative stress among other toxic mechanisms of action such as inhibition of the thymidine phosphorylation, and inhibition of the adenine nucleotide translocator (ANT)(Valenti *et al.*, 2000). This class of compounds is also responsible for the inhibition of mitochondrial complex I by cyclic adenosine monophosphate (cAMP) which leads to disturbances in the $NADH/NAD^+$ ratio and generation of free radicals. Cardiac mitochondrial DNA (mtDNA) of mice treated for 35 days with AZT had over 120% more oxo-dG (8-oxo-7, 8-dihydro-2'-deoxyguanosine, a biomarker of oxidative damage to DNA) in their mitochondrial DNA than untreated controls (Lynx *et al.*, 2006; Lynx and McKee, 2006). AZT treatment also caused an increase in mitochondrial lipid peroxidation and oxidation of mitochondrial glutathione (de la Asuncion *et al.*, 1998). Dietary supplementation with vitamins C and E protected against end-points of mitochondrial oxidative stress (Rosa *et al.*, 2009). Other drugs such as thiazolidinediones (TZD), also known as glitazones, oral antihyperglycemic compounds which increase insulin-stimulated glucose removal have been used as an adjunctive therapy in *diabetes mellitus (Petersen et al., 2000)*. These drugs cause mitochondrial dysfunction affecting NADH oxidation at complex I. The inhibition of complex I can lead to ATP depletion, oxidative stress and ultimately, cell death (Scatena *et al.*, 2004). Some compounds such as anthracyclines (doxorubicin, daunorubicin) are at the moment the most effective

antineoplastic drugs used in the clinical practice (Lefrak et al., 1973). However, their use is associated with a significant increase in cardiotoxicity. Due to its side effects, the clinical use of doxorubicin (DOX) and similar compounds are limited by cumulative, dose-related, progressive myocardial damage and may lead to congestive heart failure. Several mechanisms explain the cardiotoxicity of DOX, but the exact mechanism and metabolic consequences remain unknown (de Nigris *et al.*, 2008; Chen *et al.*, 2010; Effenberger-Neidnicht and Schobert, 2011).

Doxorubicin causes oxidative stress associated with the mitochondrial redox cycle, causing mitochondrial damage and deregulation of mitochondrial and cellular calcium homeostasis (Sardao *et al.*, 2009b; Ascensao *et al.*, 2011). Doxorubicin-induced oxidative stress can also be part of the mechanism by which increased MPTP occurs, being implicated in mitochondrial and cellular dysfunction (Oliveira *et al.*, 2006). Induction of the MPTP results into a sudden non-selective increase in the permeability of the inner mitochondrial membrane to solutes of molecular mass less than ~1,500 KDa (Martin *et al.*, 2009; Zorov *et al.*, 2009). At least *in vitro,* MPTP occurs when mitochondria are treated with an excess of calcium or several reagents that increase oxidative stress (Kowaltowski *et al.*, 2001). This phenomenon results in a loss of mitochondrial membrane potential and in a decrease of ATP production (Kroemer *et al.*, 2007). The DOX quinone moiety is reduced by mitochondrial complex I, producing superoxide anion by a redox-cycling mechanism (Figure 1.3.2). The attempts to find alternative anthracyclines with lower toxicity resulted in the discovery of epirubicin and idarubicin, which belong to the new generation of this class of drugs. Cisplatin is an anti-cancer drug whose use is limited because of cardiotoxicity. Oxidative stress and apoptosis have been postulated to contribute to this toxicity. Indeed, cisplatin-treated rats exhibit increased

cardiac lipid peroxidation as measured by malondialdehyde formation and display decreased reduced glutathione content and decreased superoxide dismutase activity (El-Awady el *et al.*, 2011). Moreover, both mitochondrial DNA and nuclear DNA of cisplatin-treated rats showed free radical-induced damage.

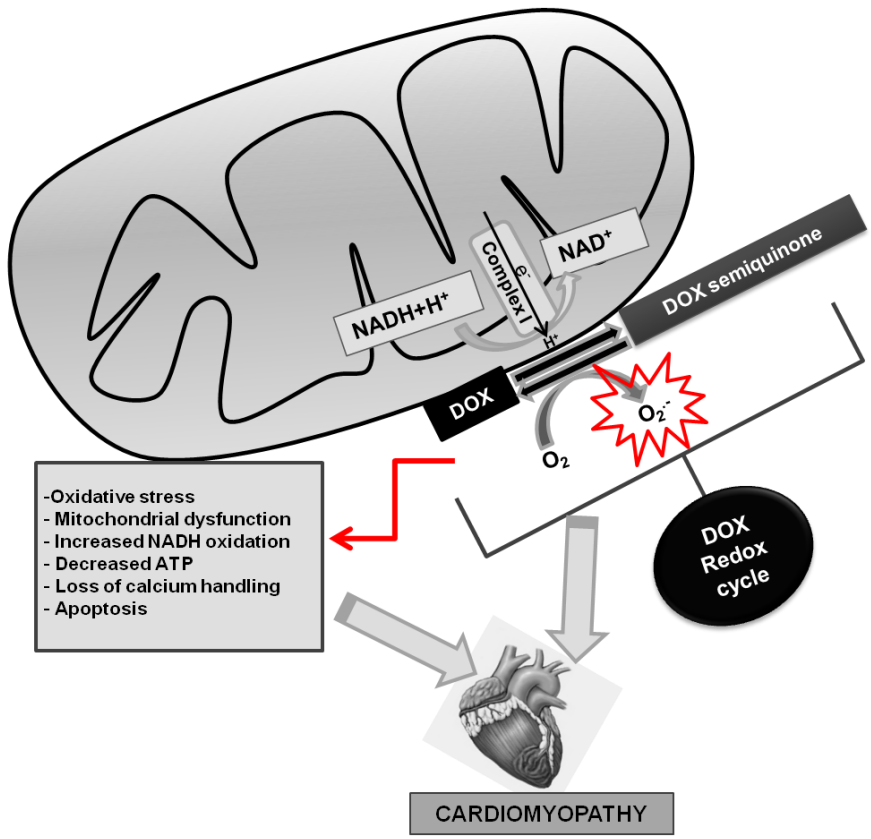


Figure 1.3.2- Doxorubicin **(**DOX) causes mitochondrial impairment through redox cycling in mitochondrial complex I, resulting in induction of the mitochondrial permeability transition pore, besides other mitochondrial consequences. DOX-induced cardiomyopathy is a serious side-effect of the treatment.

1.3.1.3 Mitochondrial drug-induced liver toxicity

The liver is one of the major targets for drug-injury since most of the biotransformation process takes place in this organ. The present section will focus on some examples of hepatotoxicants which affect mitochondrial function and cause oxidative stress.

Tamoxifen is a non-steroidal anti-estrogen agent which has been used as a post-operative adjuvant therapy for breast cancer (Floren *et al.*, 1998). Reports of tamoxifen-induced toxicity may be explained by results obtained on isolated liver mitochondria. Tamoxifen and estradiol lead to mitochondrial failure by acting on the flavin mononuceotide (FMN) site of mitochondrial complex I (Moreira *et al.*, 2006). A particular study demonstrated that tamoxifen is electroforetically accumulated inside hepatic mitochondria, where it acutely impairs β-oxidation and respiration (Larosche *et al.*, 2007). Furthermore, tamoxifen decreases fat removal from the liver and steatosis, despite a secondary down-regulation of hepatic fatty acid synthase expression (Larosche *et al.*, 2007). Tamoxifen induces oxidative stress and mitochondrial-involved apoptosis via the stimulation of mitochondria nitric oxide synthase (Nazarewicz *et al.*, 2007). Acetominophen (APAP) is another compound that induces liver toxicity and injury (Jaeschke *et al.*, 2012), especially in mice, which became an attractive model to analyze liver injury because it is both clinically relevant and experimentally convenient. It is known that the toxicity of APAP is initiated by the formation of a reactive metabolite which depletes glutathione and binds to extracellular proteins, especially in mitochondria (see Figure 1.3.3). The result is mitochondrial oxidative stress and peroxynitrite formation, in part though amplification by c-jun-N-terminal kinase activation, leading to mitochondrial DNA damage and opening of the MPTP (Jaeschke and Bajt, 2006; McGill *et al.*, 2011). The metabolism of

APAP causes the formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NADPQI) which binds to mitochondrial proteins and initiates oxidative stress. Gluthathione (GSH) traps NAPQI first and the GSH adduct is excreted. However, after GSH is depleted, NAPQI reacts with cellular proteins and forms an APAP adduct. Although the original hypothesis that the general protein binding of NAPQI causes toxicity has been questioned, it remains unquestionable that the metabolic activation of APAP is the critical initiating event of cell death (Nelson, 1990). After the initial concerns, the protein binding hypothesis was modified. With the recognition of mitochondrial oxidative stress and the fact that formation of mitochondrial protein adducts correlated with liver injury, the current concept emerged. In this hypothesis, ROS and protein binding, especially to mitochondrial proteins, is an important initiating event that by itself is not sufficient to cause cell death. In contrast, this protein binding induces mitochondrial oxidative stress with the formation of superoxide anion and peroxynitrite which in turn amplifies the original stress, eventually leading to necrotic cell death (Jaeschke *et al.*, 2003). Valproic acid (2-n-propylpentanoic acid; VPA) is a drug used to treat a wide spectrum of seizure disorders, in addition to bipolar disorder, migraine and neuropthatic pain (Chateauvieux *et al.*, 2010). VPA is relatively safe, but teratogenicity, pancreatitis and severe liver injury have been associated with its use (Chang and Abbott, 2006). VPA is metabolized in the liver, via mitochondrial β-oxidation and gluocoronic acid conjugation, producing multiple metabolites. Valproic acid is activated and binds to reduced acetyl Co-A which allows its translocation to the mitochondrial matrix and the consequent inhibition of several mitochondrial enzymes and decreased fatty acid β-oxidation. A role for oxidative stress in VPA-associated hepatotoxicity in rats is suggested. *In vitro* treatment of primary cultures of rat hepatocytes with VPA leads to ROS generation (Tong *et al.*, 2005). Also, other *in vivo* studies indicated that VPA

treatment is associated with development of oxidative stress in rats (Tong *et al.*, 2003; Lee *et al.*, 2009). It was also suggested that the therapeutic doses of VPA resulted in oxidative stress in humans. A genetic deficiency in antioxidant capacity may be a predisposing factor in the idiosyncratic type of VPA hepatotoxicity (Neyns *et al.*, 2008; Stewart *et al.*, 2010).

Finally, another example of hepatotoxicity are the non-steroidal anti-inflammatory drugs (NSAIDS) such as nimesulide, a selective cyclo-oxygenase-2 inhibitor widely used for the treatment of inflammatory and pain conditions (Boelsterli, 2002). Nimesulide is almost exclusively metabolized and cleared by the liver (Tan *et al.*, 2007). Metabolic biotransformation of nimesulide in the liver can occur at both the phenoxy ring moiety and the aromatic nitro group. The major metabolite is 4'-hydroxy nimesulide and it is normally conjugated with sulphate or glucoronic acid. Nitroreduction is also an important pathway of biotransformation and of great importance in toxicological terms since it results in the formation of an aromatic amine (Boelsterli, 2002). This amine metabolite is further metabolized by N-acetylation, catalysed by N-actyltransferases (NAT) (Li *et al.*, 2009). These intermediates can also undergo sulpho- or glucorono-conjugation and be excreted in urine. *In vitro* systems suggested that these intermediates have the potential to cause oxido-reductive stress and undergo covalent binding to selective target proteins, with mitochondria being particularly sensitive organelles. The underlying cause for nimesulide-induced oxidative stress is the relative ease by which nitroaromatic compounds can be enzymatically reduced by nitroreductase-catalysed one or two electron transfer pathways. The one electron transfer pathway produces the nitro anion radical, a highly reactive species which under aerobic conditions is able to transfer the electron to molecular oxygen, thereby producing superoxide anion radicals (Squella *et*

al., 1999). *In vivo*, however, this intermediate has not been demonstrated (Boelsterli, 2002). Redox cycling is potentially hazardous because one molecule of nitro compound can theoretically give rise to a large number of superoxide anion molecules. Also, recent studies indicated that nimesulide is able to uncouple mitochondrial oxidative phosphorylation in isolated mitochondria, mitochondrial swelling and NAD(P)H oxidation and MPT pore opening (Singh *et al.*, 2010).

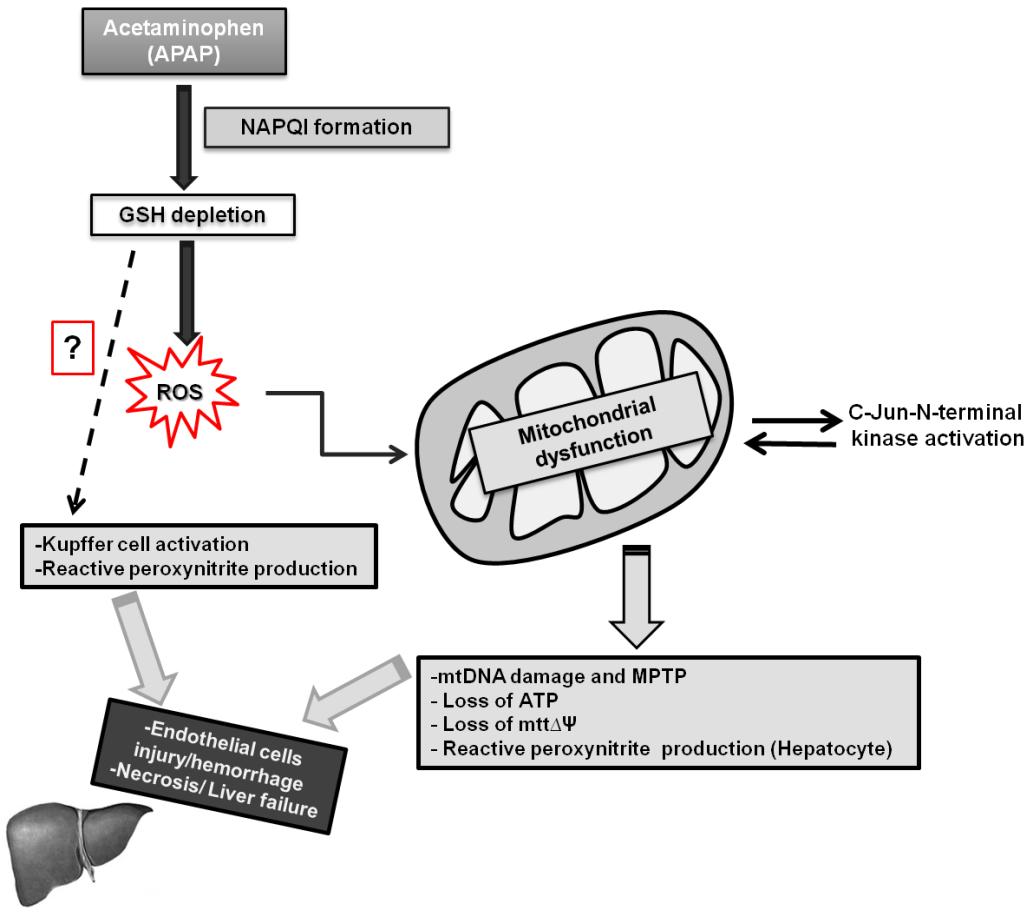


Figure 1.3.3 - Acetaminophen (APAP) metabolism causes the formation of a highly toxic metabolite NAPQ1 which binds to mitochondrial proteins and initiates an oxidative stress cascade due to glutathione depletion.

1.3.1.4 Mitochondrial drug-induced renal toxicity

In comparison with other organs, the kidney is distinctively susceptible to chemical toxicity, partially because of its disproportionately high blood flow and due to its anatomically and functionally complexity. In contrast, liver cells have a tremendous capacity to repair and regenerate, whereas, kidney cells possess a very limited repair capability (Betton *et al.*, 2005). Drug-induced acute renal failure accounts for around 20% of hospitalizations. Among older adults, the incidence of drug-induced nephrotoxicity may be as high as 66% (Naughton, 2008). Several different drug classes such as anticancer drugs, antibiotics, NSAIDs and immunosuppressants exhibit kidney injury, oxidative stress being a contributing factor to toxicity.

Regarding anti-neoplastic renal toxicity, two compounds are considered classical examples: DOX and cisplatin. Cisplatin toxicity appears to involve inhibition of protein synthesis, mitochondrial injury and DNA damage causing blockage of DNA replication and gene transcription due to the formation of single and double strand DNA breaks (Cummings and Schnellmann, 2002; Santos *et al.*, 2007). Hydroxyl radicals were found responsible for cisplatin-induced apoptosis as hydroxyl radical scavengers inhibit cytochrome c release and caspase activation (Cummings and Schnellmann, 2002; Santos *et al.*, 2007). When renal collecting duct-derived cells were incubated with inhibitors of the mitochondrial respiratory chain or ATP-synthase, cisplatin-induced apoptosis was strongly enhanced showing that intact mitochondria are essential to prevent cisplatin-induced apoptosis (Zhang and Lindup, 1994). Furthermore, *in vivo* studies demonstrated cisplatin-induced oxidative damage to mitochondrial lipids, including cardiolipin, oxidation of mitochondrial proteins and lower aconitase activity (Santos *et al.*, 2007). Another study showed that cisplatin

depleted the GSH pool and increased lipid peroxidation (Zhang and Lindup, 1993).

Aminoglycoside antibiotics including gentamicin also cause renal toxicity (Walker *et al.*, 1999; Cuzzocrea *et al.*, 2002). Gentamicin leads to the formation of hydrogen peroxide by renal cortical mitochondria(Walker and Shah, 1987); also, radical scavengers and iron chelators provided functional and histological protection against renal failure in gentamicin-treated rats. Gentamicin has been shown to release iron from renal cortical mitochondria and this was supported by *in vitro* studies (Ueda *et al.*, 1993). The results strongly support the importance of hydroxyl radicals or a similar oxidant in gentamicin-induced renal failure.

Cyclosporin-A has played a major role in the improvement of solid-organ transplantation, immunosuppression, and enhancing patient survival (Tariq *et al.*, 1999; Betton *et al.*, 2005; Holzmacher *et al.*, 2005). However, its clinical use has been hampered by frequent reports of nephrotoxicity. The renal toxicity is attributed to reduced renal blood flow which can lead to hypoxia-reoxygenation injury accompanied by excessive generation of ROS (Tariq *et al.*, 1999). It was also previously documented that cyclosporine A-induced renal toxicity due to an alteration of intracellular calcium homeostasis, including increased calcium accumulation in the matrix as well as increased ROS production (Simon *et al.*, 2001). Although many studies have been conducted, the mechanism of cyclosporin-A-induced nephrotoxicity is far from clear.

Diclofenac (DCLF) is a nonsteroidal anti-inflammatory drug that is widely used for the treatment of various diseases such as osteoarthritis, rheumatoid arthritis, and acute muscle pain conditions, among others, although diclofenac can cause nephrotoxicity in humans and experimental animals

(Hickey *et al.*, 2001). Studies showed that DCLF is a strong inducer of oxidative stress through increased levels of malondaldehyde (MDA) and kidney toxicity. Oxidative stress induced by DCLF was also coupled with massive kidney DNA fragmentation (Hickey *et al.*, 2001). These two-free radical mediated events may ultimately translate into apoptotic cell death of kidney cells *in vivo*.

Methotrexate-induced renal toxicity is thought to be due to oxidative stress since administration to rats increased malondialdehyde levels (lipid peroxidation) in the kidney and decreased superoxide dismutase, catalase and glutathione peroxidase activities in renal tissue, resulting in a decrease in the antioxidant capacity (Oktem *et al.*, 2006).

1.3.2 Mitochondria and idiosyncratic drug-induced liver injury

As described previously, liver failure is a major clinical problem in drug development as an increasing number of successfully launched drugs or new potential pro-drugs have been implicated in drug-induced liver injury, in patients (Lee, 2003). Although most drugs cause liver injury infrequently, these reactions are considered to be idiosyncratic. Nevertheless, drug-induced hepatic injury is the most frequent reason for drug withdrawal from the market and more than 75% of IDRs result in liver transplantation or even death (Ostapowicz *et al.*, 2002). Understanding the mechanisms of liver damage induced by drugs and understanding the role of mitochondria in these pathways, can lead to the prevention of this phenomenon in patient population and allow a better safety prediction of idiosyncratic drug reactions, such as DILI.

1.3.2.1 Mechanisms of liver injury by drugs

There are at least six described mechanisms for hepatocyte injury by drugs. For instance, the disruption of calcium homeostasis can lead to disassembly of actin at the surface of the hepatocyte, resulting in membrane bebbling, rupture and cell lysis (Beaune *et al.*, 1987). This happens when drugs bind covalently to intracellular proteins and there is a loss of the ionic gradient, leading to a decline in the ATP levels and cellular collapse (Yun *et al.*, 1993). Other drugs can affect transport proteins at the canalicular membrane, specialized for bile excretion, interrupting the bile flow and causing cholestasis (Trauner *et al.*, 1998). Loss of villous processes and the interruption of transport pumps such as the multidrug-resistance-associated protein 3 (MRP3) prevent the excretion of bilirubin and other compounds from the organism. In mixed forms of hepatic injury, the combined failure of canalicular pumps and other intracellular events allows toxic bile acids to accumulate, causing secondary hepatocytes injury.

Biotransformation of drugs involves high energy reactions which can result in the formation of adducts, the drugs can covalently bound to enzymes, thus creating nonfunctionating adducts. These enzyme-drug adducts migrate to the cell surface in vesicles which serve as target immunogens for T-cells responses, stimulating multifaceted immune responses involving both cytolytic T-cells and cytokines (Robin *et al.*, 1997). The secondary cytokine response may cause inflammation by additional neutrophil-mediated hepatotoxicity (Jaeschke *et al.*, 2002). Apoptosis can also occur simultaneously with immune-mediated injury activating the tumor necrosis factor (TNF) and FAS pathways. Fas may trigger the cascade of intracellular caspases which will cause cell shrinkage and fragmentation of nuclear chromatin (Reed, 2001). Several drugs inhibit mitochondrial function as mentioned previously, for instance, by interfering with β-oxidation and

OXPHOS enzymes, or by disturbing mtDNA (Pessayre *et al.*, 2001). The consequences are in general, oxidative stress induction, ensuing anaerobic metabolism, lactic acidosis, and triglyceride accumulation (microvesicular fat within cells) (Pessayre *et al.*, 2001). Steatohepatitis (fat accumulated in large vesicles outside liver cells, associated with inflammation) is commonly associated with alcohol abuse. This pattern of injury is also associated with other drugs such as nucleoside reverse-transcriptase inhibitors, which bind directly to mtDNA, as well as valproic acid, and aspirin, for instance. See figure 1.3.4 for a summary of the mentioned mechanisms.

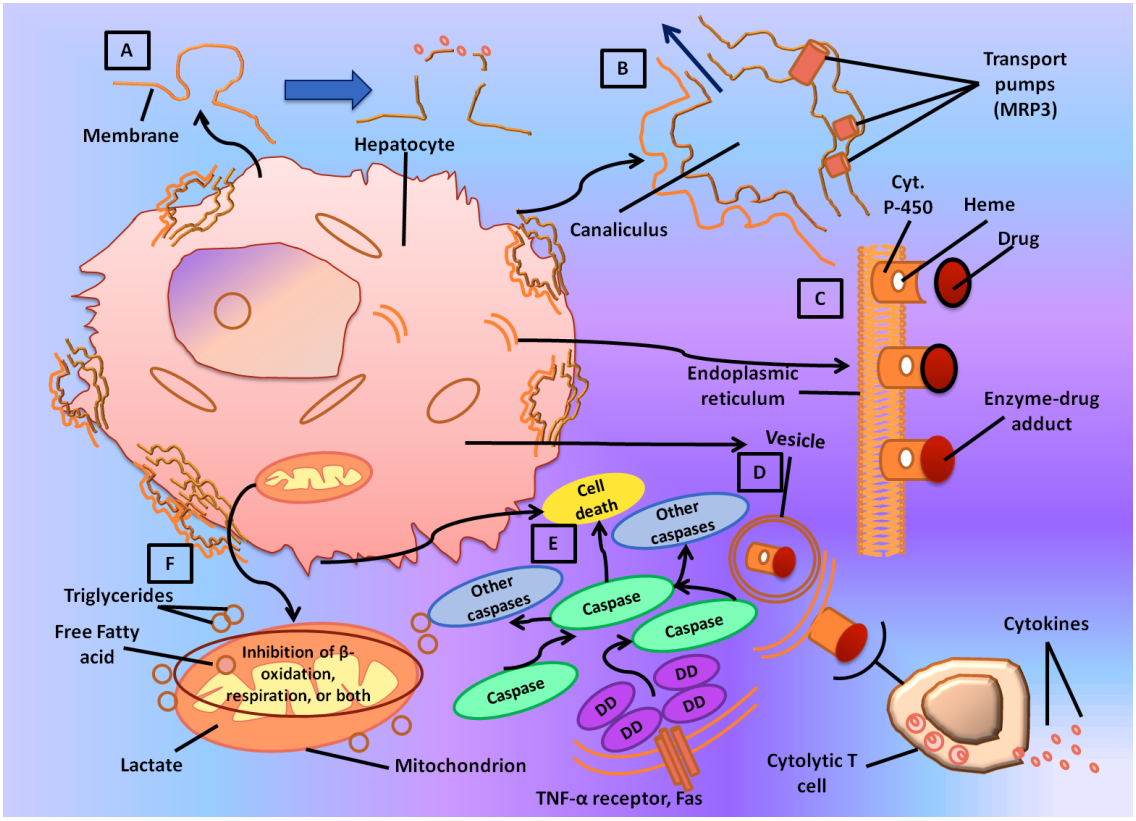


Figure 1.3.4 - Six mechanisms of drug-induced liver injury. A normal hepatocyte can be affected in at least six different ways. **A)** Covalent binding of the drug to intracellular proteins can lead to the loss of ionic gradients, decreased ATP levels, actin disruption, swelling and eventually, cell rupture. **B)** Some other drugs can bind or disable the bile salt export protein and therefore cause cholestasis. **C)** Drugs that bind covalently to enzymes form adducts and the ones which are large enough can serve as immune targets that migrate to the cell surface **D)** in vesicles to serve as immunogens for cytolytic T cells attack. **E)** The secondary cytokine response may cause inflammation and additional neutrophil-mediated hepatotoxicity. Apoptosis might occur by tumor necrosis factor receptor or Fas which as a result can trigger the cascade of caspases activation. **F)** Other drugs target mitochondria and inhibit its function through the synthesis of nicotinamide adenine nucleotide and flavin adenine dinucleotide, decresing the ATP production, inhibiting respiration and fatty acids oxidation. The free fatty acids can no longer be metabolized and might accumulate and the lack of aerobic respiration results in lactate accumulation and ROS, which can later disrupt mtDNA. Legend: DD-death domain (Adapted from Lee, 2003).

1.3.3 DILI as an idiosyncratic drug reaction

Idiosyncratic drug reactions result from a succession of unlikely events but, as discussed at Section 1, the underlying mechanisms are still not known. It is speculated that individual genetic and/or acquired factors (idiosyncrasies) may affect the susceptibility to a particular drug, but there are still some unknowns. The clinical symptoms of DILI are parenchymal necrosis or cholestatic injury in the absence of necrosis or even, a combination of both (Kaplowitz, 2005). This is generally accompanied by serum hepatic damage markers, and overt liver injury in which occurs after a variable period of time, ranging from weeks to more than a year after. Once initiated, the progression of the disease can be abrupt (Iwase *et al.*, 1999).

1.3.3.1 Mechanisms of DILI

Why only a very small group of individuals develop liver injury? It has been hypothesized that a single gene polymorphism in a particular drug-metabolizing enzyme cannot account for the increased susceptibility because simple associations were not identified so far (Boelsterli, 2003). In addition, a major role of the immune system is one of the current models to explain idiosyncratic DILI. In some, but not all cases, the mechanism underlying idiosyncratic DILI have an immune-mediated response. This is primarily based on the clinical observation that liver injury is accompanied by fever, eosinophilia, and rash as well as, proinflammatory infiltrates. The hapten model of IDR is not compatible with delayed toxicity of several months or even more than a year after treatment, so this model can be only applicable to a small portion of all drugs causing idiosyncratic DILI. However, the reason why only a few individual react could be because they develop tolerance rather than an immune response towards the hapten. A novel concept is that episodes of inflammation may predispose an

individual and modulate the state of susceptibility (Roth *et al.*, 1997; Roth *et al.*, 2003). The LPS-drug combination study with rats has been shown to cause acute hepatotoxicity using various drugs such as chlorpromazine (Buchweitz *et al.*, 2002), ranitidine (Luyendyk *et al.*, 2003), diclofenac (Deng *et al.*, 2006) or trovafloxacin (Waring *et al.*, 2006). In another study, animals were infected with reovirus and the induced inflammatory response potentiated acetaminophen-induced injury (Maddox *et al.*, 2010). This response turned out to be similar for several different drugs. This may raise the question of whether it is really the LPS that potentiate the drug effects or whether it is a drug-mediated effect in amplifying the toxic liver effects of LPS.

Taken together, these models are inadequate to explain the clinical hepatotoxicity of most drugs, thus idiosyncratic DILI underlying mechanisms remain unidentified.

1.3.3.2 The mitochondrial hypothesis of idiosyncratic DILI

There is experimental and clinical evidence for the involvement of mitochondria in idiosyncratic DILI. For instance, the "threshold effect" which implies that cumulative damage can go unnoticed until a critical time, which then leads to a rapid progression to cell death. This fits well with clinical data exhibiting delayed but sudden onset of hepatotoxicity after drug treatment in some patients (Figure 1.3.5). There is evidence that the threshold is greater than 90% for tRNA point mutations and greater than 60% for several mtDNA deletions (Pulkes and Hanna, 2001). The cell phenotype alters rapidly from normal to abnormal when the mutant mtDNA reaches a critical level (Shoffner, 1996). One of the most common alterations is in complex I. In fact, one third of all alterations are associated with genes encoding complex I (and another one third of genes involved in

complex IV) (Coon *et al.*, 2006). This observation is according to the fact that random mutations in coding mtDNA are most likely to occur in complex I because from all directly encoding base pairs, 56% encode for complex I subunits (Cortopassi and Wang, 1995). Partial complex I deficiency accounts with increased mitochondrial superoxide anion and high levels of oxidative stress. The SOD-deficient mouse model exhibited sensitivity to drugs that targeted complex I, producing mitochondrial superoxide anion (Wang and Cortopassi, 1994). Animals with heterozygous deletions of Tme (T-associated maternal effect) had only 50% of normal wild-type SOD2 activity and when treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (*MPTP*) developed massive brain toxicity. It is possible that a similar mechanism could occur for hepatic-targeting drugs. This means that exposure to mitochondrial drugs in combination with underlying deficiencies in complex I and increased oxidative stress, can result in DILI. Unfortunately, there is only scattered clinical and experimental evidence that mitochondria may be involved in idiosyncratic DILI. One important observation is that many drugs that cause DILI also impair mitochondrial function. For instance, OXPHOS uncoupling by the majority of NSAIDs, tolcapone and troglitazone; inhibition of complex I/III, by leflunomide; increased mitochondrial oxidative stress induced by diclofenac or troglitazone; increased intracellular calcium by diclofenac; or activation of JNK by troglitazone, which can lead to MPTP induction. Also, nucleoside analog reverse transcriptase inhibitors (NRTIs) clearly exhibit mitochondrial liability (Feng *et al.*, 2001; Lewis *et al.*, 2003) and can cause cardiac and skeletal muscle dysfunction, and more importantly, induce hepatic failure through the inhibition of mtDNA polymerase-γ, which leads to mtDNA depletion. The NRTIs are a class of therapeutics with an unusually high prevalence of adverse effects (15-20%) which makes them agreeable with possible genetic predisposition (Lewis *et al.*, 2001).

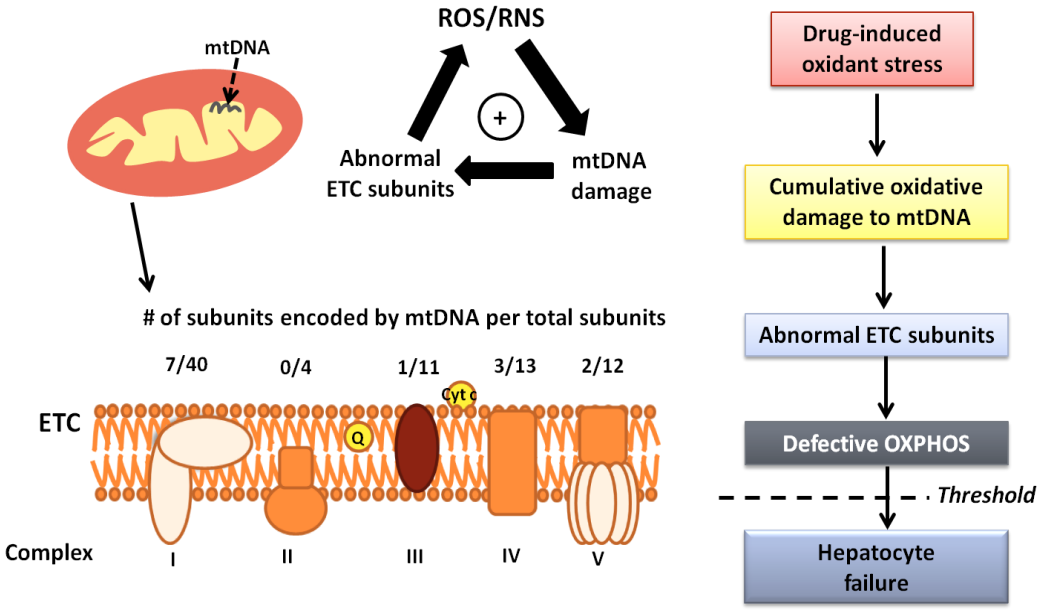


Figure 1.3.5 - Drug-induced oxidative stress and mtDNA damage. Certain subunits of the ETC are encoded by mtDNA and mutations in the mtDNA lead to a gradual increase in the abnormal function of ETC proteins and consequently, will lead to mitochondrial dysfunction. This is a vicious cycle which will also increase the oxidative stress. Legend: OXPHOS- oxidative phosphorylation, ETC- electron transport chain, mt-mitochondrial, ROS- reactive oxygen species, RNS- reactive nitrogen species (Adapted from Boelsterli and Lim, 2007).

However, no key genes have been identified so far and single mutations in mtDNA do not necessarily have to be deleterious. The fact that, there are several copies of mtDNA and a large number of mitochondria per cell, resulting in heteroplasmy, might be the clue. Nonetheless, if a sustained drug stress is superimposed on these abnormalities, the capacity of heteroplasmic mitochondria to meet the energy demands may go under a certain threshold (Lewis *et al.*, 2003). Furthermore, mitochondrial oxidative stress can also enhance drug stress, so that the equilibrium is shifted towards the pro-oxidant side and this might induce the MPTP and possibly cause cell death.

For the reasons mentioned above, there is clear evidence to believe that mitochondrial dysfunction may be linked with idiosyncratic DILI. Although ultrastructural changes in mitochondria of patients with DILI are very limited, one particular case revealed an increase in the number of mitochondria in hepatocytes obtained from a patient with tolcapone-induced DILI, simultaneously with mitochondrial swelling, loss of cristae and reduced matrix density (Spahr *et al.*, 2000). Abnormal megamitochondria were also found in human hepatocytes exposed to troglitazone (Shishido *et al.*, 2003).

If a drug causes cumulative injury to mitochondria, these changes may not become visible until the threshold effect comes into action. Once mitochondria are lethally damaged, the injury can occur even after discontinuation of the treatment. More direct evidence was seen in patients with DILI. For instance, valproate-induced liver injury was linked with an inherited dysfunction of mitochondrial electron transport chain complexes, suggesting abnormal ETC proteins (Krahenbuhl *et al.*, 2000).

An important observation that links DILI to mitochondria is the age factor. For many drugs, aging is a risk factor, and interestingly, mitochondrial DNA mutations also increase with age, which is also correlated with apoptotic markers (Suski *et al.*, 2011). Thus, it is probable that mitochondria become more susceptible to drug-induced toxicity with aging (Marchi *et al.*, 2012). Another clue is that idiosyncratic DILI is more often observed in older women, as opposed to men (Persky *et al.*, 2000; Strehlow *et al.*, 2003). Indeed, estrogens have been implicated in exerting protective effects against oxidative stress in a variety of tissues, however, to date it is still unclear to what extent such gender-specific susceptibility to oxidative stress may be involved in idiosyncratic DILI.

1.3.4 Experimental evidence of drug-induced mitochondrial dysfunction and DILI

In the majority of the cases, the experimental evidence for the involvement of mitochondria is based on the observation that a particular drug which causes DILI in patients causes mitochondrial dysfunction. Normally, the data obtained so far is usually obtained with cell cultures and isolated mitochondria. *In vivo* studies that demonstrated drug-induced mitochondrial toxicity are actually rare. Normal, disease-free animals are usually resistant to drug effects in terms of mitochondrial alterations and specifically sensitized models are needed to be able to unmask a potential mitochondrial liability. New tools became available to observe drug-induced mitochondrial toxicity in hepatocytes. For example, fluorescent probes that are accumulated by mitochondria allows for a easy determination of different mitochondrial endpoints, including ROS production, alterations of transmembrane potential and calcium or even follow the induction of the MPTP (Blattner *et al.*, 2001; Lim *et al.*, 2006).

Several alternative animal models have been used to study the toxicity caused by several drugs which are hepatotoxicants. For instance, toxicogenomics biomarkers for hepatotoxicity were investigated in a study where 36 inbred mice strains were treated with acetaminophen (300mg/Kg, ig.) (Harrill *et al.*, 2009). The use of genetically defined panel of mice has advantages over classical models used in toxicology which used a single strain, because it takes the advantage of the vast genetic diversity that is available among these mice strains. A dramatic gradient of acute hepatotoxicity across strains was observed, with liver gene expression data revealing 26 genes that correlated with liver necrosis, and were not affected by genetic differences between individual strains, which can serve as biomarkers for acetaminophen-induced toxicity responses across a

heterogeneous population (Harrill *et al.*, 2009). Another study conducted by Rusyn *et al.* used trichloroethene (TCE), an industrial contaminant, (2,100 mg/kg) in 15 strains of mice and analyzed hepatic and serum end-points 24 hours post-dosing (Bradford *et al.*, 2011). The metabolism of TCE was different between strainsm with different individual levels of oxidative and conjugative metabolites. In addition, this knowledge may not only be used to determine the extent of within-species variability but also can serve as the scientific basis for the selection of strains for assessment of organ-specific toxic effects in longer-term studies (Bradford *et al.*, 2011). Regarding this topic, many other studies were conducted with common mice mitochondrial DNA haplotypes which have been associated with various phenotypes, including learning performance and disease penetrance (Brown *et al.*, 2002; Roubertoux *et al.*, 2003). No influence of mtDNA haplotype in cell respiraton has been demonstrated so far. Enriquez *et al.* demonstrated by using cell lines carrying four different common mouse mtDNA haplotypes in an identical nuclear background (cybrids), that the respiratory capacity per molecule of mtDNA in cells with the NH3T3 or NZB mtDNA was lower than with C57BL/6J, CBA/J or BALB/cJ mtDNA. The genetic element underlying those differences was also determined (Moreno-Loshuertos *et al.*, 2006).

In the search for a good model to analyze DILI, it would be desirable to have an animal model to investigate the relationship between abnormal mitochondrial function and drug toxicity. This is not possible with healthy animals; instead animal models of human disease, that exhibit mitochondrial liabilities and compromised mitochondrial function, must be developed. So, an ideal model would be a murine model that features inherited mitochondrial changes and which would provide the proof-of-concept for the hypothesis that silent mitochondrial defects can unleash a drug's

mitochondriotoxic potential. Furthermore, the studies previously mentioned with different mice strains can also be useful platforms to predict liver injury related with genetic susceptibilities to certain drugs and allow a more realist understanding of what happens in a heterogeneous population, such as human population.

The $SOD2^{+/-}$ mouse model has been used in aging and neurodegenerative studies, in order to understand the role of mitochondrial dysfunction in drug-induced hepatoxicity (Lebovitz *et al.*, 1996). While homozygous SOD2 knockout mice die early postnatally, heterozygous mice appear to be phenotypically normal (Van Remmen *et al.*, 1999). However, those mice have manifestations of cumulative oxidative stress in mitochondria, including reduced aconitase activity, decreased complex I acivity, increased levels of 8-hydroxydeoxyguanosine and decreased ATP levels (Williams *et al.*, 1998; Ong *et al.*, 2006). For instance, long-term treatment of $SOD2^{+/-}$ mice with nimesulide induced increased oxidative mitochondrial damage, increased carbonyls and decreased aconitase activity, and increased cytochrome c release, indicative of apoptosis (Ong *et al.*, 2006). All the alterations mentioned were not present in wild-type mice under the same conditions. Similarly, this mouse model was used to test tert-butyl hydroperoxide exposure, stavudine, and troglitazone, which was removed from the market in 2000 due to an high risk of idiosyncratic DILI (Yokoi, 2010). Another study used flutamide in the same mouse model and obtained the same data pattern, decreased aconitase activity, and also decreased complexes I-III expression, by the subunits encoded by mtDNA (Kashimshetty *et al.*, 2009). Taken together, this data is in agreement with the hypothesis that hepatocytes with silent mitochondrial dysfunction are more susceptible to drug effects that may dramatically increase oxidant stress (see Figure 1.3.6).

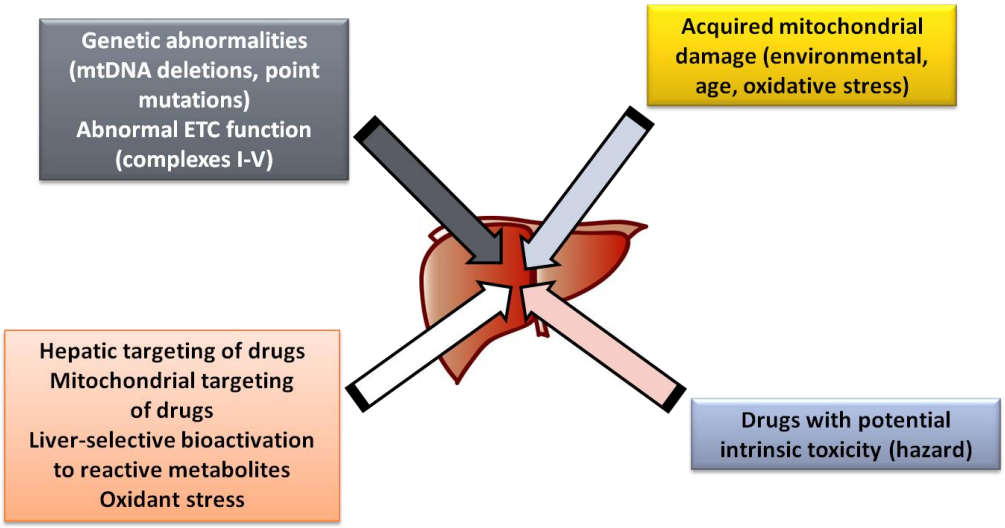


Figure 1.3.6 - The liver as the target organ for drug-induced mitochondrial toxicity and overall dysfunction. The determinants of susceptibility can affect the same organelle as the drug imposing cumulative damage to mitochondria, which can be additively or synergistically. Abbreviation- ETC- electron transport chain (Adapted from Boelsterli and Lim, 2007).

1.3.5 Final considerations

The role of underlying genetic (and/or acquired) mitochondrial abnormalities which corresponds to a high degree of heteroplasmy results in silent, not clinically detectable, mitochondrial dysfunction and oxidative stress. If individuals are exposed to mitochondria-targeting drugs that are bioactivated in the liver, and which increase local ROS formation, this may induce cumulative damage in that organ, which will eventually lead to parenchymal cell failure and tissue necrosis, once the mitochondrial threshold effect has been surpassed. We can also think that in normal individuals with no mitochondrial abnormalities, the threshold is not exceeded at therapeutic doses, while in subjects with genetically compromised mitochondria, liver injury can abruptly occur after a given lag time, once mitochondrial alterations accumulate.

This is still part of an hypothesis and it is critical to find better models to test this hypothesis and to detect the possible risk of severe liver injury, even before drugs are even launched on the market. It is not obvious that mtDNA variants may be associated with susceptibility, making it difficult to make clear associations with phenotype and genotype.

It is, therefore, critical to develop new *in vitro* and *in vivo* platforms to evaluate IDRs in a context of genetically diverse backgrounds.

Summary points of Section 3:

- Mitochondria are not only cell furnaces of the cells but are also targets for drug-induced toxicity.

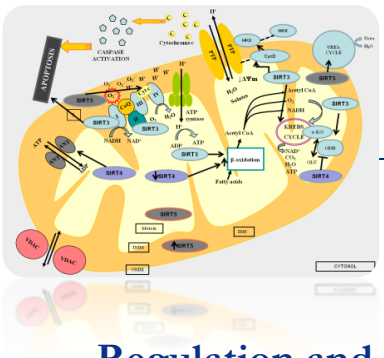
- Drug-induced mitochondrial injury can happen in different organs, especially the ones which are more metabolically active, such as the liver, heart, kidney, brain and testis.

- Idiosyncratic DILI is a major concern for pharmaceutical companies since it is responsible for the majority of drug withdrawal from the market and it is extremely difficult to predict.

- Recent findings lead to the thought that mitochondria and DILI might be linked and the knowledge on drug-induced mitochondrial toxicity can provide clues on the mechanisms for idiosyncratic DILI.

- The scarce number of animal models to study idiosyncratic DILI may not yet allow us to further understand this complex phenomenon where mitochondria may play a part, but can shed some light on the mitochondrial hypothesis of idiosyncratic DILI.

➢ New and more realist *in vitro* and *in vivo* models should be created and the diverse genetic background seen in the human population should be taken into account.



Section 4

Regulation and protection of mitochondrial physiology by Sirtuins: focus on SIRT3 controversial role

1.4 - Regulation and protection of mitochondrial physiology by sirtuins

The link between sirtuin activity and mitochondrial biology has recently emerged as an important field. This conserved family of NAD^+-dependent deacetylase proteins has been described to be particularly involved in metabolism and longevity. Recent studies on protein acetylation have uncovered a high number of acetylated mitochondrial proteins indicating that acetylation/deacetylation processes may be important not only for the regulation of mitochondrial homeostasis but also for metabolic dysfunction in the context of various diseases such as metabolic syndrome/diabetes and cancer. The functional involvement of sirtuins as sensors of the redox/nutritional state of mitochondria and their role in mitochondrial protection against stress are hereby described, suggesting that pharmacological manipulation of sirtuins is a viable strategy against several pathologies.

This section of the present thesis comprises the recent knowledge in the field of sirtuins with special focus to the mitochondrial isoform SIRT3 involved in the regulation of mitochondrial metabolism and possible role in the protection against drug-induced toxicity.

1.4.1 Classification of sirtuins

Sirtuins, or Silent information regulator proteins (Sir), and their homologs are present in a very wide range of organisms, from bacteria to humans, forming a conserved family of proteins (Denu, 2005). Up to date, seven sirtuin homologues have been identified in mammalian cells. These have different intracellular localization as well as various roles in cell physiology (Tanny *et al.*, 1999; Blander and Guarente, 2004). Based on the phylogenetic analysis of the core domain, mammalian sirtuins have been classified into four classes together with other Sir2-related proteins widely distributed in eukaryotes and prokaryotes (Frye, 2000; Smith *et al.*, 2000). Mammalian SIRT1 (120.0 kDa), SIRT2 (41.5 kDa) and SIRT3 (43.6 kDa) are considered Class I, while SIRT4 (35.2 kDa) is grouped into Class II. Class III SIRT5 (33.9 kDa) and SIRT6 (39.1 kDa) together with SIRT7 (44.8 kDa), Class IV, are other examples. All the described sirtuins contain a conserved 275 amino acid catalytic core domain together with N-terminal and/or C-terminal domains. A list of the different sirtuins, plus other details on their physiology/activity, can be seen in Table 1.1.

Table 1.1 - Characterization of mammalian sirtuins.

HDAC III	Sirtuin	Localization	Substrates	Catalytic activity	Function	Modulators		Knockout outcome	References
						Activators	*Inhibitors*		
class I	**SIRT1**	Cytosol Nucleus	PGC1α, eNOS, FOXO, p53, MyoD, NF-kB, histone H3 and H4	NAD^+-dependent protein deacetylation	Cell survival, insulin signaling, inflammation, metabolism regulation, oxidative stress response, lifespan regulation	Resveratrol, AROS, HIC1, splitomicinsquercetin dihydropyridine	DBC1, NAD^+ iso-nicotinami-de, Salermide	High pre-natal mortality, metabolic abnormalities, heart and bone defects, Shortened lifespan	(Brunet *et al.*, 2004; Borra *et al.*, 2005; Bordone *et al.*, 2006; Bordone *et al.*, 2007; Feige *et al.*, 2008; Alcain and Villalba, 2009; Aquilano *et al.*, 2010; Canto *et al.*, 2010)
	SIRT2	Cytosol Nucleus	α-tubulin, histone H4	NAD^+-dependent protein deacetylation	Cell cycle regulation, nervous system development,	Dihydropyridine	Sirtinol, NAD^+, iso-nicotinami-de, Salermide	Aberrances during mitosis, defective development	(Hiratsuka *et al.*, 2003; Garske *et al.*, 2007; Nahhas *et al.*, 2007; Southwood *et al.*, 2007; Wang *et al.*, 2007; Harting and Knoll, 2010)
	SIRT3	Mitochondria nucleus	AceCS2, IDH2, ShdhA	NAD^+-dependent protein deacetylation	Regulation of mitochondria l energetic metabolism	Dihydropyridine	NAD^+, iso-nicotinami-de, dihydrocou m-rin	Affected mitochondrial metabolism, respiratory chain complex I inhibition	(Allison and Milner, 2007; Ahn *et al.*, 2008; Alhazzazi *et al.*, 2010; Cimen *et al.*, 2010; Someya *et al.*, 2010)
class II	**SIRT4**	Mitochondria	GDH	Mono-ADP-ribosyl transferase	Regulation of mitochondria l energetic metabolism, insulin secretion		NAD^+, iso-nicotinami-de	Increased GDH activity	(Argmann and Auwerx, 2006; Haigis *et al.*, 2006; Ahuja *et al.*, 2007)

class III	**SIRT5**	Mitochondria	Histone H4, CPS1 cyt c	NAD^+-dependent protein deacetylation, NAD-depend protein lysine desuccinylase and demalonylase	Urea cycle regulation, apoptosis		NAD^+, iso-nicotinamide, suramin	Altered mitochondrial metabolism	(Nakagawa and Guarente, 2009; Nakagawa *et al.*, 2009)
class IV	**SIRT6**	Nucleus	Histone H3K9	NAD^+-dependent protein deacetylation, mono-ADP-ribosyl transferase	Genome stability, DNA repair, nutrient-dependent metabolism regulation		NAD^+, iso-nicotinamide	Chromosomal aberrations, tumorgenesis, premature senescence, decreased rate of mitochondrial respiration	(Mahlknecht *et al.*, 2006b; Mostoslavsky *et al.*, 2006; Michishita *et al.*, 2008; Kawahara *et al.*, 2009; McCord *et al.*, 2009)
	SIRT7	Nucleus	RNA pol I, p53	NAD^+-dependent protein deacetylation	Regulation of rRNA transcription, cell cycle regulation		NAD^+, iso-nicotinamide	Proliferation arrest, increased rate of apoptosis, heart abnormalities, fibrosis, shortened lifespan	(Michishita *et al.*, 2005; Ford *et al.*, 2006; Voelter-Mahlknecht *et al.*, 2006; Vakhrusheva *et al.*, 2008)

1.4.2 Sirtuin enzymatic activity

All sirtuins, with only one exception (SIRT4), catalyze protein deacetylation in which the lysine acetyl group is transferred from the target protein to the ADP-ribose component of NAD^+, which leads to their full dependence on NAD^+ availability and indicates that sirtuins can be sensors of the cellular redox state. As a consequence, sirtuin activity leads to the generation of deacetylated proteins, 2'-O-acetyl-ADP ribose and nicotinamide (Sauve, 2010). Moreover, SIRT6 additionally demonstrates ADP-ribosyl transferase activity while SIRT4 (as mentioned earlier) demonstrates ADP-ribosyl transferase activity only. As described above, sirtuin activity is regulated by NAD^+ availability. On the other hand, nicotinamide noncompetitively inhibits sirtuins suggesting that the deacetylation reaction product can also act as an endogenous regulator of sirtuin activity (Sauve, 2010). Interestingly, isonicotinamide, which binds to the nicotinamide pocket of

yeast sirtuin (Sir2), only inhibits the base exchange, with the deacetylation activity remaining unaffected or even being increased (Sauve *et al.*, 2005; Cen *et al.*, 2011). This fact indicates that chemical compounds such as isonicotinamide can act as potent sirtuins activators in mammalian cells. Additionally, it has been demonstrated that the activity of yeast sirtulin Hst2 (a Class I member) can be regulated by homo-oligomerization of the enzyme (Sauve *et al.*, 2006). Another well known and currently intensively studied sirtuin activator is resveratrol, a polyphenol present in different sources, including for example grapes skin and red wine (Lagouge *et al.*, 2006). It has been demonstrated that polyphenols and especially resveratrol, reduce K_m values for sirtuin substrates, resulting in different biological effects as increased resistance to apoptosis and to stress stimuli. Apart from nicotinamide, an inhibitory effect on sirtuins has also been described for several other compounds. The most known are sirtinol, splitomicin (specific for yeast sirtuins Hst1 and Sir2) and dehydrosplitomicin (specific mostly to sirtuin Hst1) (Grubisha *et al.*, 2005). Human sirtuins are not inhibited significantly by splitomicin as well as by dehydrosplitomicin. More about the mechanism of protein deacteylation carried out by sirtuins as well as detail on their inhibitors and activators can be found in the review of Sauve et al. (Sauve *et al.*, 2006).

1.4.3 Sirtuin protein targets

Potential sirtuins substrates are various acetylated proteins involved in cell metabolism, apoptosis and regulation of gene transcription. Sirtuins can thus determine the ability of cells to adapt to different conditions (Haigis and Guarente, 2006; Finkel *et al.*, 2009; Vaquero, 2009). Published data suggest a significant role of deacetylation in metabolic responses to fasting or caloric restriction as well as in the response to different stress stimuli,

including for example oxidative stress (Choudhary *et al.*, 2009; Schwer *et al.*, 2009).

Among the several proteins which are deacetylated by sirtuins, histones and transcription factors such as p53 (Li *et al.*, 2010), FOXO (Brunet *et al.*, 2004), peroxisome proliferator activated receptor γ (PPARγ) (Picard *et al.*, 2004), nuclear factor-κB (NFκB) (Kawahara *et al.*, 2009) and PGC-1α (Sugden *et al.*, 2010) can be found. Sirtuins are also able to deacetylate α-tubulin (Tang and Chua, 2008) and acetyl-CoA synthetase (Hallows *et al.*, 2006). Another important protein for metabolism, glutamate dehydrogenase (GDH) is a well-known example of ADP-ribosylation substrates for SIRT4 (Haigis *et al.*, 2006). The variety of sirtuin substrates, either being crucial enzymes or gene regulatory elements, indicates the possibility that multiple sirtuins may modulate cell physiology and metabolism through interacting with distinct targets regulated under a wide range of physiological conditions.

1.4.4 Tissue specific and intracellular localization of mammalian sirtuins

Molecular analysis revealed that SIRT1, 2, 3, 5 and 6 are ubiquitously expressed in different tissues and organs. The highest amount of SIRT6 was detected in muscles, brain and heart, while SIRT4 is mostly present in muscle, kidney, testis and liver (Michishita *et al.*, 2005). By its turn, SIRT7 has been found in brain, kidney, liver, lung and adipose tissue (Michishita *et al.*, 2005). Further detailed studies determined subcellular localization of several sirtuins. Among seven mammalian sirtuins, SIRT1 and SIRT2 show both cytoplasmic and nuclear localization, while SIRT6 and SIRT7 are only located in the nucleus and nucleoli, respectively (Michishita et al., 2005).

SIRT3, SIRT4 and SIRT5 are mitochondrial proteins, although SIRT3 can be also found in the nucleus (Schwer and Verdin, 2008; Shoba *et al.*, 2009).

1.4.5 Mitochondrial sirtuins: characterization and activity

1.4.5.1 Localization and processing

Among the seven sirtuins already here described, three (SIRT3, SIRT4 and SIRT5) are present inside mitochondria, regulating several metabolic pathways through protein desacetylation (Fig. 1.4.1). About 1/5 of all mitochondrial proteins undergo acetylation, being one of the largest pools in the cell. Proteomic studies of mice liver mitochondria revealed almost 300 lysine residues that can be acetylated in 133 proteins involved in different metabolic pathways, including Krebs and urea cycle, fatty acid beta-oxidation and oxidative phosphorylation (Kim *et al.*, 2006). Lysine acetylation profile of mitochondrial proteins demonstrates some similarities with prokaryotic organisms, which appears to support the symbiotic origin of mitochondria hypothesis (Huang *et al.*, 2010). All three mitochondrial sirtuins are located in the mitochondrial matrix (Huang *et al.*, 2010); however, SIRT5 was also found in the mitochondrial intermembrane space (Nakamura *et al.*, 2008; Schlicker *et al.*, 2008). Interestingly, SIRT5 overexpression does not increase the mitochondrial intermembrane pool of this protein but results instead into a preferential accumulation in the mitochondrial matrix (Nakagawa *et al.*, 2009). There are some controversial data showing that in COS7 cells co-expressing SIRT3 and SIRT5, the 44kDa isoform of SIRT3 can be found in the nucleus, although its function still waits to be elucidated (Scher *et al.*, 2007; Nakamura *et al.*, 2008). It was speculated that under normal conditions, both a mitochondrial and nuclear localization of SIRT3 is possible, although under stressful conditions, most of the nuclear pool moves to mitochondria (Scher *et al.*, 2007).

Mitochondrial sirtuins, especially SIRT3 and SIRT4, are responsible for sensing NAD^+/NADH balance, which by being disrupted by several factors may affect cellular redox homeostasis (Yang *et al.*, 2007; Yu and Auwerx, 2009). All three mitochondrial sirtuins are encoded by nuclear DNA and contain a mitochondrial targeting sequence located on their N' terminal end, which is immediately cleaved after reaching the mitochondrial matrix. Tissue expression profile of SIRT3, SIRT4 and SIRT5 is similar; however, relatively higher levels of SIRT5 have been observed in muscles (Hallows *et al.*, 2008; Nakamura *et al.*, 2008).

SIRT3 is a 28kDa deacetylase, with, as mentioned before, a 44kDa isoform found in nucleus, which requires proteolitic processing to achieve its maximal deacetylase activity. After reaching mitochondria, premature SIRT3 is cleaved at the N'-terminus targeting peptide by the mitochondrial processing peptidase (MPP) (Schwer *et al.*, 2002).

SIRT4 is a 35kDa protein with ADP-ribosylotransferase activity present in mitochondrial matrix. SIRT4 transfers ADP-ribose from NAD^+ to target proteins. In vitro studies showed no detectable deacetylase activity of SIRT4 but it cannot be excluded that a very specific substrate would have to be present in order to observe some relevant activity (Huang *et al.*, 2010). Similarly to SIRT3, SIRT4 is also processed in the mitochondrial matrix where a 28 amino acid sequence is cleaved when pro-SIRT4 reaches its destination (Schwer *et al.*, 2002).

The 33.0 kDa SIRT5 is present in the mitochondrial matrix, but when overexpressed, it can also be found in the mitochondrial intermembrane space. SIRT5 N-terminal 36 amino acids sequence is immediately cleaved after import into the mitochondrial matrix (Michishita *et al.*, 2005; Mahlknecht *et al.*, 2006a).

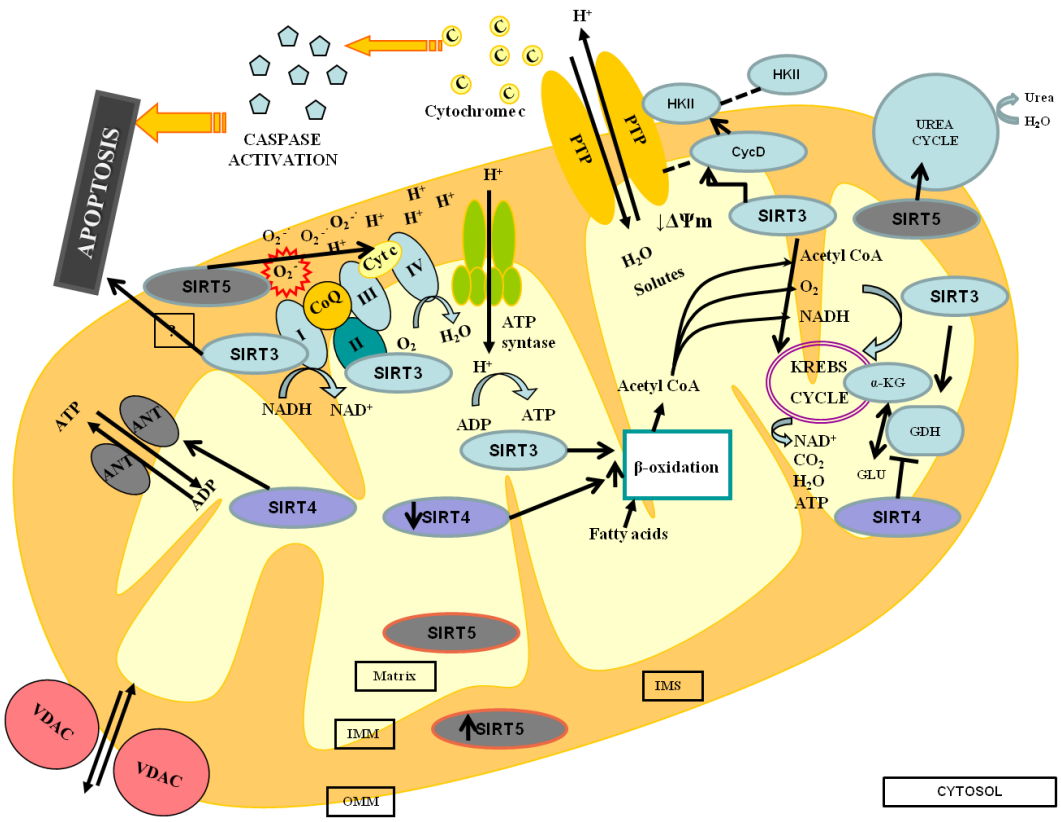


Figure 1.4.1 - In-house mitochondrial sirtuins and their targets. SIRT3 is the major deacetylase inside mitochondria and it has been showed to phisically interact with one of the subunits of complex I (NDFA9) and also seems to interact with ShdhA, one of the flavoprotein subunits of complex II of the respiratory chain. SIRT3 plays a major role in fatty acid oxidation and the homeostasis of ATP levels. This sirtuin is also important to Krebs cycle once it deacetylates Acetyl-CoA synthase 2, which is the enzyme that catalizes the conversation of acetyl-CoA in acetate that undergoes the Kreb's cycle. Another target is the enzyme isocitrate dehydrogenase 2 which catalyzes a key regulation point of the Krebs cycle. SIRT3 can also deacetylate cyclophilin D and induce hexokinase II dissociation from the mitochondrial outer membrane. SIRT4 is a NAD-dependent mono-ADP-ribosyltransferase that is known to target glutamate dehydrogenase (GDH), which converts glutamate into α-ketoglutarate and allows it to undergo the Krebs cycle. GDH can also be deacetylated by SIRT3. SIRT4 also seems to interact with the ANT isoforms 2 and 3 and it seems like SIRT4 is a negative regulator of oxidative metabolism in contrast with SIRT3. Finally, SIRT5 is known to play an important role in the urea cycle deacetylating and activating the enzyme that catalyses the first step of this cycle, carbomoyl phosphatise synthase 1 (CPS1). Also, SIRT5 can deacetylate cytochrome c, pointing a pro-apoptotic function for this sirtuin as it was also pointed to SIRT3. Legend: OMM, Outer Mitochondrial Membrane; IMM, Inner Mitochondrial Membrane; IMS, Inter membrane space.

1.4.6 Regulation of mitochondrial metabolism by sirtuins

As described above, reversible protein acetylation allows cells to adjust to a changing of the environment conditions. A key question is why and where do mitochondrial proteins become acetylated? This question has not been answered in detail yet but it has been confirmed that protein acetylation does occur in mitochondria (Guan and Xiong, 2010) and, in fact, it is also been suggested that the acetylation mechanism of mitochondrial proteins is different from cytosolic and nuclear proteins (Kim *et al.*, 2006). Studies in knockout SIRT4 and SIRT5 mice showed that there was no change in the acetylation level of mitochondrial proteins, but studies on SIRT3$^{-/-}$ showed a significant increase in protein acetylation (Lombard *et al.*, 2007; Huang *et al.*, 2010). This finding suggests that SIRT3 seems to play a central role in protein deacetylation in mitochondria. It is known that sirtuins are directly or indirectly involved in many different metabolic pathways including lipid metabolism (Lomb *et al.*, 2010), calorie restriction conditions/ insulin uptake (Ahuja *et al.*, 2007; Guarente, 2008; Qiu *et al.*, 2010c), urea cycle (Nakagawa and Guarente, 2009; Nakagawa *et al.*, 2009), glycolysis, gluconeogenesis and Krebs cycle (Huang *et al.*, 2010; Verdin *et al.*, 2010). Nowadays, major threats to human health are cancer, neurodegenerative diseases, immune dysfunction and certainly, the metabolic syndrome (Guarente, 2006). In the US, around 32% of adults and 17% of children and adolescents are obese (Elliott and Jirousek, 2008). The human body is not adapted to process the excess of calorie intake per day. Also, in many regards, all of these diseases are also associated with the aging process. When organisms are calorie restricted (30-40% decrease in food intake), it was shown to be possible to increase the lifespan up to 50%, having sirtuins a very important role in the adaptation process (Guarente, 2007; Qiu *et al.*, 2010c). When food is scarce, mammals may have to adapt to the limited

resources in order to maintain the basic functions such as growth, synthesis and reproduction, having to shut down unnecessary energy expenditures in order to survive. Thus, tissues that are crucial for survival such as the muscle, the heart and certain parts of the brain are protected from starvation and go through mild calorie restriction. On the other hand, tissues that are normally associated with endocrine and exocrine activity, such as the liver, pancreas and the reproductive system, are very likely to experience severe calorie restriction (Qiu *et al.*, 2010c). Upon abundance of resources, energy is stored as fat. During calorie restriction, animals up-regulate fatty-acid oxidation and switch fuel usage from glucose to fatty-acids. At a cellular level, increased mitochondrial biogenesis in calorie-restricted tissues suggests a tissue-specific increase in metabolic rate of the affected animals (Qiu *et al.*, 2010c). Calorie-restricted animals have low levels of blood insulin in response to limited food intake. Low insulin levels suppress glycolysis and maintain blood glucose levels. As described above, SIRT1 suppresses UCP2 in pancreatic β-cells, which allows for a more efficient ATP production (Bordone *et al.*, 2006). Nevertheless, the fulcrum of calorie restriction response seems to be mitochondrial activation which allows a metabolic adaptation to chronic energy deficit demand (Qiu *et al.*, 2010c). Here is where sirtuins enter the spotlight.

1.4.6.1 SIRT3 and regulation of mitochondrial metabolism

SIRT3 is a unique mitochondrial sirtuin since it is the only one that was related with extended lifespan in humans (Rose *et al.*, 2003). SIRT3 may be critical in sensing NAD^+ levels in the mitochondria since increased NAD^+ would trigger a regulatory pathway that would activate SIRT3 leading to the deacetylation of specific targets. It has been demonstrated that mice deficient in SIRT3 present protein hyperacetylation (Lombard *et al.*, 2007), including of the metabolic enzyme glutamate dehydrogenase (GDH)

suggesting that this sirtuin may have an important impact in metabolic control (Schlicker *et al.*, 2008). Lombard et al. concluded that SIRT3 deficient mice are metabolically unremarkable under both fed and fasted conditions, including normal thermogenesis (Lombard *et al.*, 2007). Another study demonstrated that SIRT3 was able to deacetylate and activate the mitochondrial enzyme acetyl-CoA synthase 2 (Hallows *et al.*, 2006), an enzyme that catalyzes the formation of Acetyl-CoA from acetate (Hallows *et al.*, 2006; North and Sinclair, 2007). Under ketogenic conditions, such as during calorie restriction, the liver releases a large amount of acetate into the blood. The heart and the muscles express acetyl-CoA synthase 2 and use acetate in an efficient way as an energy source. Deacetylation of acetyl-CoA synthetase switches on its activity so it is relevant that SIRT3 is indeed activated during CR (Hirschey *et al.*, 2010). A study by Ahn *et al.* (Ahn *et al.*, 2008) stressed the importance of NAD^+- dependent deacetylation in the regulation of energy homeostasis and also provided evidence for SIRT3-dependent regulation of global mitochondrial function. The authors of this paper showed that SIRT3 is an important regulator of basal ATP levels and observed that SIRT3 can physically interact with at least one of the subunits of complex I, the 39-KDa protein NDUFA9, although in a reversible manner (Ahn *et al.*, 2008). Mitochondria from $SIRT3^{-/-}$ animals display a selective inhibition of complex I activity and altered basal ATP content (Ahn *et al.*, 2008). Another target for SIRT3 is also the enzyme isocitrate dehydrogenase 2 (Schlicker *et al.*, 2008), which promotes regeneration of antioxidants and catalyzes a key regulation point of the citric acid cycle. Schilicker C et al. reported that the N- and C-terminal regions of SIRT3 regulate its activity against glutamate dehydrogenase and a peptide substrate, indicating roles of these regions in substrate recognition and sirtuin regulation (Schlicker *et al.*, 2008). Cimen *et al.* identified that the succinate dehydrogenase flavoprotein (ShdhA) subunit is also another SIRT3 target in

the mitochondrial respiratory chain (Cimen *et al.*, 2010). SdhA is highly acetylated in SIRT3 knockout mice and also that the activation of complex II was dependent on SIRT3 both in wild-type mice and in cells over-expressing SIRT3. The regulation of complex II by reversible acetylation is actually an important point of control since is a crossroad between oxidative phosphorylation and the Krebs cycle, acting on the regulation of metabolism in mammalian mitochondria. The skeletal muscle is a metabolically active organ and crucial for insulin-mediated disposal and lipid catabolism. Exercise signals can regulate SIRT3 in skeletal muscle, with a dynamic response of that sirtuin to coordinate downstream molecular responses (Palacios *et al.*, 2009). Exercise training increases SIRT3 level and CREB phosporylation and up-regulates PGC-1α. Interestingly, SIRT3 is more highly expressed in slow oxidative type I soleus muscle in comparison with fast type II or gastrocnemius muscles (Palacios *et al.*, 2009). In addition, SIRT3 levels in skeletal muscle are sensitive to diet, with SIRT3 decreasing by a high-fat diet, and increased by short-term fasting (24-hour) or long term nutrient deprivation (12-month CR) and exercise training. Since caloric restriction regiment leads to phospho-activation of AMPK in the muscle and that SIRT3$^{-/-}$ mice have low phosphorylation levels of both AMPK/CREB and low expression of PGC-1α, it was proposed that SIRT3 levels may respond to various nutritional/energetic and physiological challenges by regulating muscle energy homeostasis via AMPK and PGC-1α (Palacios *et al.*, 2009). Another important study by Hirschey et al. strengths the role of SIRT3 in the regulation of fatty acid oxidation (Hirschey *et al.*, 2010). SIRT3 expression is up-regulated during fasting in liver and brown adipose tissues (Hirschey *et al.*, 2010). Mass spectrometry results showed that the long chain acyl coenzyme A dehydrogenase (LCAD) is hyperacetylated at lysine 42 in the absence of SIRT3, with LCAD hyperacetylation reducing its enzymatic activity (Hirschey *et al.*, 2010). Mice

lacking SIRT3 presented hallmarks of fatty-acid oxidation disorders during fasting, including reduced ATP levels and intolerance to cold exposure (Hirschey *et al.*, 2010). This study identified acetylation as a novel regulatory mechanism for mitochondrial fatty acid oxidation and demonstrates that SIRT3 modulates mitochondrial intermediary metabolism and fatty-acid use during fasting. Interestingly, it was also reported that SIRT3 deacetylates cyclophilin D (Shulga *et al.*), diminishing its activity and inducing its dissociation from the adenine nucleotide traslocator (ANT). In addition, SIRT3-induced interaction with cyclophilin D also induces the detachment of hexokinase II from mitochondria. These findings might be important for the role of SIRT3 in the metabolism of cancer cells and their susceptibility to toxicity by foreign agents. SIRT3 can regulate the MPTP through the deacetylation of CypD at lysine 166, suppressing age-related cardiac hypertrophy (Hafner *et al.*, 2010a). In an elegant study, the authors reported that SIRT3 deacetylates the regulatory component of the MPTP, cyclophilin D (CypD), at lysine 166, which is adjacent to the binding site of cyclosporin A, a CypD inhibitor. Cardiac myocytes from SIRT3 knockout mice showed increased mitochondrial swelling due to the mPTP opening and accelerated signs of cardiac aging including cardiac hypertrophy and fibrosis at 13 months of age (Hafner *et al.*, 2010a). All together, the data shows that SIRT3 controls the MPTP and that a loss of SIRT3 promotes mitochondrial alterations resulting in enhanced ROS production and cell dysfunction. The results are also a clear evidence that SIRT3 activity is necessary to prevent mitochondrial dysfunction and cardiac hypertrophy during aging (Hafner *et al.*, 2010a). Although it is not new that under certain conditions SIRT3 mediates the reduction of oxidative damage, a study from Someya et al. relates age-related hearing loss and calorie restriction with this topic (Someya *et al.*, 2010). Under caloric restriction conditions, a reduction of oxidative damage in multiple tissues and a decrease of age-related hearing

loss in WT mice were observed, as opposed to mice lacking SIRT3. The authors of this study identified SIRT3 as an essential player in enhancing the mitochondrial glutathione antioxidant defense system during caloric restriction and concluded that mitochondrial adaptations and aging retardation in mammals may be dependent on SIRT3 (Someya *et al.*, 2010). The protective effects of CR against oxidative stress and damage are diminished in mice lacking SIRT3, with the effects of this protein being mediated by deacetylation of two critical lysine residues in SOD2, enhancing its antioxidant activity (Qiu *et al.*, 2010a). Several other SIRT3 targets involved in metabolism have been recently described. Shimazu et al. identified 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2) as another SIRT3 deacetylated protein (Shimazu *et al.*, 2010). Mice lacking SIRT3 present a decrease in β-hydroxybutyrate levels during fasting, which can demonstrate that SIRT3 regulates ketone body production during fasting (Shimazu *et al.*, 2010). Also, Hallows et al. identified the urea cycle enzyme ornithine transcarbomoylase (OTC) and other enzymes involved in β-oxidation as likely SIRT3 targets. Fasted mice lacking SIRT3 revealed alterations in β-oxidation and in the urea cycle, demonstrating a direct role of SIRT3 in the regulation of the two important metabolic pathways during CR (Hallows *et al.*, 2011). Under low energy input conditions, SIRT3 modulates mitochondria by promoting amino acid catabolism and β-oxidation (Hallows *et al.*, 2011). Livers from mice maintained on a high fat diet (HFD) exhibited reduced SIRT3 activity, a 3-fold decrease in hepatic NAD+ levels, and increased mitochondrial protein oxidation. In contrast, neither SIRT1 nor histone acetyltransferase activities were altered, suggesting SIRT3 as a crucial factor contributing to the observed phenotype (Kendrick *et al.*, 2011). In $SIRT3^{-/-}$ mice, HFD further increased the acetylation status of liver proteins and reduced the activity of respiratory complexes III and IV. This study identified acetylation patterns in liver

proteins from HFD-fed mice and the results suggest that SIRT3 is an integral regulator of mitochondrial function, with its depletion resulting in hyperacetylation of critical mitochondrial proteins that protect against liver lipotoxicity under conditions of nutrient excess (Kendrick *et al.*, 2011).

1.4.7 Protection of mitochondrial function by sirtuins

The role of mitochondria in cell physiology and survival, as well as in drug-induced toxicity is well established (Sections 2 and 3). Besides control of mitochondrial metabolism, sirtuins are also involved in the lines of defense of that organelle. In fact, there is actually evidence that at least SIRT3 can protect mitochondria from exogenous and endogenous stresses (Scher *et al.*, 2007; Sundaresan *et al.*, 2008; Kong *et al.*, 2010; Pillai *et al.*, 2010). Mitochondria are an important site of reactive oxygen species (ROS) production in a cell, which is why a tight ROS production by that organelle must be exerted by different mechanisms in order to prevent structural damage and accelerated aging (Guarente, 2008). The role of mitochondrial sirtuins in the protection against mitochondrial damage is now beginning to be clarified. One starting point was the finding that NAD^+ levels dictate cell survival (Yang *et al.*, 2007). One of the major causes for cell death due to genotoxic stress is the hyperactivation of PARP-1 that depletes nuclear and cytosolic NAD^+ causing the translocation of the AIF from the mitochondrial membrane to the nucleus. Yang et al. identified Nampt as a stress- and nutrient-responsive gene that increases mitochondrial NAD^+ levels (Yang *et al.*, 2007). Increased mitochondrial NAD^+ levels improve cell survival during genotoxic stress and that this protection is dependent on SIRT3 and SIRT4, which means that Nampt-mediated cell protection requires mitochondrial sirtuins (Yang *et al.*, 2007). Nampt increased SIRT3 activity since Nampt overexpression decreased the acetylation level of

AceCS2. Allison et al. exploited apoptosis trough Bcl-2/p53 regulation and verified a SIRT3 involvement in this process (Allison and Milner, 2007). Bcl-2 and SIRT3 were silenced separately and in combination in human epithelial cancer and non-cancer cells. It was demonstrated that SIRT3 is required for apoptosis under basal conditions, by selective silencing of Bcl-2 in HCT116 human epithelial cancer cells. SIRT3 is dispensable for stress-induced apoptosis in HCT116 human epithelial cancer cells but it is an essential proapoptotic mediator for both Bcl-2/p53-regulated apoptosis. Interestingly, SIRT3 functions in JNK2-regulated apoptosis but it is dispensable for both SIRT-1 regulated and stress-induced apoptosis. SIRT3 is a pro-apoptotic protein that participates in distinct basal apoptotic pathways (Allison and Milner, 2007). Nevertheless, this paper does not really point to a direct link between SIRT3 and protection against apoptosis under stress conditions in contrast to many other papers. For example, it has been shown that SIRT3 protects the mouse heart by blocking the cardiac hypertrophic response (Sundaresan *et al.*, 2008). In cardiomyocytes, SIRT3 prevent cardiac hypertrophy activation by activating the fork head box O3a-dependent (Foxo3a-dependent), anti-oxidant superoxide dismutase and catalase genes and decreasing cellular levels of ROS (Sundaresan *et al.*, 2008). The results demonstrate that SIRT3 is an endogenous negative regulator of cardiac hypertrophy by the suppression of ROS levels. Another study showed that SIRT3 levels are increased under stress not only in mitochondria but also in the nuclei of cardiomyocytes (Sundaresan *et al.*, 2008). This particular paper identified Ku70 as a new SIRT3 target, since SIRT3 physically binds to ku70 and deacetylates the latter, promoting its interaction with Bax. Under stress, SIRT3 overexpression protects cardiomyocytes by partially preventing the translocation of Bax to mitochondria. This study points an essential role of SIRT3 in the survival of cardiomyocytes under stress conditions. Recently,

SIRT3 was pointed out as a tumor-suppressor mitochondrial-localized protein (Kim *et al.*, 2010). The expression of a single oncogene (Myc or Ras) in SIRT3 knockout MEFs results in *in vitro* transformation and altered intracellular metabolism. In addition, SIRT3 knockout mice developed ER/PR-positive mammary tumors (Kim *et al.*, 2010). In this work, it is also shown that several human cancer tissues exhibit reduced SIRT3 levels, thus leading the authors to propose SIRT3 as a mitochondrial fidelity protein. In this regard, loss of SIRT3 results in a decrease in the antioxidant defenses, resulting in an increase of ROS production. A prooxidant environment may be permissive for in vivo carcinogenesis. PGC-1α is an important inducer of detoxifying enzymes, even though the molecular mechanism is not clearly understood. PGC-1α activated the mouse SIRT3 promoter mediated by an estrogen-related receptor binding element (ERRE) (Kong *et al.*, 2010). The knockdown of ERR α reduced the induction of SIRT3 by PGC-1α in C2C12 myotubes. SIRT3 was crucial for PGC-1α-dependent induction of components of the mitochondrial respiratory chain, such as ATP synthase 5c and cytochrome c and antioxidant enzymes glutathione peroxidase-1 and, MnSOD (or SOD2) (Kong *et al.*, 2010). Both overexpression of SIRT3 and PGC-1α decreased basal ROS levels in myotubes. SIRT3 stimulated mitochondrial biogenesis and might provide a novel target for treating ROS-related diseases. Another interesting study focused on SIRT3 capacity of reducing lipid accumulation via AMKP activation in human hepatic cells (Shi *et al.*, 2010). The knockdown of SIRT3 downregulated the phosphorylation of AMPK and acetyl coenzyme A carboxylase (ACC) promoting increased lipid accumulation. It was concluded that the capacity of SIRT3 to activate AMPK is dependent on its deacetylase activity (Shi *et al.*, 2010). A very interesting paper correlates p53, SIRT3 and protection of *in vitro*-fertilized mouse against oxidative stress (Kawamura *et al.*, 2010). During pre-implantation development, mitochondrial dysfunction or

increased levels of oxidative stress adversely affect the developmental outcome. In this study, it was shown that SIRT3 inactivation increases mitochondrial ROS production leading to p53 up-regulation and alterations in downstream gene expression. The findings indicate that SIRT3 may play a protective role in pre-implantation of embryos under stress conditions during *in vitro* fertilization and culture, pointing out a new and interesting future clinical application related with manipulation of SIRT3 and post-implantation success. One particular study involved SIRT3 in oral cancer (Alhazzazi *et al.*, 2011a). The authors demonstrated for the first time that SIRT3 is overexpressed in oral squamous cell carcinoma (OSCC) *in vitro* and *in vivo*, when compared with other sirtuins. Down-regulation of SIRT3 inhibited cell growth, increased cell sensitivity to chemotherapy and reduced tumor burden in vivo, demonstrating that SIRT3 can be a novel potential therapeutic target for oral cancer (Alhazzazi *et al.*, 2011a). SIRT3-mediated deacetylation of evolutionary conserved lysine 122 in MnSOD results into increased activity in response to stress. SIRT3-knockout results in increased mitochondrial superoxide, formation of a tumor-permissive environment and finally, enhanced mammary carcinogenesis (Tao *et al.*, 2010). To the best of our knowledge, there is not as much information about SIRT4 or even SIRT5 but it seems very likely that both sirtuins can also be important in mitochondrial protection against genotoxic stress suggesting that they can contribute to apoptosis in tumor-supressive or stress resistant conditions (Verdin *et al.*, 2010). Interestingly, SIRT4 is apparently involved in mitochondrial oxidative metabolism. SIRT4 KO primary hepatocytes and myotubes were used in order to study fatty acid oxidation and oxygen consumption under these conditions (Nasrin *et al.*, 2010). Evidences suggest that SIRT4 is a negative regulator of oxidative metabolism, in contrast with SIRT3 and SIRT1, which means that a functional interplay between different sirtuins must exist in order to coordinate the flow of energy during

a given metabolic state. SIRT4 inhibition may improve hepatic insulin sensitivity via increased fat oxidative capacity and hence may be beneficial for the treatment of type 2 diabetes. Despite the limited knowledge on *in vivo* substrates for SIRT5, the contribution of this sirtuin in disease has been suggested. Repetitive elements in its gene structure suggested possible genomic instabilities and malignant diseases (Gertz and Steegborn, 2010). Furthermore, one particular study indicates that SIRT5 is decreased after alcohol exposure in rats, which increased hepatic mRNA expression of FoxO1 and p53 (Lieber *et al.*, 2008). Thus, alcohol consumption compromises nuclear mitochondrial interactions by post-translational modifications which contribute to alteration of mitochondrial biogenesis through the newly discovered decrease in SIRT5. SIRT5 contributes to liver damage induced through chronic exposure to alcohol but would also be interesting to understand the role of this sirtuin under other stress conditions and understand its role in mitochondrial protection against various stressors.

1.4.8 SIRT3: a spotlight in the protection against drug-induced oxidative stress?

Several studies showed that SIRT3 enhances the ability of mitochondria to deal with ROS generated as OXPHOS by-products (Figure 1.4.2). First, SIRT3 deacetylates and also activates isocitrate dehydrogenase (IDH_2), helping to replenish the mitochondrial pool of NADPH. Elevated NADPH is necessary for glutathione reductase, which converts oxidized glutathione (GSSG) into reduced glutathione (GSH). Normal mice develop hearing loss which is an age-related disease due to ROS-induced cochlear damage, which will eventually become better by calorie restriction via SIRT3-dependent deacetylation of IDH_2 decreasing ROS levels (Someya *et al.*, 2010). Another

evidence showed that SIRT3 can also deacetylate and activate SOD2, thereby reducing oxidative damage in the liver. SIRT3 appears to present antioxidant activity. Qiu *et al.* (Qiu *et al.*, 2010b) showed that calorie restriction (CR) results in the deacetylation and activation of SOD2, presumably by upregulating SIRT3. This finding may explain why CR has been associated with a reduction in ROS levels in mitochondria. It is important to note that the role of SIRT3 on ROS detoxification through the generation of NADPH and activation of SOD2 occurs in the mitochondrial matrix and does not represent a boost in antioxidant capacity in the nucleus or cytosol. However, it has been showed that SIRT3 is capable of suppressing ROS responsive pathways that are induced by cardiac hypertrophy in the cytosol such as RAS, AKT, and MAPK signalling (Sundaresan *et al.*, 2009). Since SIRT3 deacetylates so many components of the respiratory chain, it may directly affect ROS production. In addition, SIRT3 deacetylates cyclophilin D to inhibit apoptosis induced by MPTP opening (Hafner *et al.*, 2010b). A controversial role of SIRT3 in the mechanism of acetaminophen toxicity has been shown. Lu *et al.* (Lu *et al.*, 2011) showed that liver failure caused by the reactive metabolite (NAPQI) from APAP is exacerbated by fasting. Fasting can promote SIRT3-mediated mitochondrial-protein deacetylation thus acetaminophen metabolites bind to lysine residues. The authors investigated whether deacetylation predisposes mice to toxic metabolite-mediated disruption of mitochondrial proteins. $SIRT3^{-/-}$ mice were protected from acetaminophen hepatotoxicity, with mitochondrial aldehyde dehydrogenase 2 as a direct SIRT3 substrate. Furthermore, aldehyde dehydrogenase 2 deacetylation increased acetaminophen toxic-metabolite binding and enzyme inactivation (Lu *et al.*, 2011). Therefore, protein deacetylation enhanced xenobiotic liver injury by modulating the binding of a specific toxic metabolite to mitochondrial proteins. Thus, SIRT3 may have a dual role and caution is required when

looking for new therapeutic strategies which aim to potentiate SIRT3 activity.

SIRT3 may have a dual role as participating in antioxidant protection, but can also increase ROS generation. Furthermore, SIRT3 can also act as a tumor suppressor or a tumor promoter (for a comprehensive review on this topic see (Alhazzazi *et al.*, 2011b), depending on the cell type and tumor type, and also, the presence of different stress stimuli or cell death. In a similar way to what happened in the controversial study presented above, the role of SIRT3 is still missunderstood.

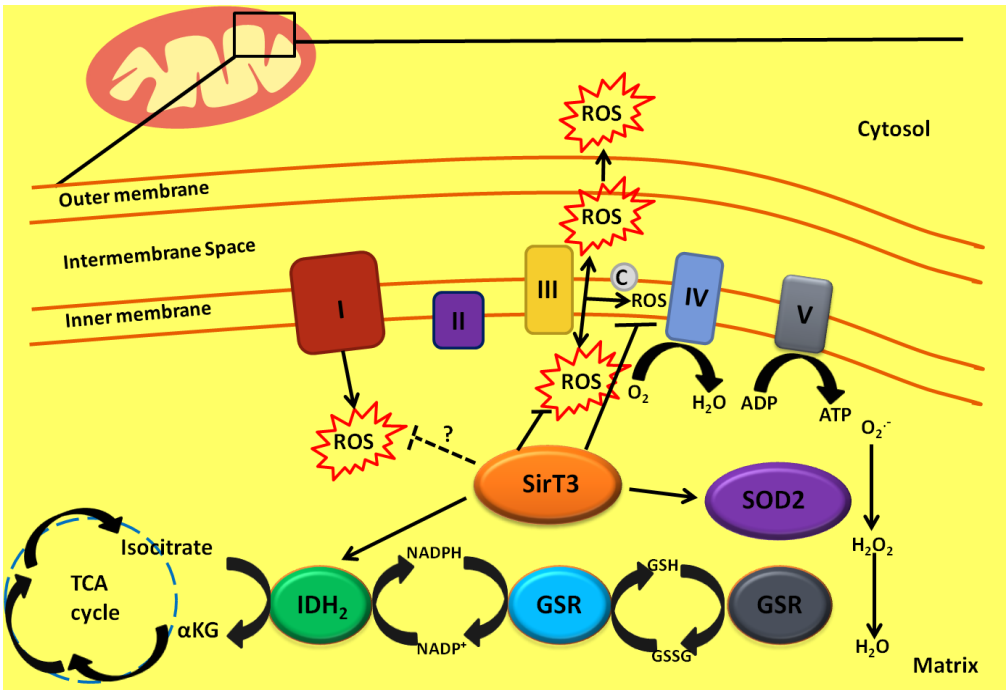


Figure 1.4.2 - SIRT3 protection against ROS production in mitochondria. ROS are mainly formed in complex I and III. The superoxides are detoxified by SOD2 and converted into hydrogren peroxide (H_2O_2), which will be converted into water by glutathione peroxidase (GPX). GSSH is reduced by glutatione reductase which is dependent on NADPH. NADPH is generated from NAD^+ via isocitrate dehydrogenase (IDH2). SIRT3 can activate SOD2 and IDH2 and this will inhibit ROS production (Adapted from Bell and Guarente, 2011).

1.4.9 Future perspectives: are sirtuins good therapeutic targets?

It is plausible that drugs aimed at modulating sirtuins activity and/or expression may have important consequences in cellular responses to stress and life span. It is extremely important to fully identify and characterize targets of this exciting class of proteins and understand how the manipulation of their enzymatic activity or protein expression can be involved in the protection, not only of mitochondria but also of the entire cell. It seems that sirtuins are strategically positioned along different organs and cellular compartments and that they may play distinct roles ((Smith *et al.*, 2002; Schwer and Verdin, 2008; Vaquero, 2009; Yu and Auwerx, 2009; Verdin *et al.*, 2010). Furthermore, it would be very interesting to understand how sirtuins communicate within each other and form a network that should in theory, contribute to protect the cell. What do these proteins have in common? How do they interact with each other? Is the stoichiometry of the different sirtuins in mitochondria constant? How does it change there and in other cellular locations upon different stimuli? Many of these questions remain unanswered. Mitochondrial sirtuins may contribute to the decrease of "unhealthy" mitochondria and some of them (e.g. SIRT3) might even be protective against drug-induced toxicity (Scher *et al.*, 2007; Sundaresan *et al.*, 2008; Sundaresan *et al.*, 2009), which is why it is fundamental to understand the mechanism behind sirtuin-mediated mitochondrial protection and how targeting sirtuins can be a valid future therapeutic approach for several different diseases. Although many recent reviews have provided new directions in this field, there is still much to be elucidated. There is still a gap of information regarding SIRT4 and SIRT5 functions that warrants further studies in order to understand the roles of these important players in the regulation and protection of mitochondrial function. This review provided an overall view of sirtuin function with a

special relevance on the most recent findings on the function and relevance of mitochondrial sirtuins. Furthermore, the new and exciting data opens a completely new road for novel pharmaceutical applications of inhibitors and inducers of this class of proteins.

Summary points of Section 4:

- Mammalian sirtuins are a family of NAD^+-dependent deacetylases and act as molecular sensors of cellular energy balance, and regulators of metabolic responses to nutritional alterations.

- There are seven members of sirtuins in mammals. SIRT 1, 6 and 7 are found in the nucleus, whereas SIRT2 can be find in the cytoplasm and SIRT 3, 4 and 5 exist in mitochondria.

- SIRT3 is the major deacetylase in mitochondria and in its absence, proteins are hyperacetylated. SIRT4 has ADP-ribosyl-transferase activity and SIRT5 has robust demalonyase and desuccinylase activities instead of deacetylase activity.

- SIRT3 KO mice in contrast with SIRT4 and SIRT5 null mice have constitutive deficits of ATP production.

- SIRT3 has been shown to play a role in the protection against oxidative stress besides playing a role in the regulation of intermediary, lipid, and carbohydrate metabolism.

- Recent studies suggest that the role of SIRT3 against oxidative stress and in cancer is controversial. Thus, further work is needed to clarify how modulation of SIRT3 activity has therapeutic applications.

1.5 Hypothesis and specific aim of the present work

A major concern of pharmaceutical companies is drug withdrawal from the market due to toxic and unpredictable drug toxicity. Although rare, IDRs can sometimes lead to severe liver injury and fatalities. Mitochondria can be not only the target for many different compounds but also be the cause of their toxicological effects, being perhaps an explanation for IDR and organ injury. With this in mind, the aim of our study was to investigate whether two intrinsic mitochondrial factors can regulate drug toxicity. The present thesis is thus aimed at investigating two distinct questions (Figure 1.5.1) a) Are mtDNA single nucleotide polymorphisms related with alterations of mitochondrial bioenergetics and drug-induced toxicity? b) Is SIRT3 an important determinant in mitochondrial susceptibility to drug-induced toxicity?

The hypothesis for the first aim was that different cell strains with distinct mtDNA SNPs have different mitochondrial bioenergetic profiles and may therefore vary in their response towards drug-induced toxicity. In order to achieve our goal, we used an *in vitro* platform composed by four strains of mouse embryonic fibroblasts (MEFs) with mtDNA polymorphisms. We sequenced the mtDNA from C57BL/6J, MOLF/EiJ, CZECHII/EiJ and PERA/EiJ, with the latter two being sequenced for the first time. Further, we characterized and compared the bioenergetics profile of four mice strains along two different passages, 3 and 10, not only to understand the bioenergetic differences between strains but also along cell culture aging (see Section 3.1). Furthermore, in order to validate the model, we chose seven different drugs which are also known to cause not only IDR but also mitochondrial toxicity and tested them in terms of toxicity (see Section 3.2).

The second specific aim in this thesis aimed to understand the possible role of SIRT3 in the regulation of drug-induced toxicity by doxorubicin (DOX). As described above, DOX is a widely used anti-cancer drug, although presenting cardiotoxicity. Our hypothesis was that over-expression of SIRT3 on H9c2 cardiomyoblasts decreases the toxicity of DOX (see Section 3.3).

The present thesis is relevant as an approach to investigate the role of mitochondrial toxicity in IDR, possibly aimed at predicting and preventing IDR in clinical practice.

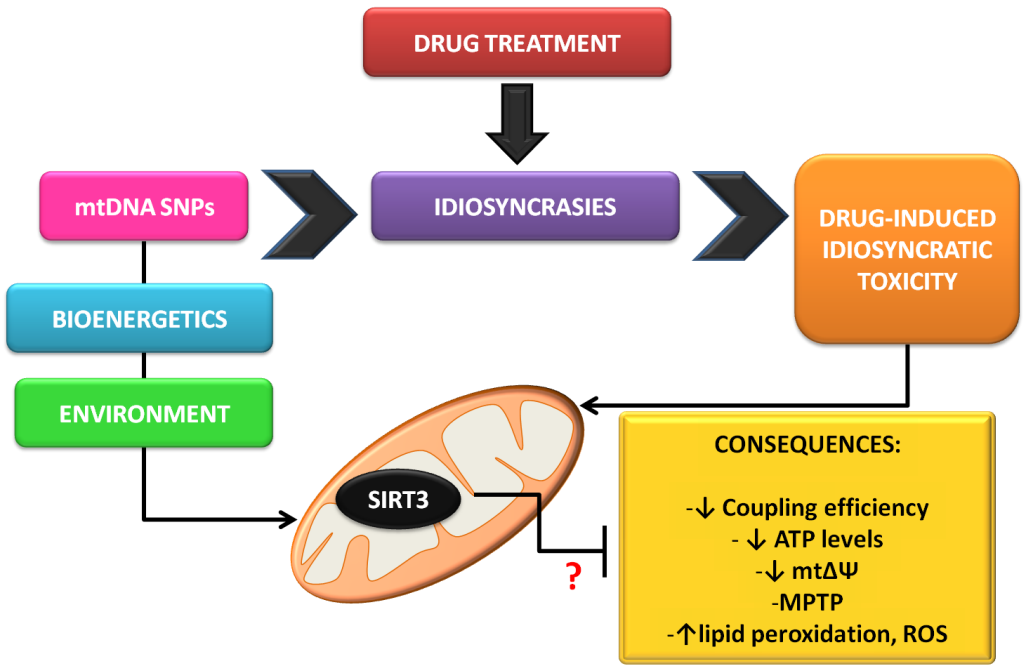


Fig. 1.5.1 - An schematic overview of the two main topics of this thesis. The possible involvement of mitochondria in both drug-induced idiosyncratic reactions and as a SIRT3 containing compartment against-drug-induced toxicity. Legend: MPTP- mitochondrial permeability transition pore, ROS- reactive oxygen species, SIRT3- sirtuin 3, mtΔΨ- mitochondrial transmembrane potential, ATP- adenosine triphosphate.

2- Materials and Methods

2.1- Mitochondrial bioenergetics and drug-induced toxicity in a panel of mouse embryonic fibroblasts with mitochondrial DNA single nucleotide polymorphisms (Chapters 3.1 and 3.2)

2.1.1 Materials

Cell culture media and supplements for mouse embryonic fibroblasts (MEFs) were purchased from Invitrogen (Carlsbad, CA). All chemicals were purchased from Sigma Aldrich (St. Louis, MO) and were of the highest purity available. The CellTiter-Glo® kit was purchased from Promega (Madison, WI). The T175 cm^2 cell culture flasks were purchased from BD Biosciences (Bedford, MA). The 96-well black walled clear bottom plates used for high content imaging were purchased from Becton Dickinson (Bedford, MA). The 96-well white walled, opaque bottom plates used for luminescence readings were from Becton Dickinson (Bedford, MA). The BCA kit for protein determination was from Pierce (Rockford, IL). XF96 sensor cartridges, XF96-well plates, calibration buffer and calibration plates were obtained from Seahorse Bioscience (Billerica, MA). Low-buffered serum-free RPMI medium was purchased from Molecular Devices (Sunnyvale, CA).

2.1.2 Cell culture conditions

Mouse embryonic fibroblasts (MEFs) were purchased at passage 0 from the Jackson Laboratory (Bar Harbor, ME) and were grown in T175 cm^2 cell culture flasks in a humidified atmosphere at 37°C, 5% CO_2 in 1X Knockout DMEM (Invitrogen 10829-018) containing high glucose (17.5 mM) and sodium pyruvate (110 mg/L) and also supplemented with 2 mM L-glutamine, 15% fetal bovine serum, 1X non-essential amino acids (Invitrogen 11140), 0.25 mg/mL of gentamicin and 0.1 mM of β-

mercaptoethanol. We refer to this medium as "Cell culture medium" in the rest of section 2.1. Cells were grown until passage 3 or passage 10 before analysis.

2.1.3 mtDNA sequencing

mtDNA sequencing was performed essentially as described in Goios *et al* (Goios *et al.*, 2007) with some modifications. Total DNA from the four strains of MEFs, at passage 3 and passage 10, was extracted following a standard phenol-chloroform protocol (digested with proteinase K in TE (10 mM Tris, 1 mM-EDTA, pH 7.5) buffer containing 0.5% SDS, purified with phenol-chlorophorm-isoamyl alcohol, and precipitated with ethanol. Thirty four overlapping fragments of approximately 500 bp covering the entire mtDNA genome were then amplified by PCR (polymerase chain reaction) with the appropriate oligodeoxynucleotides and annealing temperatures. The primers used for sequencing the mtDNA of C57BL/6J and PERA/EiJ were as described in Goios *et al* (Goios *et al.*, 2007). The primers used for sequencing the mtDNA of CZECHII/EiJ and MOLF/EiJ are shown in Table 1. The PCR products were purified using solid-phase reversible immobilization. DNA sequencing reactions were carried out using a BigDye Terminator v3.1 kit (Applied Biosystems, Life Technologies, Carlsbad, CA) and post-reaction dye terminator removal was done using Agencourt CleanSEQ (Beckman Coulter Genomics, Danvers, MA). Automated DNA sequencing was performed on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). All fragments were sequenced from both strands.

Table 2.1 - Oligonucleotide pairs used for amplification and sequencing of the mtDNA from fibroblasts of the mouse strains, CZECHII/EiJ and MOLF/EiJ.

Name	**Sequence** (5'-3')	**Location** (AY172335)	**Annealing temp.** (ºC)
M1F	CAC CAC CAG CAC CCA AAG CT	15378-15397	62
M1R	GTC ATG GGC TGA TTA GAC CCG	15885-15865	
M2F	GGG CCA TCA AAT GCG TTA TCG C	15806-15827	62
M2R	GGC CAG GAC CAA ACC TTT GTG	87-67	
M3F	CCG TGA ACC AAA ACT CTA ATC ATA	16250-16273	61
M3R	TTT AGG TCC TAA CAA TGA ATT TTC	432-409	
M4F	CTT CGG CGT AAA ACG TGT CAA C	348-369	63
M4R	GCG GTG TGT GCG TAC TTC ATT	909-889	
M5F	AAA CTA AAG GAC TAA GGA GGA TTT	826-849	61
M5R	TTT GCC ACA TAG ACG AGT TGA TT	1390-1368	
M6F	ACT AAA AGA ATT ACA GCT AGA AAC	1307-1330	61
M6R	GGT GTT GGG TTA ACA GAG AAG	1871-1851	
M7F	CAA CTC GGA TAA CCA TTG TTA GT	1781-1803	61
M7R	ATC TGG GTC AAT AAG ATA TGT TG	2342-2320	
M8F	TGA CCT CGG AGA ATA AAA AAT CC	2256-2278	61
M8R	CTA CTA ATG TTA GGA AGG CTA TG	2820-2798	
M9F	AAT ATC CTA ACA CTC CTC GTC C	2763-2784	61
M9R	ATT ATG GCT ATG GGT CAG GCT	3301-3281	
M10F	GGA TCC TAC TCT CTA CAA ACA C	3222-3243	58
M10R	CTA TTG TCC TAG AAA TAA GAG GG	3783-3761	

M11F	CGG GAG TAC CAC CAT ACA TAT	3685-3705	61
M11R	TAA TCA GAA GTG GAA TGG GGC	4258-4238	
M12F	CAA ACA AAC GGT CTT ATC CTT AA	4178-4200	61
M12R	CTA TGA GTG TTG CTA TAA TTA GAC	4760-4737	
M13F	CCA CTA ACA GGA TTC TTA CCA A	4679-4700	61
M13R	GTG ATA TTC ATG TCG AAT TGC AAA	5248-5225	
M14F	TGG CGG TAG AAG TCT TAG TAG	5183-5203	61
M14R	CCT GCA TGG GCT AGA TTT CC	5746-5727	
M15F	CTC CTT CTC CTA GCA TCA TCA A	5655-5676	58
M15R	TGT CTA CAT CTA ATC CTA CTG TG	6228-6206	
M16F	GGA ATA GTA TGA GCA ATA ATG TC	6141-6163	58
M16R	GAT GAG AAC AGC TGT TAG TGA A	6728-6707	
M17F	CGC TAC TCA GAC TAC CCA GA	6661-6642	61
M17R	GGT TTC AAC TTC TTG TGC ATC TA	7201-7179	
M18F	CCT TAG TCC TCT ATA TCA TCT C	7119-7140	58
M18R	TAT TTT AGT GGA ACC ATT TCT AGG	7665-7642	
M19F	GGT TAT TCT ATG GCC AAT GCT C	7581-7602	61
M19R	ATT ATT AGG GTT CAT GTT CGT CC	8141-8119	
M20F	AAC AAC CGT CTC CAT TCT TTC	8041-8061	58
M20R	GTG GGT CAT TAT GTA TTA TCA TG	8615-8593	
M21F	TCA AGC CTA CGT ATT CAC CCT	8553-8573	61
M21R	TTT ATG TGG TTT CGT TTA CCT TC	9085-9063	
M22F	AGT ACT TCT AGC ATC AGG TGT TT	9011-9033	60

M22R	TTG TAG GGT CGA ATC CGC ATT	9591-9571	
M23F	GCA TTC TGA CTC CCC CAA ATA A	9519-9540	61
M23R	ATC TGT TCC GTA CGT GTT TGA A	10140-10119	
M24F	ACC ATC TTA GTT TTC GCA GCC	10060-10080	60
M24R	GAA CCG ATT AGG GTA TAA AAT AGG	10636-10613	
M25F	GAT GAG GGA ACC AAA CTG AAC	10590-10570	60
M25R	TTA GTA TTG TTG CTC CTA TGA AG	11112-11090	
M26F	CTA ATC GCC TAC TCC TCA GTT	11019-11040	58
M26R	GTC AGA TTC ACA GTC TAA TGT TT	11585-11563	
M27F	ACT TAT TCT TCT AAC TAC CAG TC	11495-11517	58
M27R	GAT TGA TGT TTG GGT CTG AGT G	12087-12066	
M28F	GCC CTT TTT GTC ACA TGA TCA AT	12018-12040	61
M28R	CTC CGA GGC AAA GTA TAG TTG	12585-12565	
M29F	TTT TCC TAC TGG TCC GAT TCC A	12511-12532	62
M29R	AGG TCT GGG TCA TTT TCG TTA AT	13090-13068	
M30F	AGC ATA CGA ATC ATT TAC TTC GT	13030-13008	62
M30R	GCG GGT ATT TTT ATT ATT ATC GAG	13590-13567	
M31F	GTA CTT TAT ATC ATT CCT AAT TAA C	13499-13523	58
M31R	TTT TTG GTT GGT TGT CTT GGG T	14037-14006	
M32F	CCC ACT AAC AAT TAA ACC TAA AC	13985-13963	62
M32R	ATA AAT GCT GTG GCT ATG ACT G	14530-14509	
M33F	TCA TGT CGG ACG AGG CTT ATA	14432-14452	62
M33R	GGG GAT TGA GCG TAG AAT GG	14999-14980	

M34F	TAA TCC ACT AAA CAC CCC ACC	14920-19440	58
M34R	AAT GTA CTA GCT TAT ATG CTT GG	15507-15485	

2.1.4 Sequence analysis

The mtDNA sequences of the four strains of MEFs at passage 3 and passage 10 were compared with the published mtDNA sequence of the mouse strain, C57BL/6J (NC_005891.1; (Goios *et al.*, 2007)), using the software, SeqMan Pro (DNAStar Lasergene 8.0, Madison, WI). The software, Simbiot (www.simbiot.net), was used to analyze the synonymous and nonsynonymous substitutions caused by the single nucleotide polymorphisms (SNPs).

2.1.5 Cell proliferation measurements

MEFs were seeded in cell culture medium at a density of 160,000 cells/well in 12-well plates for measuring cell number after 6 hours and 18 hours. Cells were also seeded at the same density in 6-well plates for measuring the cell number after 48 hours. At each time point, the cells were trypsinized, stained with trypan blue and the viable cells counted with a Countess® Automated Cell Counter (Invitrogen, Life Technologies, Carlsbad, CA).

2.1.6 Mitochondrial membrane potential assessment

MEFs were seeded in black-walled clear-bottom 96-well plates at 8,000 cells/100 µl/well in cell culture medium and kept overnight in a humidified atmosphere at 37C°, 5% CO_2. The following day, the cell culture medium in the two last rows of the 96-well plates was replaced with 100 µl of 75 µM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) dissolved in cell culture medium. The MEFs were then incubated for 4 h in a humidified

atmosphere at 37°C, 5% CO_2. Hundred micro liters of cell culture medium containing Hoechst 33342 (8 μg/ml) and Tetramethyl Rhodamine Methyl Ester- TMRM (200 nM) was then added to each well and the cells incubated for 30 min in a humidified 37°C, 5% CO_2 atmosphere. Cells were rinsed three times with Hanks Balanced Salt Solution (HBSS; 200 μl/well); the final HBSS rinse was not aspirated. Automated live-cell image acquisition was performed on a Thermo Fisher Scientific Cellomics® ArrayScan® VTI High Content Screening Reader using a 10x objective. The ArrayScan® Reader was equipped with a Thermo Fisher Scientific Cellomics® Live Module so that the cells could be maintained in a humidified 37 °C, 5% CO_2 atmosphere. The fluorescence resulting from Hoechst (representing the nuclei) and TMRM (representing the mitochondrial membrane potential) was measured in channels 1 and 2, respectively using a filter set equipped with an XF93-Hoechst filter and an XF93-TRITC filter. Image analysis was done using the Compartmental Analysis Bioapplication (Thermo Fisher Scientific). Image acquisition was set to a minimum of 500 objects per well or to a maximum of three fields per well. The exposure settings for channels 1 and 2 were set to be at 25% of the dynamic range based on the vehicle-treated C57BL/6J MEFs. MEF staining due to TMRM was defined as the punctate signal and was measured as the Mean CircSpot Average Intensity in channel 2.

2.1.7 Cellular ATP measurements

MEFs were seeded in 96-well white walled opaque bottom plates at 8,000 cells/100 μl/well in cell culture medium, at both passage 3 and 10. Basal ATP levels were measured 2 hours and 24 hours after seeding using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's instruction. In the other set of experiments, MEFs were again seeded in 96-well white walled opaque bottom plates at

8,000 cells/100 µl/well in cell culture medium, at passage 10. The final media volume was 100 µl. Compound stock solutions were prepared in DMSO and then diluted in (a) 1X Knockout DMEM (Invitrogen 10829-018) containing 17.5 mM glucose (17.5 mM) and sodium pyruvate (110 mg/L) and supplemented with 2 mM L-glutamine, 15% fetal bovine serum, and 1X non-essential amino acids (Invitrogen 11140) or (b) glucose-free DMEM (Invitrogen 11966-025) supplemented with 10 mM galactose, 2 mM glutamine, 5 mM HEPES, 10% FBS, 1 mM sodium pyruvate. Final DMSO concentration was 0.5%. Cellular ATP concentrations were assessed 24 hours after compound addition using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's instruction.

2.1.8 Cellular oxygen consumption measurements

Oxygen consumption was measured at 37°C using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). All four MEF strains were seeded within the same plate for each experiment and incubated in a 37°C, 5% CO_2 humidified atmosphere for 24 hours. The cells were seeded at a density of 16,000 cells/80µl/well since the absolute rate of oxygen consumption was linearly related to the cell number between 8000 – 32,000 cells/well. In addition, an XF96 sensor cartridge for each cell plate was placed in a 96-well calibration plate containing 200 µL/well calibration buffer and left to hydrate overnight at 37°C.

The cell culture medium from the cell plates was replaced the following day with 150 µl/well of pre-warmed low-buffered serum-free RPMI medium and incubated at 37°C for 30 min to allow the temperature and pH of the medium to reach equilibrium before the first rate measurement. Rotenone, oligomycin, FCCP and antimycin-A were prepared as 700X stock solutions

in DMSO. 2-Deoxy-D-Glucose (2-DG) was prepared as a 7X stock solution in serum-free low-buffered RPMI medium and its pH adjusted to 7.4 with 1 M NaOH. Test compounds which had to be injected into reagent delivery port A of each well were diluted to 7X in low-buffered serum-free RPMI medium and the pH adjusted to 7.4 with 1 M NaOH. Test compounds which had to be injected into port B of each well following injection of compound in port A were diluted to 8X in low-buffered serum-free RPMI medium. 25 µL of compound or vehicle (1% (v/v) DMSO) in low-buffered serum-free RPMI medium was then pre-loaded into the ports of each well in the XF96 sensor cartridge. Since each well in the cell plate contained 150 µl of medium, the final concentration of DMSO in each well after reagent injection was 0.14% (v/v).

The sensor cartridge and the calibration plate were loaded into the XF96 Extracellular Flux Analyzer so that the cartridge could be calibrated. When the calibration was complete, the calibration plate was replaced with the cell plate. Four baseline rate measurements of the oxygen consumption rate (OCR) of the MEFs were made using a 2 minute mix, 5 minute measure cycle. The compounds were then injected pneumatically by the XF96 Analyzer into each well, mixed, and OCR measurements were made using the 2 minute mix, 5 minute measure cycle. All oxygen consumption rates were calculated as a percentage of the fourth baseline rate measurement obtained in the same well, ie the rate obtained just before addition of compound in port A.

2.1.9 Complex I activity assay

Cell pellets containing approximately $1x10^6$ cells were harvested and submitted to 3 freeze-thaw cycles and dissolved in 100 µl of 25 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4. NADH oxidation was assessed

spectrophotometrically in a cuvette at 340nm in a dual beam JASCO V-660 spectrophotometer at room temperature. 20 µl of sample was added to 200 µl of reaction buffer containing 25 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4, 1 µM antimycin-A, 0.2 mM NADH, 0.4% BSA. A baseline rate in the absence of the substrate, ubiquinone, was first recorded for 2 minutes. Ubiquinone was then added to a final concentration of 70 µM and the rate of NADH oxidation recorded for 3 minutes. NADH oxidation is due to mitochondrial Complex I as well as other cellular NADH dehydrogenases. In order to determine Complex I activity, rotenone, an inhibitor of Complex I, was added to a final concentration of 2 µM and the rate determined for another 3 minutes. Complex I activity was calculated by subtracting the rotenone-insensitive NADH oxidation rate in its linear phase from the total NADH oxidation rate in its linear phase. The protein concentration was determined by the BCA method (Pierce, Rockford, IL).

2.1.10 Complex IV activity assay

Cell pellets containing approximately $1x10^6$ cells were collected and resuspended in 100 µl of 50 mM KH_2PO_4, pH 7.2, 1.5% dodecyl maltoside and were kept on ice for 30 minutes before centrifugation at 14,000g for 15 minutes at 4°C. The supernatants were transferred to new centrifuge tubes and the protein concentration determined by the BCA method (Pierce, Rockford, IL). Complex IV activity was determined spectrophotometrically in 96-well format in a Spectramax plate reader for 20 min at 550 nm in kinetic mode at room temperature. The reaction was started by adding 5 µg of protein to 200 µl/well of reaction solution containing 20 mM KH_2PO_4, pH 7.2, 0.015% dodecyl maltoside, 70 µM reduced cytochrome c. Complex IV activity was calculated in the linear phase.

2.1.11 Citrate synthase activity assay

Cell pellets containing approximately $1x10^6$ cells were collected and resuspended in 100 µl of 50 mM KH_2PO_4, pH 7.2, 1.5% dodecyl maltoside and were kept on ice for 30 minutes before centrifugation at 14,000g for 15 minutes at 4°C. The supernatants were transferred to new centrifuge tubes and the protein concentration determined by the BCA method (Pierce, Rockford, IL). Citrate synthase activity was determined spectrophotometrically in 96-well format in a Spectramax reader at 412 nm in kinetic mode at room temperature. The reaction in the absence of the substrate, oxaloacetate, was first determined for 7 minutes by adding 5 µg of protein to 200 µl/well of reaction solution containing 10 mM Tris-HCl, pH 8.1, 200 µM acetyl CoA, and 500 µM DTNB. Oxaloacetate was then added to a final concentration of 1 mM and the rate was measured for 30 minutes. Citrate synthase was calculated in the linear phase after subtracting the rate obtained in the absence of oxaloacetate from the rate obtained in the presence of oxaloacetate.

2.1.12 Statistical analysis

Statistical analysis was performed with GraphPrism (La Jolla, CA) and Microsoft Office Excel 2007. Data are presented as Means±SEM or ±SD. Statistical analysis was performed using one-way ANOVA followed by the Bonferroni Multiple Comparison test for inter-strain comparison at the same passage or 2-way ANOVA when comparing MEFs at passage 3 versus passage 10. For dose response experiments, a two-tail Student t-test was performed. A p value of <0.05 was considered significant.

2.2- SIRT3 and regulation of Doxorubicin-induced mitochondrial toxicity (Chapter 3.3)

2.2.1 Materials

Cell culture media and supplements were received from Invitrogen (Carlsbad, CA). Doxorubicin (DOX) hydrochloride, the In Vitro Toxicology Assay Kit Sulforhodamine B based and other chemical reagents were purchased from Sigma Aldrich (St. Louis, MO) and were of the highest purity available. The CellTiter-Glo® kit, the Caspase-Glo® 3/7 Assay and, MultiTox-Glo Multiplex Cytotoxicity Assay were purchased from Promega (Madison, WI). The 96-well white walled, opaque bottom plates used for luminescence readings were from Becton Dickinson (Bedford, MA). The RT-qPCR primers were obtained by Quiagen (Valencia, CA). The BCA Protein Assay Kit was from Pierce (Rockford, IL) and the SIRT3 antibody was from Cell Signaling (Danvers, MA).

2.2.2 Cell culture conditions

H9c2, rat heart myoblasts (ATCC® Number:CRL-1446), MRC-5, human lung fibroblasts (ATCC® Number:CCL-177, BJ, human skin fibroblasts (ATCC® Number:CRL-2522) cells were purchased from American Type Cell Culture (ATCC®, Manassas, VA) and were grown in DMEM and 10% fetal bovine serum (FBS) and 1%P/S in a humidified atmosphere at 37 °C, 5% CO_2. H9c2 and MRC-5 cells were treated with doxorubicin (1 μM) during 24 hours and either harvested for protein and/or mRNA extraction, or platted for functional studies. Human mammary fibroblasts (HMF) (Catalog Number: 7630) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and cell pellets were a gift from Dr. Vilma Sardão.

2.2.3 Transformation of competent cells and plasmid constructs purification

One Shot® TOP10 chemically competent *E. coli* (Life Technologies, Grand Island, NY) was used to transform cells, allowing high-efficiency cloning and plasmid propagation, according to the manufacturer's protocol. Then, the bacterial cells were harvested by centrifugation at 6,000g for 15 min at 4ºC and the supernatant was collected. After, purification step with the Hispeed Plasmid Midi kit (Qiagen, Valencia, CA) was performed by following the manufacturer's protocol. The DNA concentration was determined with a Nanodrop spectrophotometer (Thermo Scientific, Lanham, MD). The purity of the sample was also analyzed through the 260/280 ratio. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8.

2.2.4 SIRT3 overexpression in H9c2 cells

To evaluate the role of SIRT3 deacetylase, the wild type (hSIRT3–Flag), the deacetylase catalytically inactive constructs (hSIRT3–H248Y–HA) and empty vectors were overexpressed in H9c2 cells during 48h at 4μg of DNA/32μl of Fugene HD (Roche).

2.2.5 Gene expression analysis

Total RNA was isolated from H9c2 cells using RNeasy Mini Kit (QIAGEN). Reverse transcription of total RNA was performed using SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and ran on an Opticon2 DNA engine (Bio-Rad). All reactions were normalized using an

18S ribosomal endogenous control. Primers were purchased from predesigned QuantiTect primer assays (QIAGEN, Valencia, CA).

2.2.6 Western-blot analysis

Total protein from cells was extracted using RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.5% deoxycholic acid, 1% NP-40, 0.1% sodium dodecyl sulfate, and 0.5 M NaCl) supplemented with protease inhibitor cocktails and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). After denaturation at 95ºC for 5 min in a *Laemmli* sample loading buffer (from Bio-Rad) an equivalent amount of protein (20μg) was separated by 10% Tris-glycine gel (Invitrogen) and transferred to nitrocellulose membranes or PVDF membranes. After blocking with 5% milk in TBST (50mM Tris-HCl, pH 8; 154 mM NaCl and 0.1% tween 20) for 2 h at room temperature, membranes were incubated overnight at 4ºC with antibodies. The SIRT3 antibody was purchased from Cell Signaling (rabbit anti-SIRT3 1:1000 Catalog number: #2627) and the β-actin antibody was purchased by Sigma-Aldrich (rabbit anti-β-actin). The membranes were further incubated with a secondary antibody, goat anti-rabbit IgG (1:5000), during 2 hours at room temperature. Before blocking, the membranes were stained with Ponceau reagent to ensure the complete transfer of proteins from the gel to the membrane.

2.2.7 Intracellular ATP levels

H9c2 cells were seeded in 96-well white-walled, opaque bottom plates at a density of 40,000 cells/ml/well with cell culture medium after having been previously transfected for 24h. Then, the cells were treated with 1μM of DOX. Basal ATP levels were measured 24 hours after seeding using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's instructions.

2.2.8 Sulforhodamine B (SRB) colorimetric cytotoxicity assay

Transfected and non-transfected H9c2 cells were seeded at a density of 40,000 cells/ml and treated with 1 μM of doxorubicin during 24 hours. Cell growth was measured by the In Vitro Toxicology Assay Kit (Sulforhodamine B based - SRB) (Sigma Aldrich, St. Louis, MO), which measures the total biomass by staining cellular proteins with SRB. An increase or decrease in the number of cells (total biomass) results in a concomitant change in the amount of dye incorporated by the cells in culture, which is measured at 565 nm in a spectrophotometer, according to the manufacturer's instruction. MRC-5 cells were also plated at a density of 40,000 cells/ml in 24 well-plates and treated with DOX (1μM) during 24 hours and cell proliferation was assessed using the SRB assay as previously described.

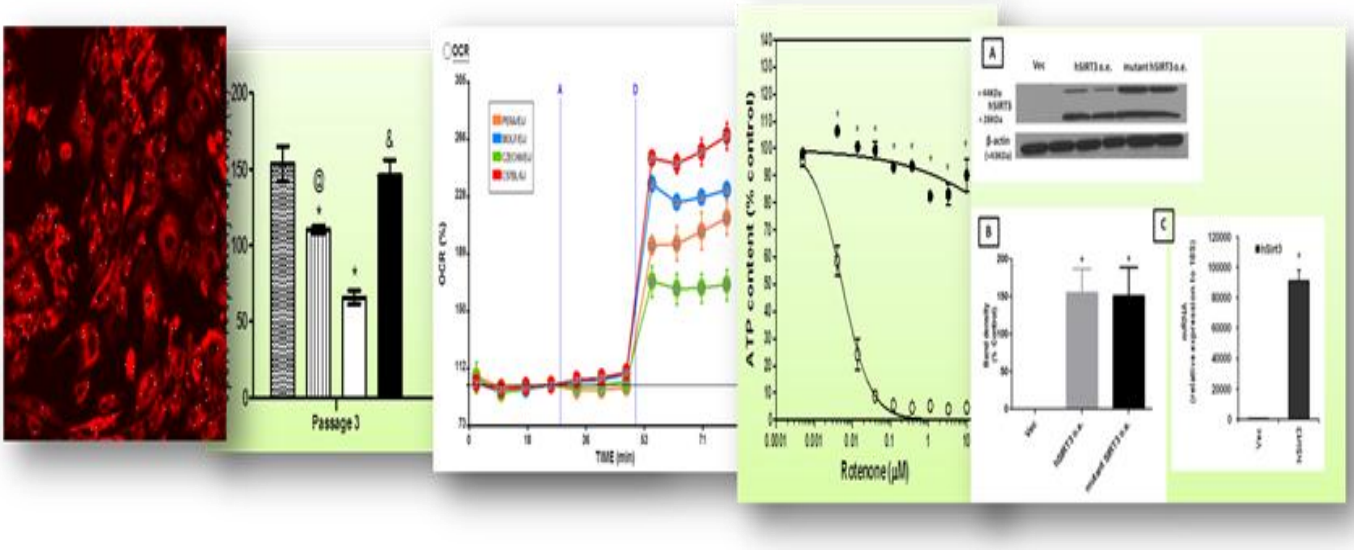
2.2.9 Caspase activity and cell death determination

H9c2 cells were seeded in 96-well white-walled opaque bottom plates at a density of 40, 000 cells/ml/well in suitable cell culture medium after having been previously transfected for 24h. Next, cells were treated with 1μM DOX. Caspase 3/7 activity and cell death were measured 24 hours after cell seeding by using the Caspase-Glo® 3/7 Assay and MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI), respectively, according to the manufacturer's instruction. The data from both cell death and caspase 3/7-like activity assays were normalized with the SRB results, %Cell deat/% Cell mass ratio and % Caspase activity/% Cell mass, respectively.

2.2.10 Statistical analysis

Statistical analysis was performed with GraphPrism. Data are presented as Means±SEM. Statistical analysis was performed using one-way ANOVA

followed by the Bonferroni Multiple Comparison test. For mRNA expression experiments, a two-tail Student t-test was performed. A p value of <0.05 was considered significant.


Passage 3
OCR
OCR (%)
TIME (min)
ATP content (% control)
Rotenone (μM)
A
B
C
Vec
hSIRT3 a.e.
mutant hSIRT3 a.e.
β-actin
hSirt3

3- Results

3.1. Interstrain differences in the bioenergetic profile of embryonic fibroblasts from four strains of mice with mtDNA single nucleotide polymorphisms

3.1.1 Summary

Mitochondrial DNA variations including single nucleotide polymorphisms (SNPs) have been proposed to be involved in idiosyncratic drug toxicity by affecting the bioenergetic capacity of cells. The existing *in vitro and in vivo* models used to evaluate compound toxicity are not suitable to predict idiosyncratic reactions since they lack the genetic diversity seen in the human population. Therefore, we introduce here a new framework that could be used to evaluate compound toxicity. The model involves the use of four strains of mouse embryonic fibroblasts (MEFs) with mtDNA polymorphisms. Our hypothesis is that different cell strains with distinct mtDNA SNPs may have different mitochondrial bioenergetic profiles and, therefore, can be used to investigate different predispositions to drug-induced toxicity. We sequenced mtDNA from embryonic fibroblasts isolated from C57BL/6J (the reference strain), MOLF/EiJ, CZECHII/EiJ and PERA/EiJ mice, with the latter two being sequenced for the first time. The mtDNA sequences suggest that PERA/EiJ is more closely related to C57BL/6J than to CZECHII/EiJ and MOLF/EiJ, while CZECHII/EiJ is closely related to MOLF/EiJ. The bioenergetic profile of the four strains of MEFs were investigated at passage 3 and also at passage 10, in order to determine differences between strains and differences caused by cell aging. Our results showed that there were clear differences among the four strains of MEFs at both passages, with CZECHII/EiJ having a lower mitochondrial robustness when compared to C57BL/6J, followed by

MOLF/EiJ and PERA/EiJ. We propose that this model is a useful starting point to study compounds that may cause idiosyncratic drug toxicity.

3.1.2 Introduction

Mitochondria are pivotal players in the development of drug toxicity, including drug-induced liver injury (DILI), due to their critical role in cell bioenergetics (Boelsterli and Lim, 2007). It has been hypothesized that mitochondrial DNA (mtDNA) alterations which may cause clinically silent abnormalities may predispose individuals to idiosyncratic DILI when exposed to certain drugs (Boelsterli and Lim, 2007). Episodes of drug-induced injury have been associated with the concept of a mtDNA threshold effect in which a phenotype resulting from accumulation of mutated mtDNA is not evident until a certain degree of heteroplasmy is observed, after which rapid mitochondrial degeneration occurs (Boelsterli and Lim, 2007). Thirteen proteins are encoded by mtDNA and all of them are subunits of the complexes involved in oxidative phosphorylation. In addition, mtDNA encodes 2 rRNAs and 22 tRNAs which are necessary for the synthesis of the 13 mtDNA-encoded proteins (Wallace and Fan, 2010). Mutations within some of the mtDNA-encoded genes have been known to cause serious diseases such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), and Leber hereditary optic neuropathy (LHON) (Gomez-Duran *et al.*, 2010; Wallace, 2010b). Nevertheless, not all nucleotide changes within mtDNA are harmful and, in fact, several mtDNA single nucleotide polymorphisms (SNPs) are known to occur within the human population (Wallace, 2010b). However, accumulation of mtDNA SNPs could, over time, lead to a decrease in the respiratory capacity of cells. If the accumulation of mtDNA SNPs is compounded with the effect of drugs that have off-target effects on mitochondrial function, this could lead to

mitochondrial bioenergetic impairment and hence to idiosyncratic drug reactions (Boelsterli and Lim, 2007).

Drugs that cause idiosyncratic drug reactions are difficult to identify in pre-clinical rodent models since these *in vivo* systems do not display the diverse genetic variation seen in the patient population. In an effort to overcome this limitation, a panel of 36 strains of genetically diverse mice was used to study the toxicity of acetominophen (Harrill *et al.*, 2009). In addition, a panel of inbred mouse strains has been used to study inter-individual variability in the metabolism of warfarin (Guo *et al.*, 2006). Furthermore, hepatocytes from 15 genetically different mouse strains have been used to evaluate strain-specific responses to different toxicants, such as acetominophen, WY-14,643 (a peroxisome proliferator activator receptor (PPAR) agonist) and rifampin (Martinez *et al.*, 2010).

The widespread use of the laboratory mouse in research is mainly due to the existence of a variety of inbred mouse strains derived by repeated inter-sibling mating, which also generates an everlasting population of genetic clones (Szatkiewicz *et al.*, 2008). Since mice within a single strain share the same genetic background, it is possible to compare genetic and phenotypic differences among different mouse strains (Svenson *et al.*, 2003). Furthermore, the existence of a definite set of genetic differences among inbred mouse strains allows one to explore the effect of genetic diversity on many phenotypes of interest (Wade and Daly, 2005). Much effort has been made to understand and characterize SNPs in inbred mouse strains ever since the nuclear genomic sequence of the C57BL/6J strain was reported (Frazer *et al.*, 2004; Szatkiewicz *et al.*, 2008).

To the best of our knowledge, no bioenergetic characterization has been performed on murine embryonic fibroblasts cells which vary in their mtDNA sequence. It is conceivable that cells isolated from mice which

differ in their mtDNA sequence may have different bioenergetic profiles and, therefore, vary in their response to drugs which cause mitochondrial impairment. To this end, the objective of this work was to compare the bioenergetic profile of embryonic fibroblasts harvested from four strains of mice, C57BL/6J, PERA/EiJ, CZECHII/EiJ and MOLF/EiJ. C57BL/6J is a common inbred mouse strain whereas PERA/EiJ, CZECHII/EiJ and MOLF/EiJ are wild-derived inbred mouse strains (Goios *et al.*, 2007). The published mtDNA sequence of MOLF/EiJ shows that it has 393 SNPs in comparison with that of C57BL/6J (Goios *et al.*, 2007). Since the mtDNA sequences of PERA/EiJ and CZECHII/EiJ have not been reported before, we sequenced the entire mtDNA of embryonic fibroblasts from these two strains of mice at passage 3. Furthermore, since cells may accumulate mutations in mtDNA with increased passage number, we also compared the mtDNA sequence of each of the four strains used in our study at passage 3 and passage 10. Although an unequivocal relationship between mtDNA SNPs and bioenergetic profiles is still hard to establish, the present work is a clear framework to further understand the relationship between idiosyncratic drug reactions and mitochondrial bioenergetic phenotypes generated by a combination of mtDNA SNPs.

3.1.3 Results

3.1.3.1 Mitochondrial DNA (mtDNA) from embryonic fibroblasts at passages 3 and 10 from four strains of mice had single nucleotide polymorphisms

Mitochondrial DNA (mtDNA) from embryonic fibroblasts at passage 3 and passage 10 from the mouse strains, C57BL/6J, PERA/EiJ, CZECHII/EiJ and MOLF/EiJ, was sequenced as described in Methods and Materials.

The mtDNA sequences of PERA/EiJ and CZECHII/EiJ have not been reported before.

The mtDNA sequence of C57BL/6J from passage 3 embryonic fibroblasts was identical to that of the published sequence of this strain (Goios *et al.*, 2007). Single nucleotide polymorphisms (SNPs) were detected in the mtDNA of fibroblasts from PERA/EiJ (106 SNPs), CZECHII/EiJ (390 SNPs) and MOLF/EiJ (393 SNPs) at passage 3 when compared with the published mtDNA sequence of the reference strain, C57BL/6J.

The mtDNA sequence of C57BL/6J fibroblasts at passage 10 was identical to that of C57BL/6J fibroblasts at passage 3. Similarly, the mtDNA sequence of PERA/EiJ fibroblasts and MOLF/EiJ fibroblasts at passage 10 was identical to that of PERA/EiJ fibroblasts and MOLF/EiJ fibroblasts at passage 3, respectively. In contrast, the mtDNA of CZECHII/EiJ fibroblasts at passage 10 had three more SNPs than the mtDNA of CZECHII/EiJ fibroblasts at passage 3. These extra SNPs were located in *mt-Nd5,* a gene which encodes a subunit of mitochondrial Complex I. However, since these 3 SNPs were synonymous nucleotide substitutions, there were no amino acid changes.

We next analyzed mtDNA SNPs that were nonsynonymous substitutions (ie SNPs that cause amino acid changes) in the 13 polypeptide-encoding genes that code for subunits of the mitochondrial respiratory chain. PERA/EiJ differed from C57BL/6J by nine amino acids (Table 3.1.1). Seven of these amino acids were in subunits of Complex I (ND1, ND4L, ND4 and ND5), one amino acid was in a subunit of Complex IV (COII) and one amino acid was in a subunit of Complex V (ATP synthase Fo subunit 6). CZECHII/EiJ differed from C57BL/6J by 35 amino acids (Table 3.1.1). Thirty of these amino acids were in subunits of Complex I

(ND1, ND2, ND3, ND4, ND4L, ND5 and ND6), one amino acid was in a subunit of Complex III (Cyt b), one amino acid was in a subunit of Complex IV (CO1), and three amino acids were in subunits of Complex V (ATP synthase Fo subunits 6 and 8). MOLF/EiJ differed from C57BL/6J by 34 amino acids (Table 3.1.1). Twenty nine of these amino acids were in subunits of Complex I (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6), two amino acids were in a subunit of Complex III (Cyt b), and three amino acids were in subunits of Complex V (ATP synthase Fo subunits 6 and 8). CZECH/EiJ and MOLF/EiJ had 27 amino acid changes in common when compared with C57BL/6J (Table 3.1.1). In contrast, only 3 amino acid changes found in PERA when compared with C57BL/6J were also found in CZECHII/EiJ and MOLF/EiJ. When compared with the C57BL/6J, the majority of amino acid changes that occurred in PERA/EiJ, CZECHII/EiJ and MOLF/EiJ occurred in subunits of Complex I.

Table 3.1.1 -Nonsynonymous substitutions found in the mtDNA polypeptide-encoding genes within the four strains of mouse embryonic fibroblasts (MEFs) at passage 3 and passage 10, and the corresponding amino acid changes.

mtDNA gene	Nucleotide substitution	Amino acid in C57BL/6J	Amino acid in PERA/EiJ	Amino acid in CZECHII/EiJ	Amino acid in MOLF/EiJ
ND1	G3207A	V	V	I	V
	A3220G	N	S	S	S
ND2	G3965A	V	V	M	M
	G4187A	G	G	S	S
	A4500T	Y	Y	F	Y
	G4550A	A	A	T	A
	C4551T	A	A	V	V
	A4581G	N	N	S	S
	A4596G	N	N	S	N
	T4884C	L	L	P	P
	C4947T	T	T	I	I
Co1	G6789A	A	A	T	A
Co2	G7505A	V	I	V	V

Atp8	C7807A	I	I	M	M
Atp6	C7977A	F	F	F	L
	G8074A	V	V	I	I
	A8131G	T	T	A	T
	A8224C	M	L	M	M
ND3	A9504G	T	T	A	A
ND4L	G9985A	V	M	M	M
ND4	T10246C	V	A	V	V
	A10432G	N	N	S	S
	A10464G	I	I	V	I
	T10525C	I	I	I	T
	A10597G	N	N	S	S
	A10605G	I	V	I	I
	T10707C	F	L	F	F
	A11037A	V	V	V	I
	G11217A	V	V	I	I
	A11249T	M	M	I	I
ND5	A11778G	I	I	V	V
	C11826T	H	H	Y	Y
	C11907T	H	H	Y	Y
	T12555C	F	F	F	L
	C12573T	L	L	F	F
	T12835C	I	T	T	T
	G13168A	S	S	N	N
	G13176A	V	V	I	I
	T13225C	V	V	A	V
	T13240C	I	T	I	I
	A13282G	N	N	S	S
	G13287C	A	A	P	P
ND5 (cont)	C13409A	I	I	I	M
	C13471T	T	T	T	M
ND6	A13757G	V	V	A	A
	C13760T	C	C	Y	Y
Cytb	G14259A	V	V	I	I
	A15027G	I	I	I	V

Nucleotide substitutions in genes encoding tRNAs, rRNAS and the D-loop are summarized in Tables 3.1.2, 3.1.3 and 3.1.4, respectively. In the tRNA

encoding genes, PERA/EiJ had 7 SNPs, CZECHII/EiJ had 15 SNPs, and MOLF/EiJ had 17 SNPs when compared with the reference strain, C57BL/6J (Table 3.1.2).

Table 3.1.2 - Nucleotide substitutions found in the mtDNA tRNA-encoding genes within the four MEF strains at passage 3 and passage 10.

mtDNA gene	Nucleotide position	Nucleotide in C57BL/6J	Nucleotide in PERA/EiJ	Nucleotide in CZECHII/EiJ	Nucleotide in MOLF/EiJ
tRNA-Phe	18	A	T	A	A
	55	G	C	T	T
tRNA-Val	1077	A	G	A	A
	1084	A	A	G	G
tRNA-Ile	3757	C	C	T	T
tRNA-Gln	3817	C	C	T	T
tRNA-Trp	4967	T	T	C	T
tRNA-Cys	5204	A	A	A	AA
tRNA-Tyr	5276	T	G	T	T
	5277	A	-	A	A
tRNA-Asp	6991	A	A	A	G
tRNA-Gly	9406	T	C	T	T
	9439	A	A	G	A
	9441	T	T	A	G
tRNA-Arg	9828	A	A	A	AA
	9858	G	G	A	A
tRNA-His	11606	A	A	G	G
tRNA-Ser_2	11650	G	A	G	G
tRNA-Glu	14088	C	C	T	T
tRNA-Thr	15295	A	A	G	G
	15304	A	A	G	G
	15311	T	T	T	C
	15339	C	C	A	A
tRNA-Pro	15369	G	G	A	A
	15400	T	T	C	C

In the two rRNA encoding genes, PERA/EiJ had 9 SNPs, CZECHII/EiJ had 29 SNPs and MOLF/EiJ had 27 SNPs when compared with C57BL/6J (Table 3.1.3).

Table 3.1.3 - Nucleotide substitutions found in the mtDNA rRNA-encoding genes within the four MEF strains at passage 3 and passage 10.

mtDNA gene	Nucleotide position	Nucleotide in C57BL/6J	Nucleotide in PERA/EiJ	Nucleotide in CZECHII/EiJ	Nucleotide in MOLF/EiJ
12S rRNA	109	A	A	G	G
	158	A	A	A	G
	165	A	A	C	C
	348	C	C	T	T
	350	T	T	C	T
	455	G	A	A	A
	456	T	C	T	T
	716	A	A	T	T
	817	C	C	T	T
16S rRNA	1248	G	G	A	G
	1493	A	A	AT	A
	1494	A	AA	AA	A
	1495	C	C	C	T
	1498	C	C	A	C
	1519	G	A	A	A
	1585	A	A	G	G
	1590	G	A	A	A
	1626	T	T	C	C
	1673	T	T	C	C
	1675	T	C	T	T
	1692	C	C	A	A
	1715	C	C	T	C
	1756	C	C	T	T
	1773	G	G	A	A
	1775	G	G	A	A
	1826	C	C	C	T

16S rRNA (cont)	1840	T	C	T	T
	1841	C	C	T	T
	1893	C	C	T	T
	2185	A	A	G	G
	2205	T	T	T	TT
	2238	T	T	A	A
	2340	G	A	A	A
	2344	T	T	C	C
	2525	C	C	T	T
	2647	T	T	C	T
	2656	C	C	C	T
	2661	A	T	A	A

In the D-loop, PERA/EiJ had 9 SNPs, while CZECHII/EiJ and MOLF/EiJ each had 34 SNPs when compared with C57BL/6J (Table 3.1.4). As in the case with the nonsynonymous substitutions occurring in the polypeptide-encoding genes, many of the SNPs found in the genes encoding tRNAs, rRNAs and the D-loop of CZECHII/EiJ were also found in MOLF/EiJ.

Table 3.1.4 - Nucleotide substitutions found in the mtDNA D-Loop region withi strains at passage 3 and passage 10.

mtDNA gene	Nucleotide position	Nucleotide in C57BL/6J	Nucleotide in PERA/EiJ	Nucleotide in CZECHII/EiJ	Nucleotide in MOLF/EiJ
D-Loop	15423	A	A	T	C
	15447	C	C	T	T
	15480	T	C	T	T
	15499	T	A	A	A
	15508	A	A	T	T
	15517	G	G	A	A
	15519	T	T	A	A
	15520	C	C	T	T
	15524	T	T	C	T
	15532	T	T	C	C

D-Loop (cont)	15536	C	C	T	T
	15548	T	T	C	C
	15549	C	C	T	T
	15550	A	A	G	G
	15552	T	T	C	T
	15554	T	T	C	C
	15556	T	T	T	A
	15558	T	C	T	-
	15559	A	A	A	-
	15573	C	C	A	A
	15574	T	T	C	C
	15578	A	A	T	-
	15579	C	C	C	T
	15584	T	T	C	C
	15588	C	C	T	T
	15594	T	C	C	C
	15596	A	A	T	T
	15600	G	G	A	A
	15603	C	T	C	C
	15687	A	A	G	A
	15917	C	T	T	T
	16099	A	A	A	AA
	16142	G	G	A	G
	16154	A	A	T	T
	16184	A	A	G	G
	16211	T	T	C	C
	16248	T	T	C	C
	16249	T	T	T	C
	16254	G	G	A	G
	16256	A	G	A	A
	16272	T	C	C	C
	16275	T	C	T	T
	16276	C	C	T	C
	16291	C	C	T	T

3.1.3.2 Cell growth and morphology of the four strains of mouse embryonic fibroblasts (MEFs) at passages 3 and 10

In order to characterize the cell growth of the embryonic fibroblasts from the four mouse strains, cell numbers were determined at three time-points (6h, 18h and 48h) post-seeding. The data was normalized to time zero (t0) which corresponds to a seeding density of 160, 000 cells/mL. Overall, at both passages 3 and 10, during the first 6 hours post-seeding, cell mass increased for all of the strains, after which cell growth slowed down between t = 6h and t = 18h, for all of the strains. After 48 hours, the cell growth showed differences between passages and among strains. At passage 3, 48 hours post-seeding, cell proliferation varied among the strains in the rank order, CZECHII/EiJ > C57BL/6J, MOLF/EiJ > PERA/EiJ (Fig 3.1.1 A), whereas at passage 10, the rank order was PERA/EiJ > CZECHII/EiJ > C57BL/6J > MOLF/EiJ (Fig 3.1.1 B).

The morphological features also differed from passage 3 (Fig 3.1.2, 1st column) to passage 10 (Fig 3.1.2, 3rd column). At passage 3, the cell population was more heterogeneous, there being different cell sizes and different shapes within the same strain (Fig 3.1.2, 1st column). Overall, PERA/EiJ had apparent smaller nuclei and a smaller cell size than the other strains when in a 2-D monolayer culture. At passage 10, the cell population was more homogeneous in all four strains (Fig 3.1.2, 3rd column), with PERA/EiJ showing an apparent smaller size and smaller nuclei when compared to the other strains.

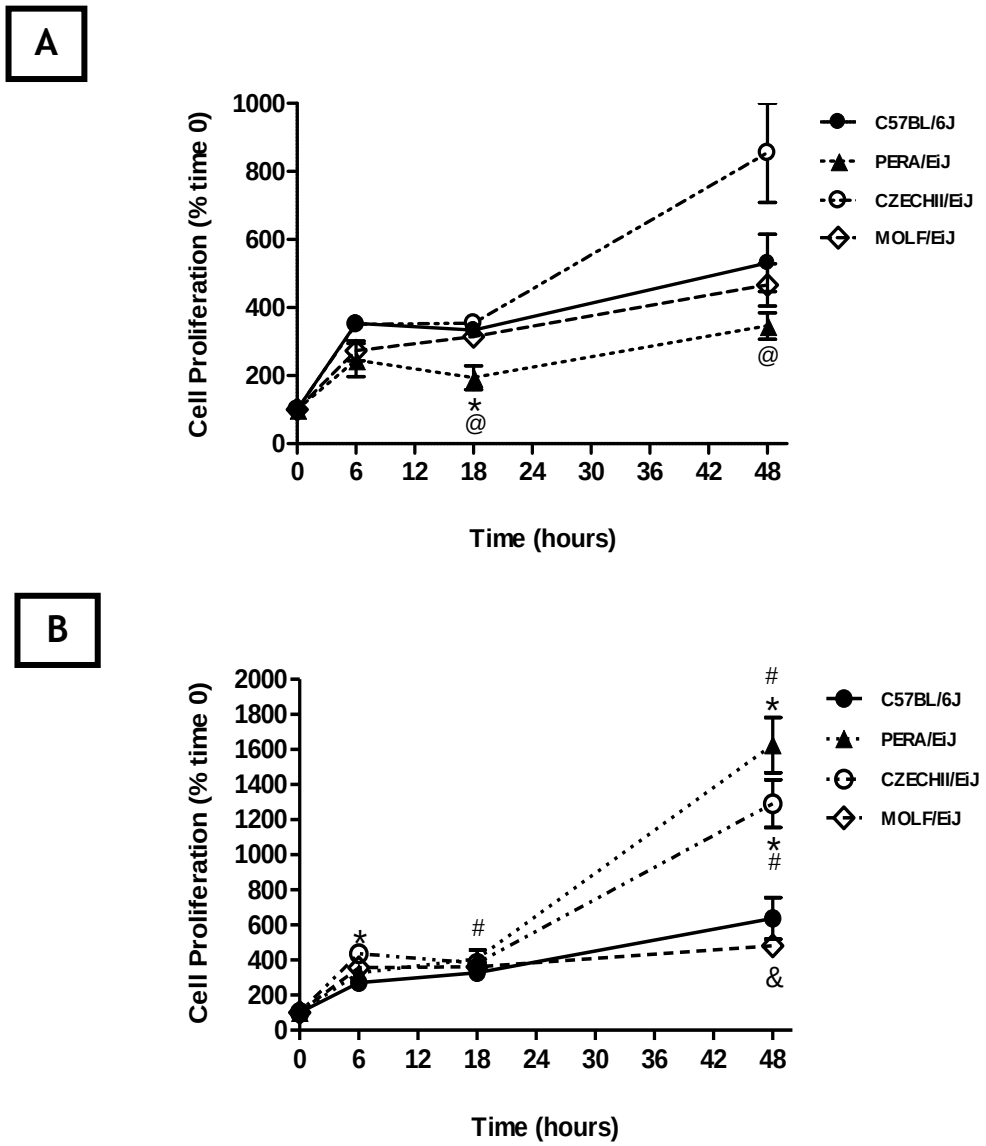


Fig. 3.1.1 - Cell growth curves of the four strains of MEFs at passages 3 and 10. Cells were seeded at a density of 160,000 cells/ml in cell culture medium. Cells were trypsinized and counted at 3 time points: 6, 18 and 48 hours. The data is expressed as % of time 0. **A)** The four strains of MEFs at passage 3. **B)** The four strains of MEFs at passage 10. Each data point represents Mean±SEM of 3 independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant. * versus. reference strain C57BL/6J at the same passage, # versus. same strain for passage 3, @ versus. CZECHII/EiJ, &versus. PERA/EiJ and CZECHII/EiJ.

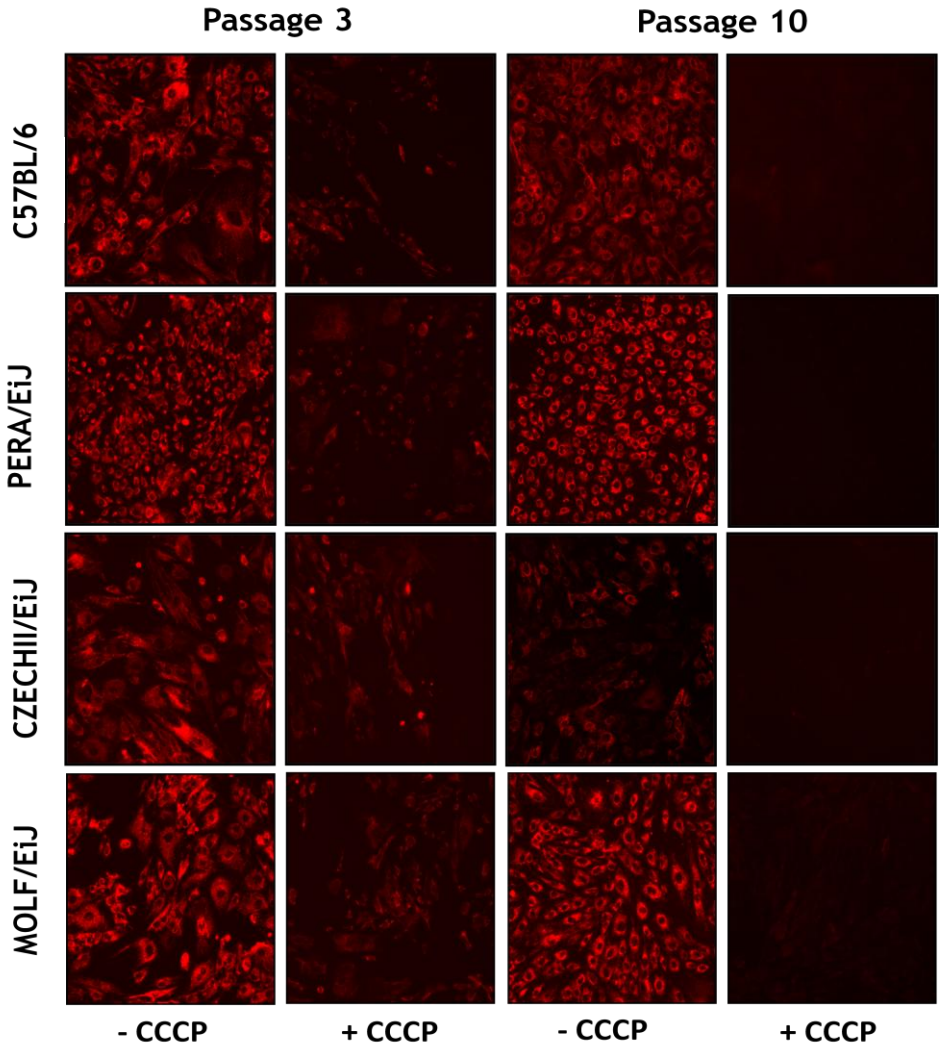


Fig. 3.1.2 - Measurement of mitochondrial membrane potential with the fluorescent probe TMRM. MEFs were seeded at 8,000 cells/100 µl/well in cell culture medium and incubated overnight. 75 µM of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was dissolved in cell culture medium, incubated with the cells for 4 hours and used as a positive control to depolarize the mitochondrial membrane potential. Cell culture medium supplemented with TMRM (200 nM) was then added to each well and the cells were incubated for a further 30 minutes. Automated live-cell image acquisition was performed on a Thermo Fisher Scientific Cellomics® ArrayScan® VTI High Content Screening Reader using a 10x objective after TMRM labeling (which is dependent on the mitochondrial membrane potential) for both passage 3 and passage 10.

3.1.3.3 Mitochondrial transmembrane electric potential and ATP content of the four strains of MEFs.

We assessed the mitochondrial membrane potential (mtΔΨ) of all four strains by using TMRM staining which was semi-quantified in terms of % relative to the reference strain, C57BL/6J, upon subtracting the fluorescence value after CCCP treatment and normalizing by citrate synthase activity. Fig 3.1.2 shows TMRM staining for all the strains at passages 3 and 10. At passage 3, PERA/EiJ was the cell strain with the highest mean mtΔΨ when compared to the other strains, being significantly different from the reference strain, C57BL/6J (around 1.4 fold higher) and MOLF/EiJ (Figure 3.1.3). CZECHII/EiJ also showed statistically significant higher mtΔΨ when compared to C57BL/6J (Figure 3.1.3). MOLF/EiJ showed the lowest mean value when compared to PERA/EiJ and CZECHII/EiJ for mtΔΨ although still significantly higher than C57BL/6J (Figure 3.1.3). At passage 10, PERA/EiJ mtΔΨ was significantly higher when compared with C57BL/6J and CZECHII/EiJ while MOLF/EiJ mtΔΨ was also significantly higher than C57BL/6J and also, significantly different from the other strains (Figure 3.1.3). In contrast to what was observed at passage 3, CZECHII/EiJ mtΔΨ was significantly lower than C57BL/6J at passage 10. From passage 3 to passage 10, PERA/EiJ showed a 1.4 fold increase in the mtΔΨ, while MOLF/EiJ showed a 1.2 fold increase. CZECHII/EiJ showed a 50% decrease in mtΔΨ (Fig. 3.1.3).

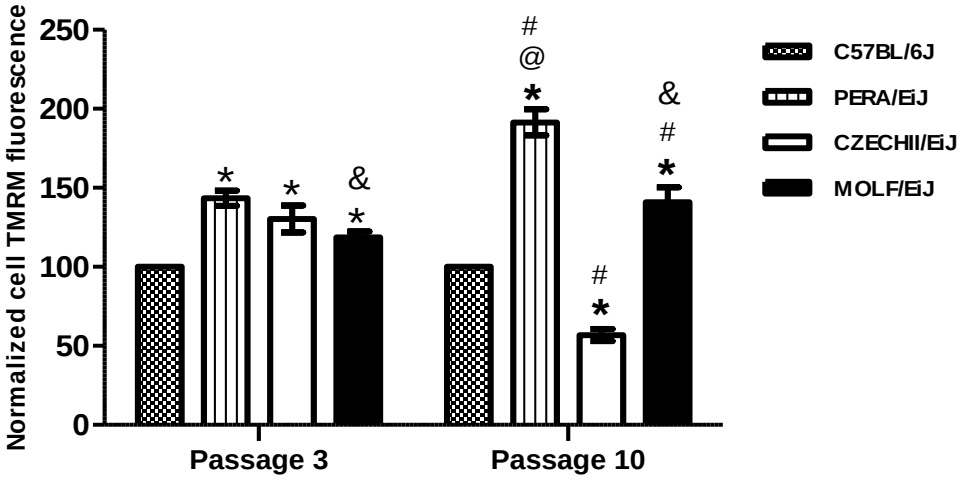
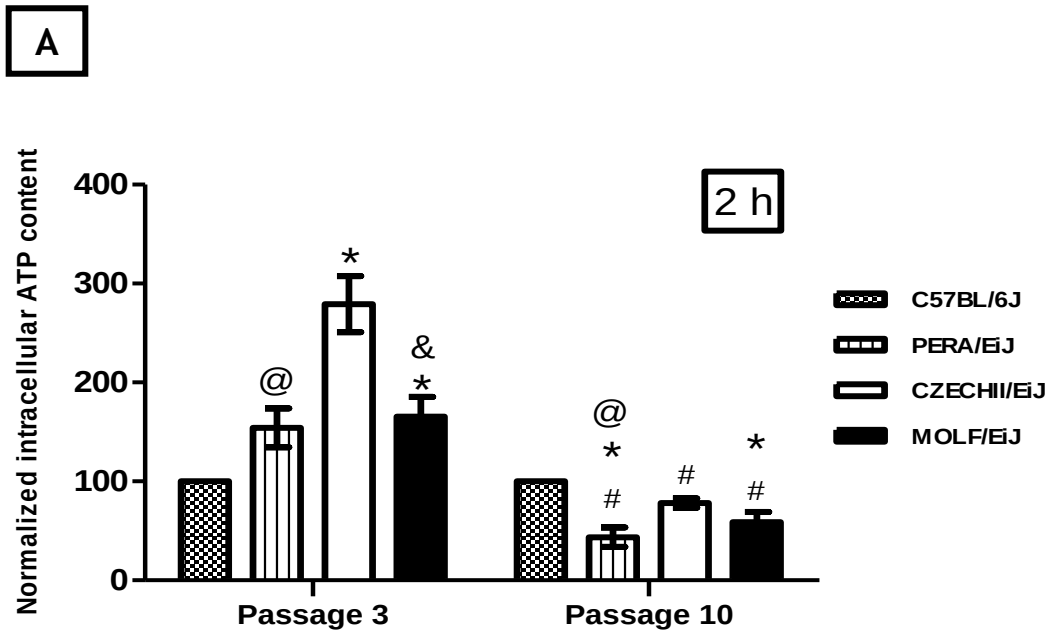


Fig. 3.1.3 - Quantitative analysis of mitochondrial membrane potential with the fluorescent probe TMRM. The bar graphs represent the CircSpot average intensity with TMRM divided by the citrate synthase activity of each strain and after normalization with CCCP. The data is presented as % relative to C57BL/6J, the reference strain. Each bar represents the Mean± SEM, N=3-5 independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 versus passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, # vs. same strain for passage 3, & vs. PERA/EiJ (passage 3 and 10) and CZECHII/EiJ (passage 10), @ vs. CZECHII/EiJ.

We next determined the ATP content of the MEFs at two different time points, 2h and 24h after seeding and compared the intracellular ATP at passages 3 and 10 (Fig 3.1.4A and B). The results were calculated as % of the ATP measured in the reference strain, C57BL/6J, and taking into account citrate synthase activity to normalize mitochondrial content. At passage 3, CZECHII/EiJ and MOLF/EiJ showed statistically significant higher ATP levels when compared with the reference strain, as measured 2 h after seeding. Furthermore, MOLF/EiJ and PERA/EiJ were significantly

different from CZECHII/EiJ. At passage 10, the pattern was different and PERA/EiJ and MOLF/EiJ had a statistically significant decrease in intracellular ATP levels when compared to the reference strain C57BL/6J and also, when compared to CZECHII/EiJ. At 24hours, the strains showed the same pattern described for passage 3. At passage 10, all three cell strains, MOLF/EiJ, PERA/EiJ and CZECHII/EiJ had decreased ATP content when compared with the reference strain. When comparing passage 10 with passage 3, MOLF/EiJ, PERA/EiJ and CZECHII/EiJ strains were statistically different at the two different time points.



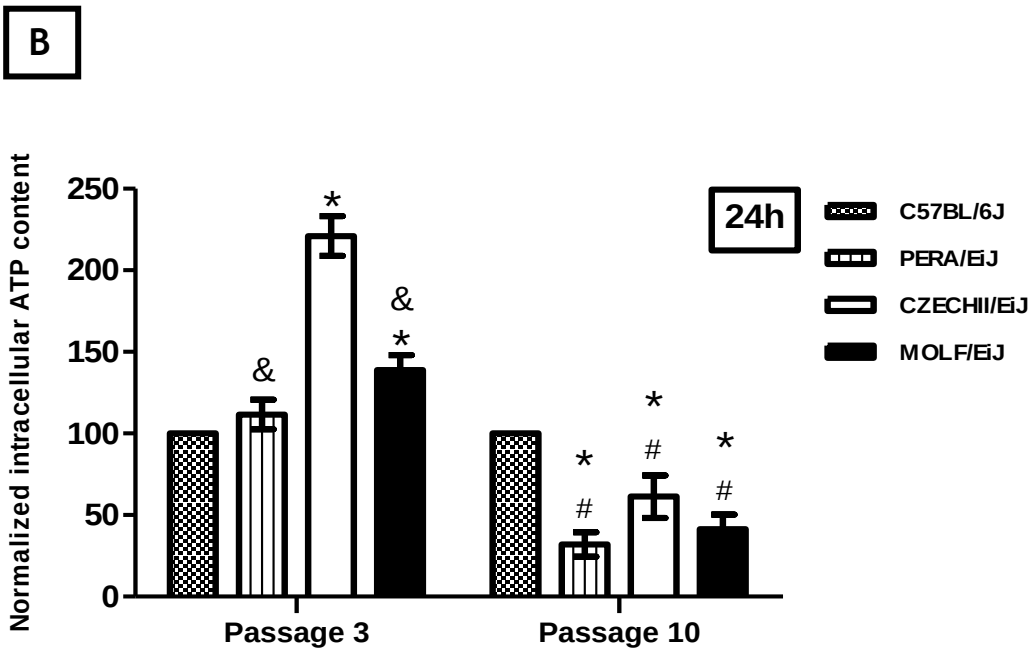


Fig. 3.1.4 - Cellular ATP levels of four strains of MEFs, in two different passages. MEFs were seeded in 96-well white opaque plates at 8,000 cells/well in 100 µl in cell culture medium. Basal ATP levels were determined at 2 (A) and 24 hours (B) after seeding, for both passages 3 and 10. The data is presented as % relative to C57BL/6J, the reference strain and normalized with the citrate synthase activity. Each bar represents the Mean ± SEM, N=5-7 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant. For 2h: * vs. reference strain C57BL/6J at the same passage, #vs. same strain for passage 3, & vs. CZECHII/EiJ, @ vs. CZECHII/EiJ (passage 3 and 10). For 24h: * vs. reference strain C57BL/6J at the same passage, # vs. same strain for passage 3, & vs. CZECHII/EiJ.

3.1.3.4 Bioenergetics profile of the four MEFs strains at passages 3 and 10

We next characterized the cellular respiration profile of all four strains of MEFs at passage 3 and passage 10. In order to compare the mitochondrial

bioenergetics of the four MEF strains used, the ATP-linked respiration, proton-leak, coupling efficiency, maximum respiratory capacity, spare respiratory capacity, and non-mitochondrial respiration were determined at passages 3 and 10 using the XF96 extracellular flux analyzer. The percentage change in the oxygen consumption rate (OCR) upon addition of compound was calculated considering that the baseline OCR (ie the OCR just before compound addition) was 0%.

To assess the component of basal oxygen consumption that is used for ATP synthesis (ATP-linked respiration), oligomycin was injected into the samples to inhibit the ATP synthase. At passage 3, there were no differences among the four strains (Table 3.1.5). At passage 10, PERA/EiJ showed a statistically higher ATP-linked respiration when compared with the other strains (Table 3.1.5). Furthermore, comparison of the ATP-linked respiration for each strain between passage 3 and passage 10 showed that there were no differences between cell passages, with the exception of PERA/EiJ, which showed increased ATP-linked respiration from passage 3 to passage 10 (Table 3.1.5). The proton leak for all the strains was similar at passage 3. At passage 10, MOLF/EiJ had the highest proton leak among the four strains and was statistically significantly different from PERA/EiJ which had the lowest proton leak (Table 3.1.5). Comparison of the proton leak for each strain between passage 3 and passage 10 showed that there were no differences except for PERA/EiJ which had a statistically significant lower proton leak at passage 10 than at passage 3 (Table 3.1.5). The coupling efficiency was determined by calculating the ratio between the ATP-linked respiration and mitochondrial respiration (ie the ATP-linked respiration plus the proton leak). At passage 3, no significant differences in the coupling efficiency were found among the strains (Table 3.1.5). At passage 10, PERA/EiJ showed significantly higher coupling efficiency when

compared with C57BL/6J and MOLF/EiJ. Comparison of the coupling efficiency for each strain between passage 3 and passage 10 showed that PERA/EiJ was significantly higher at passage 10 than at passage 3 (Table 3.1.5). Non-mitochondrial respiration was similar for all of the strains at passage 3. At passage 10, PERA/EiJ had the lowest non-mitochondrial respiration and was statistically significantly different from CZECHII/EiJ which had the highest non-mitochondrial respiration among the four strains (Table 3.1.5). Comparison of the non-mitochondrial respiration for each strain between passage 3 and passage 10 showed that there were no significant differences.

Table 3.1.5 - Bioenergetic parameters of the four MEF strains at passage 3 and passage 10.

Strains / Bioenergetic parameters	C57BL/6J		PERA/EiJ		CZECHII/EiJ		MOLF/EiJ	
Passage number	**3**	**10**	**3**	**10**	**3**	**10**	**3**	**10**
Mitochondrial respiration (% of basal respiration)	84.0 ±0.7	80.0 ±1.0	81.0 ±1.2	85 ±1.0@	77.7 ±2.8	75 ±1.0	85 ±1.3	81 ±2.0

ATP-linked respiration (% of basal respiration)	63.0± 2.0	62.0 ±1.0	59.0 ±2.0	70.0 ±1.0*#@	61.0 ±3.0	58.2 ±3.0	64.0 ±2.0	62.0 ±2.0
Proton-leak (% of basal respiration)	21.0 ±1.0	18.0 ±0.2	22.0 ±2.0	15.0 ±0.5#	16.7 ±2.0	16.8 ±2.0	21.0 ±2.0	19.0 ±1.0&
Non-mitochondrial respiration (% of basal respiration)	16.0 ±1.0	20.0 ±1.0	19.0 ±1.0	15.0 ±1.0@	22.3 ±2.0	25.0 ±2.0	15.0 ±1.0	19.0 ±2.0

Data is represented as Mean ± SEM, N = 5 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test to compare strains within each passage. A 2-way ANOVA was performed to compare strains between passage 3 and passage 10. $p<0.05$ is considered statistically significant: * vs. reference strain C57BL/6J at the same passage. & vs. PERA/EiJ (passage 10). @ vs CZECHII/EiJ (passage 10). # vs MOLF (passage 10).

The spare respiratory capacity (or reserve capacity) was determined by measuring the maximum uncoupled respiration upon FCCP injection (Fig. 3.1.5A, B, and C). Titration with FCCP showed that C57BL/6J, CZECHII/EiJ and MOLF/EiJ showed maximum uncoupling with 1 μM FCCP, whereas PERA/EiJ showed maximal uncoupling with 0.5 μM FCCP (Figure 3.1.6). Overall, at both cell passages 3 and 10, PERA/EiJ and CZECHII/EiJ showed a significantly lower spare respiratory capacity when compared to C57BL/6J. In addition, PERA/EiJ showed an even lower spare respiratory capacity at passage 10 (Fig 3.1.5C) than at passage 3 (Fig 3.1.5B), this decrease being statistically significant.

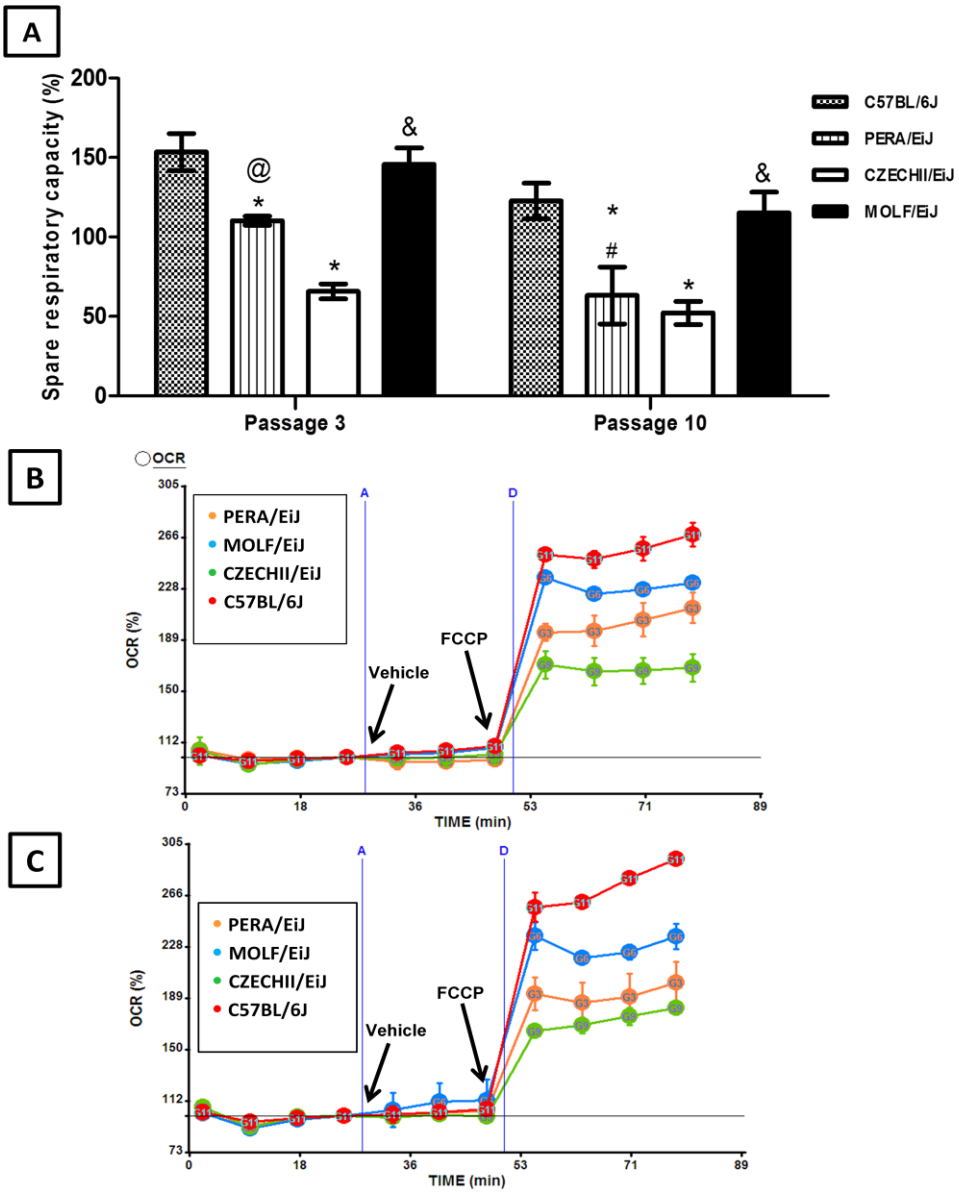


Fig. 3.1.5 - Spare respiratory capacity of the four strains of MEFs at passages 3 and 10. The spare respiratory capacity (or reserve capacity) was determined by measuring the maximum uncoupled respiration upon FCCP injection. **A)** Spare respiratory capacity at passage 3 and passage 10. Each bar represents the Mean ± SEM, N=4-5 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, # vs. same strain for passage 3, & vs. CZECHII/EiJ (passage 3 and 10), @ vs. CZECHII/EiJ (passage 3). Representative recordings of the percentage change in the oxygen consumption rate of the four strains of MEFs at passage 3 and passage 10 after FCCP injection are shown in Fig 5 **B)** and Fig 4 **C)**, respectively.

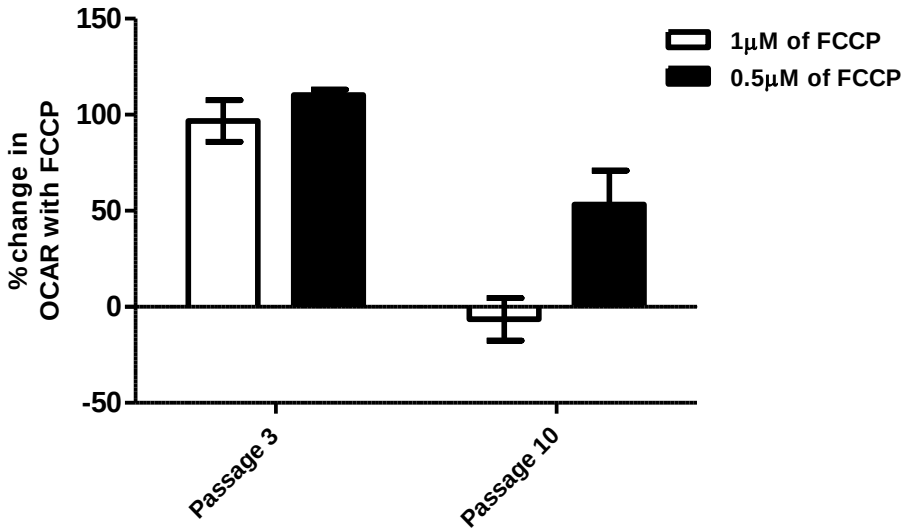


Fig. 3.1.6 - FCCP titration in PERA/EiJ at both passages 3 and 10. The data represents the % change in the OCR after the injection of FCCP (1 and 0.5 μM). Each bar represents the Mean ±SEM, N=4-5 separate experiments.

The maximum respiratory capacity is the sum of the (a) respiration that results in proton leak and (b) the respiration that occurs upon sequential addition of oligomycin and FCCP. At passage 3, PERA/EiJ and CZECHII/EiJ showed significantly lower maximum respiratory capacity when compared with both the reference strain and MOLF/EiJ (Fig 3.1.7 A and B). At passage 10, the maximum respiratory capacity of PERA/EiJ, CZECHII/EiJ and MOLF/EiJ were significantly lower than that of C57BL/6J, in the order, PERA/EiJ, CZECHII/EiJ, < MOLF/EiJ < C57BL/6J (Fig 3.1.7 A and 3.1.7 C). No significant differences between passages within each strain were observed (Fig. 3.1.7A).

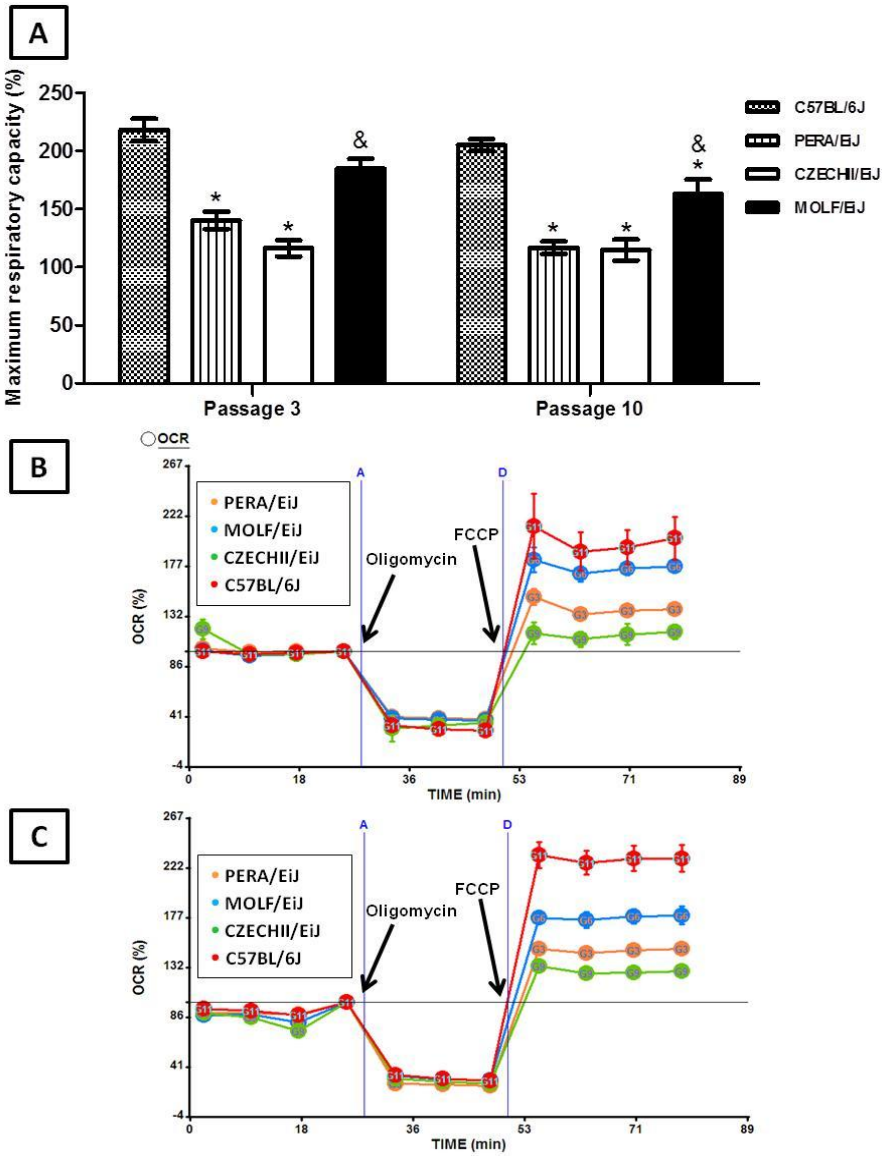


Fig. 3.1.7- Maximum respiratory capacity of the four strains of MEFs at passages 3 and 10. The maximum respiratory capacity is the sum of the respiration that results in proton leak and the respiration that occurs upon sequential addition of oligomycin and FCCP. **A)** Maximum respiratory capacity at passage 3 and passage 10. Each bar represents the Mean±SEM, N=4-5 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, & vs. PERA/EiJ and CZECHII/EiJ (passage 3 and 10). Representative recordings of the percentage change in the oxygen consumption rate of the four strains of MEFs at passage 3 and passage 10 following sequential addition of oligomycin and FCCP are shown in Fig 3.1.7 **B)** and 3.1.7 **C)**, respectively.

In order to detect variations in the OCR caused by respiratory chain inhibitors, we used rotenone and antimycin-A, complex I and complex III inhibitors, respectively. Rotenone (1 μM final concentration) and antimycin-A (1 μM final concentration), each reduced the OCR by ~70-78% in the MEFs from each of the strains, at both passages 3 and 10, with no inter-strain or inter-passage differences (Figure 3.1.8).

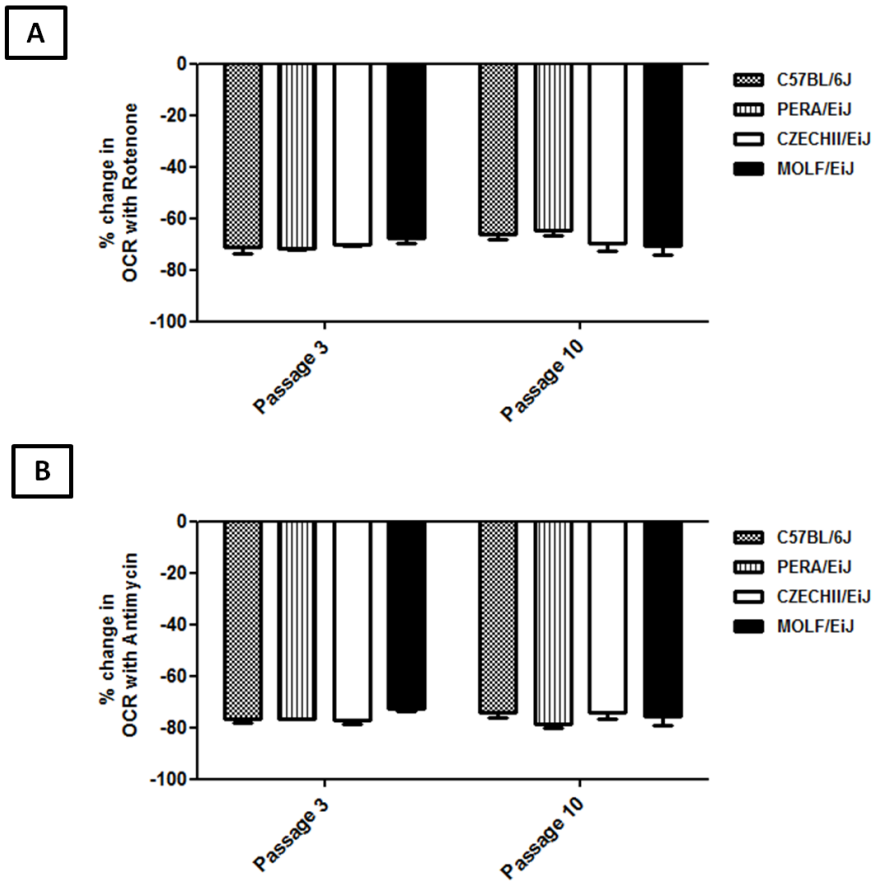


Fig. 3.1.8 - Effects of rotenone and antimycin-A, complex I and III inhibitors, respectively, on the OCR of the four strains of MEFs at passages 3 and 10. The data represents the % change in the OCR after the injection of rotenone (1 μM) **(A)** and antimycin-A (1 μM) **(B)**, respectively. Each bar represents the Mean±SEM, N=3-5 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. There were no statistically significant differences observed.

We also tested the effect of the glycolytic inhibitor, 2-deoxy-D-glucose (2-DG), on the OCR of the cells. Inhibition of glycolysis should increase the OCR of cells since they are forced to rely on oxidative phosphorylation for their ATP demands. At passage 3, C57BL/6J and PERA/EiJ increased their basal OCR by 54% and 53%, respectively (Figure 9), while MOLF/EiJ and CZECHII/EiJ were significantly less responsive to 2-DG, showing only a 33% and 32% increase in their basal OCR, respectively. At passage 10, the response to 2-DG was decreased in all of the strains although this decrease was not statistically significant between strains due to data variability (Figure 3.1.9). Furthermore, C57BL/6J and CZECHII/EiJ showed a significantly decreased response to 2-DG from passage 3 to 10.

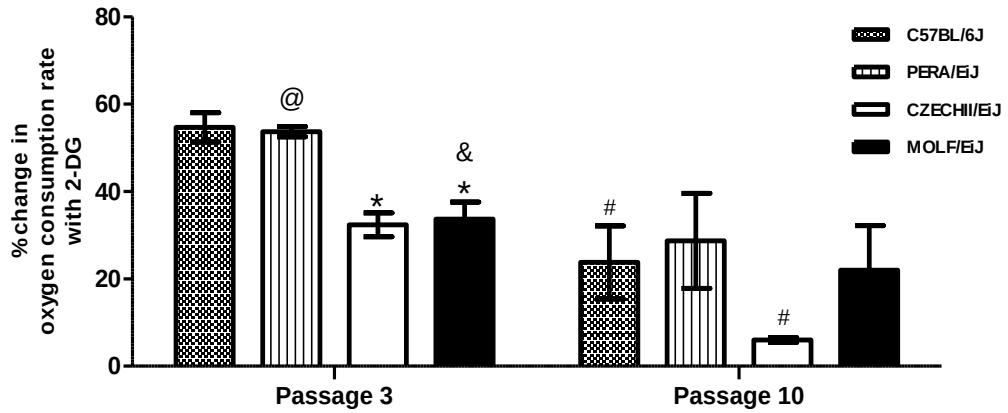


Fig. 3.1.9 - Effects of the glycolysis inhibitor, 2-deoxyglucose (2-DG), on the OCR of the four strains of MEFs at passages 3 and 10. The data represents the % change in the OCR after the injection of 2-DG (75mM). Each bar represents the Mean±SEM, N=3-5 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, # vs. same strain for passage 3, & vs. PERA/EiJ (passage 3), @ vs. CZECHII/EiJ (passage 3).

3.1.3.5 Complex I, Complex IV and citrate synthase activity in the four strains of MEFs.

Complex I activity of the MEFs from the four strains was determined since some of the mtDNA SNPs were found in genes encoding subunits of Complex I (see section 3.1.2). At passage 3, there were no differences between the strains in Complex I activity with the exception of PERA/EiJ which was statistically significantly higher than that of both C57BL/6J and CZECHII/EiJ (Fig 3.1.10A). At passage 10, both CZECHII/EiJ and MOLF/EiJ showed a statistically significant decrease in Complex I activity when compared to both C57BL/6J and PERA/EiJ (Fig 3.1.10B). Comparison of each strain between passage 3 and passage 10 showed that only MOLF/EiJ showed a significant decrease in the enzymatic activity of Complex I, while the other strains did not show any significant inter-passage differences.

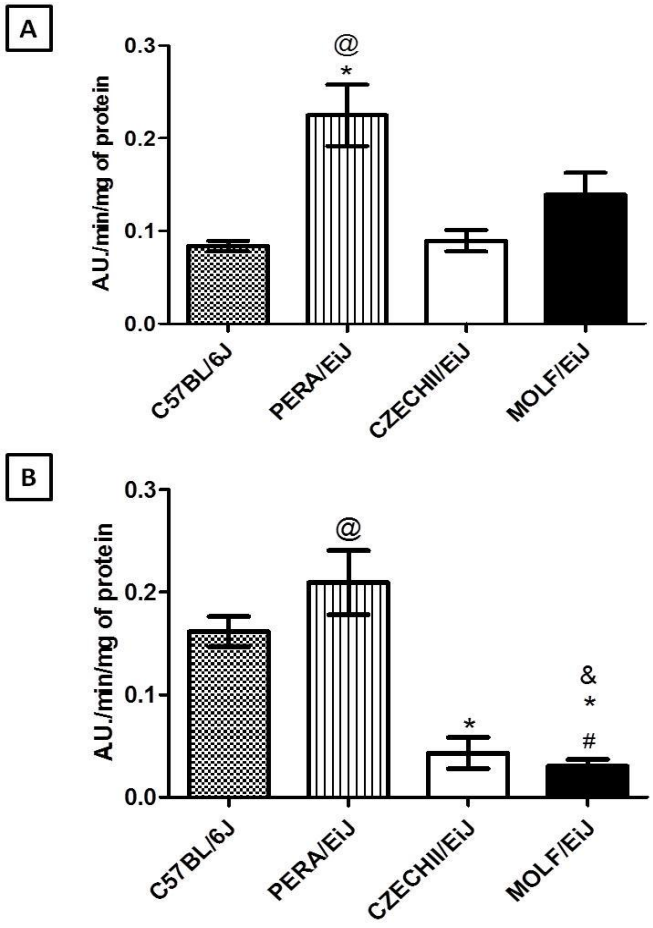


Fig. 3.1.10 - Complex I activity of the four strains of MEFs at passages 3 and 10. Complex I activity was measured as rotenone-dependent NADH oxidation, and was assessed spectrophotometrically as described in Methods and Materials. The bar graphs represent Complex I activity at passage 3 (Fig 3.1.10A) and at passage 10 (Fig 3.1.10B). Each bar represents the Mean ±SEM, N=3-5 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, # vs. same strain for passage 3, @ vs. CZECHII/EiJ (passage 3 and 10), & vs. PERA/EiJ (passage 10).

Complex IV activity of the MEFs from the four strains was also determined since some of the mtDNA SNPs were found in genes encoding subunits from Complex IV (see section 3.1.2) Overall, there were no significant differences in the complex IV activity among the strains at both passage 3 (Fig. 3.1.11A) and passage 10 (Fig. 3.1.11B) and when comparing each strain between passages.

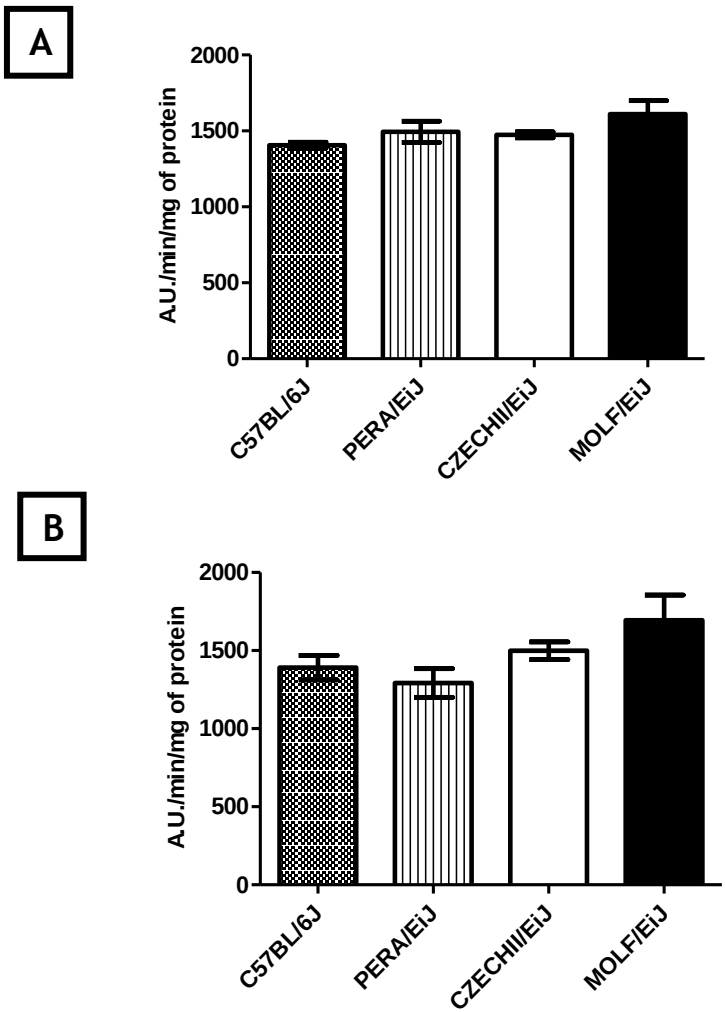


Fig. 3.1.11 - Complex IV activity of the four strains of MEFs at passages 3 and 10. Complex IV activity was determined spectrophotometrically as described in Methods and Materials. The data represents complex IV activity at passage 3 (A) and passage 10 (B). Each bar represents the Mean ± SEM, N=3-4 separate experiments.

Citrate synthase activity of the MEFs was also determined for all of the strains at both passages and used as a quantative marker of mitochondrial content. The results indicated that CZECHII/EiJ showed significantly lower citrate synthase activity at passage 3 (Fig. 2.1.12A) when compared to C57BL/6J and MOLF/EiJ. At passage 10 (Fig. 3.1.12 B), there were no statistically significant differences in the citrate synthase activity of PERA/EiJ, CZECHII/EiJ and MOLF/EiJ when compared to C57BL/6J. However, PERA/EiJ was significantly lower than MOLF/EiJ and CZECHII/EiJ. Comparison of each strain between passage 3 and passage 10 showed that C57BL/6J had a statistically significant lower citrate synthase activity at passage 10 than at passage 3, whereas CZECHII/EiJ showed a significantly higher activity at passage 10 than at passage 3. After determining citrate synthase activity, the ratios of Complex I/citrate synthase activities (CI/CS) and Complex IV/citrate synthase activities (CIV/CS) were also determined (Table 3.1.6). At passage 3, PERA/EiJ showed significantly higher CI/CS, when compared to C57BL/6J and CZECHII/EiJ. At passage 10, only MOLF/EiJ showed a statistically significantly lower CI/CS when compared to C57BL/EiJ and PERA/EiJ, even though CZECHII/EiJ CI/CS was also very low and significantly different from PERA/EiJ. CIV/CS was significantly higher for all of the strains at passage 3 while at passage 10, no differences were observed among the strains. CZECHII/EiJ CIV/CS was significantly lower at passage 10 when compared to passage 3 (Table 3.1.6).

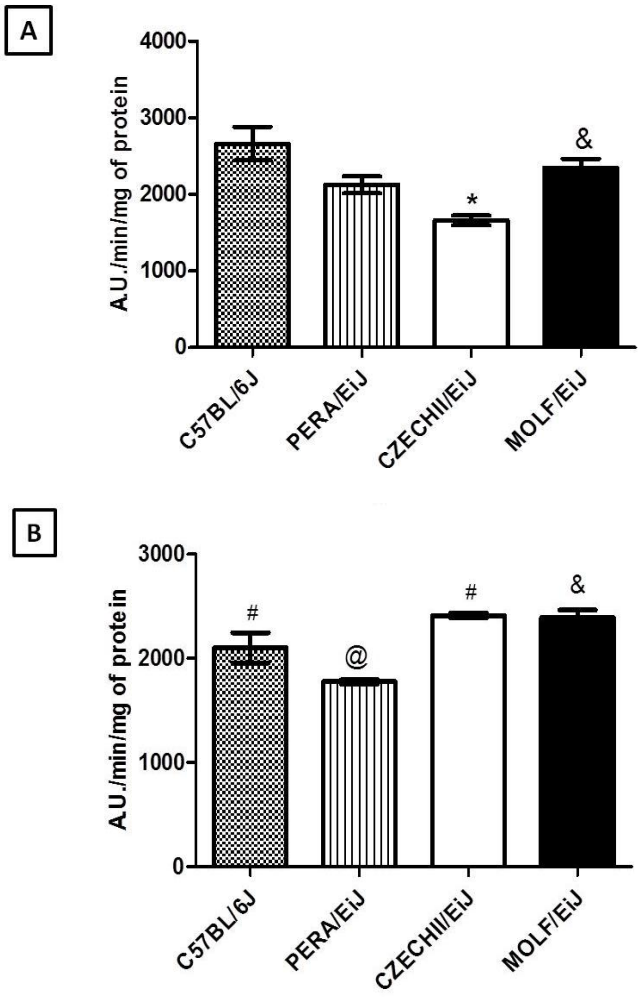


Fig. 3.1.12 - Citrate synthase activity of the four strains of MEFs at passages 3 and 10. Citrate synthase activity was determined spectrophotometrically as described in Methods and Materials. The bar graphs represent the citrate synthase activity at passage 3 (**A)** and passage 10 (**B).** Each bar represents the Mean± SEM, N=3-4 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, # vs. same strain for passage 3, & vs. CZECHII/EiJ (passage 3) and PERA/EiJ (passage 10), @ vs. CZECHII/EiJ (passage 10).

Table 3.1.6 - Normalization of Complex I and IV activities with citrate synthase activity of 4 strains MEFs at passage 3 and 10.

Strains/passage	C57 #3	C57 #10	PERA #3	PERA #10	CZE #3	CZE #10	MOLF #3	MOLF #10
ComplexI / citrate synthase (%)	0.0031 ±0.0002	0.0079 ±0.0007	0.0106 ±0.0016*@	0.0106 ±0.0018@	0.0054 ±0.0007	0.0018 ±0.0006	0.0059 ±0.0010	0.0017 ±0.0005*&
Complex IV / citrate synthase (%)	53.4649 ±3.9224	75.9871 ±2.6000	75.8174 ±2.6345*@	67.6519 ±2.1401	92.2838 ±1.7620*	61.3162 ±2.5886#	68.6087 ±2.3183*@	66.8948 ±8.09059

Data are means ± SEM of 3-5 independent experiments. All the presented values were multiplied by 100. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test and 2-way ANOVA in order to compare passage 3 vs passage 10. $p<0.05$ is considered statistically significant, * vs. reference strain C57BL/6J at the same passage, & vs. PERA/EiJ (passage 10), @ vs CZECHII/EiJ (passage 3 and 10). Abbreviations: C57- C57BL, PERA- PERA/EiJ, CZE- CZECHII, MOLF- MOLF/EiJ, #-number.

3.1.4 Discussion

Genetic or acquired mitochondrial deficiencies have been suggested to be factors that contribute to increased susceptibility to drug-induced toxicity that may trigger idiosyncratic responses (Boelsterli and Lim, 2007). The genetic drift between a mostly normal mtDNA population and an increased heteroplasmy caused by accumulation of mutated mtDNA within a cell (DiMauro and Schon, 2003) may lead to the appearance of a drug toxicity phenotype over time.

So far, there are no validated and appropriate animal models to study idiosyncratic drug responses that can be translated to humans. The studies are usually done in only one or two strains of animals and therefore the

animal models do not represent the genetic diversity that is seen in the human population. Hence, identifying drugs that cause idiosyncratic responses has been difficult. For this reason, a valid model which could simulate the heterogeneous genetic background existent in a human population must be developed to predict drug safety and different drug responses. An *in vitro* approach using a suitable biological model mimicking a genetically diverse population has the advantage of reducing the costs that *in vivo* studies entail (O'Shea *et al.*, 2011). Furthermore, there is still a large gap in our understanding of the role of mtDNA SNPs and their relationship with mitochondrial bioenergetics and how this can translate into altered mitochondrial drug liabilities.

In an effort to create a new and suitable model to study idiosyncratic drug responses, we characterized the mitochondrial bioenergetic parameters of embryonic fibroblasts isolated from four different strains of mice, C57BL/6J, PERA/EiJ, CZECHII/EiJ and MOLF/EiJ. These strains of mice have distinct mtDNA polymorphisms that could potentially simulate different sub-populations of mice that could later be extrapolated for different human genetic pools. The studied strains were chosen since they are part of the Mouse Phenome Database project collection (Maddatu *et al.*, 2012). The mtDNA sequences of C57BL/6J and MOLF/EiJ have been reported previously (Goios *et al.*, 2007). Our results showed that the mtDNA sequence of the fibroblasts, at passage 3, of the strains, C57BL/6J and MOLF/EiJ, were identical to that found by Goios *et al* (2007) (Goios *et al.*, 2007). The mtDNA of PERA/EiJ and CZECHII/EiJ were sequenced for the first time in our study. We then performed a comparative study of mtDNA SNPs in PERA/EiJ, CZECHII/EiJ and MOLF/EiJ, using C57BL/6J as the reference strain. Our results showed that, at passage 3, mtDNA from fibroblasts of PERA/EiJ had 106 SNPs including 9

nonsynonymous substitutions, CZECHII/EiJ had 390 SNPs including 35 nonsynonymous substitutions, and MOLF/EiJ had 393 SNPs including 34 nonsynonymous substitutions when compared with C57BL/6J (Tables 3.1.1-4). These results suggest that PERA/EiJ is more closely related to C57BL/6J than are either CZECHII/EiJ or MOLF/EiJ. In addition, many of the SNPs found in CZECHII/EiJ were also found in MOLF/EiJ, suggesting that these two strains may be phylogenetically related. Overall, the majority of SNPs which caused amino acid changes occurred in genes encoding subunits of Complex I, which has been described as the most affected respiratory chain enzyme during mitochondrial diseases (Wallace, 2010b).

Nonsynonymous substitutions in the polypeptide-encoding genes cause amino acid changes which could potentially affect the function of Complex I, III, IV and V. Furthermore, SNPs in the rRNA, tRNA and D-loop regions have also been reported to be associated with human diseases (Wallace *et al.*, 1988b). For instance, the mtDNA SNP, A3243G, is associated with diabetes and affects the $tRNA^{leu}$ gene in humans (Maassen *et al.*, 2006). In addition, when present in at high levels within a cell, this SNP can often lead to MELAS (Wallace, 1999). Furthermore, the mtDNA SNP, A1555G, in the 12S rRNA gene has been reported to be associated with cases of familial progressive deafness and non-syndromic hearing loss (Ferraris *et al.*, 2002; Pacheu-Grau *et al.*, 2011). Other studies using transmitochondrial cybrid lines (identical nuclear brackground) reported that 15 transformants derived from symptomatic individuals carrying the A1555G mutation have lower oxygen consumption rates and lower mitochondrial proteins synthesis when compared to control transformants (Guan *et al.*, 2001). In addition, the substitution of T to C in the position 16186 in the D-loop region has been reported to be associated with diverse

multifactorial disorders such as metabolic syndrome, dilated cardiomyopathy, and lacunar cerebral infarction (Mueller *et al.*, 2011). Moreover, the role of synonymous substitutions (SNPs which do not result in a change in the amino acid in the translated protein, due to the degeneracy of the genetic code) was recently revised (Sauna and Kimchi-Sarfaty, 2011). The importance of these silent mutations emerged with the evidence that these mutations can result in aberrant mRNA slicing, which can lead to human disease (Sauna and Kimchi-Sarfaty, 2011).

The second part of our study was aimed at characterizing the bioenergetic profile of the four strains of MEFs. We measured the OCR (oxygen consumption rate) of the MEFs, determined their mitochondrial membrane potential and cellular ATP levels, measured the activity of Complex I, Complex IV and citrate synthase in cell lysates, and also investigated alterations in cell proliferation. A summary of the main findings of our study are shown in Table 3.1.7.

When measuring the various components of oxygen consumption (eg ATP-linked respiration, proton leak etc) of the MEFs, we did not consider the *absolute* values of basal respiration of each strain. In accordance with a recent report by Brand and Nicholls (Brand and Nicholls, 2011), comparing absolute values of respiration between cells of different strains poses challenges since different strains can proliferate at different rates or may have different morphologies. For instance, if cells double in size but remain the same number, the respiration rate per cell will double, but the respiration rate per unit of cell protein or per nucleus or per amount of DNA may remain the same (Brand and Nicholls, 2011). Our results showed that the different strains of MEFs proliferated at different rates (Fig 3.1.1 A and 1B) and had different morphologies (Fig 3.1.2). Hence, the endpoints, ATP-linked respiration, proton leak, non-mitochondrial respiration,

maximum respiratory capacity and spare respiratory capacity, were measured as percentages of basal respiration for each strain.

The spare respiratory capacity of cells can be defined as the ability of substrate supply and electron transport to respond to an increase in energy demand (Yadava and Nicholls, 2007; Hill *et al.*, 2009; Brand and Nicholls, 2011). The maximum respiratory capacity of cells indicates the maximal speed at which the respiratory chain can operate. Substrate supply and respiratory chain electron transfer being the limiting steps. When determining the spare respiratory capacity and the maximum respiratory capacity, addition of the uncoupler, FCCP, is required. When using FCCP, one should titrate it carefully, in order to add the minimum amount necessary to induce fully uncontrolled respiration, without causing inhibition of respiration (Brand and Nicholls, 2011). We found that a concentration of 0.5 μM FCCP was enough to achieve maximum uncoupling in MEFs of PERA/EiJ, whereas 1 μM FCCP was necessary for the other strains. Our study showed that both PERA/EiJ and CZECHII/EiJ had a decreased maximum respiratory capacity and a decreased spare respiratory capacity at passage 3 as well as at passage 10 when compared to the reference strain, C57BL/6J, indicating that under the conditions of our experiments, their mitochondria operate closer to their bioenergetic limit.

Surprisingly, although PERA/EiJ MEFs had a lower maximum respiratory capacity and a lower spare respiratory capacity than the reference strain, at both passages, this strain also showed higher ATP-linked respiration and a higher coupling efficiency in comparison with the reference strain, at passage 10. Furthermore, the proton leak of this strain was lower at passage 10. Using oligomycin to determine ATP turnover or ATP-linked respiration results in slight hyperpolarization due to blockage of proton reflux through

the ATP synthase. Since the proton-leak is voltage-dependent and this approach stimulates proton influx, the ATP synthesis is underestimated (Brand and Nicholls, 2011). Given that oligomycin induces a collapse of ATP synthesis, cells increase their glycolysis rate by ~10-fold in order to sustain survival (Brand and Nicholls, 2011). Therefore, a change in the basal rate caused by a change in ATP turnover is most likely to be a response of mitochondria to altered ATP demand elsewhere in the cell. This unexpected observation also correlates with the fact that PERA/EiJ had a higher coupling efficiency and a lower proton leak at passage 10, as mentioned previously. A modest alteration in the proton leak might be a possible change in the mitochondrial transmembrane potential caused by substrate oxidation, although we cannot exclude that membrane alterations may account for differences in the passive proton influx. Therefore, these differences in the ATP-linked respiration, coupling efficiency and proton-leak are likely due to normal cellular adjustments to substrate oxidation and ATP demands in the cells. Moreover, we also did not find any differences regarding non-mitochondrial respiration, which mainly results from cell surface oxygen consumption, peroxisomes and substrate oxidation (Abe *et al.*, 2010).

We also found that there were no differences between the strains with respect to the decrease in OCR after addition of either rotenone, a complex I inhibitor, or antimycin-A, a complex III inhibitor. It is possible that these cells would reveal more differences if they had been grown in a galactose/glutamine-containing medium (Pelicano *et al.*, 2006). Other cell types such as HepG2 cells are more susceptible to drug-induced mitochondrial dysfunction when grown in a galactose/glutamine-containing medium since they are forced to use oxidative phosphorylation for making ATP (Marroquin *et al.*, 2007). Therefore, future studies with these four

MEF strains could be performed in different cell media, such as galactose/glutamine-containing medium or fatty acid-enriched medium, in order to explore the underlying mechanisms of drug-induced toxicity and metabolism. In the present work, we also inhibited glycolysis with the glucose analogue, 2-DG. Our results suggested that C57BL/6J and PERA/EiJ were more reliant on mitochondria than on glycolysis for their ATP production in comparison with CZECHII/EiJ and MOLF/EiJ (Figure 3.1.9). Interestingly, at passage 10, all four strains of MEFs were less able to increase their mitochondrial respiration. This could be due to decreased substrate delivery or decreased electron transfer.

Another experimental end-point we measured was the mitochondrial membrane potential of the MEFs at passages 3 and 10, taking into account their citrate synthase activity to normalize for mitochondrial content. We found that at passage 3, all strains had higher TMRM fluorescence per citrate synthase activity than C57BL/6J. At passage 10, both PERA/EiJ and MOLF/EiJ were more polarized than CZECHII/EiJ and C57BL/6J. Fibroblasts of CZECHII/EiJ at passage 10 showed a significant decrease in the mitochondrial membrane potential (depolarization) when compared with the other strains, which is in accordance with a possible lower mitochondrial activity, already described above. There are many factors that regulate mitochondrial polarization. For example, a decrease in mitochondrial $\Delta\Psi$ (mt$\Delta\Psi$) can have different causes: a) increase in energy demand and supply can result in decreased mt$\Delta\Psi$ through increased activity of the mitochondrial ATP synthase, b) decreased flux of electrons through the respiratory chain and concomitant decreased proton ejection and c) higher proton influx through the ATP synthase. This excludes, of course, the possibility that different cells may have different abilities to load fluorescent probes, which we do not believe is the case in our assays. When

we measured intracellular ATP levels, PERA/EiJ and MOLF/EiJ had a lower ATP content at passage 10 when compared with the other two strains. Although other factors may account for this, the observation can be evidence that ATP production is decreased in these two strains and hence the protonmotive gradient is not being used. The measurement of intracellular ATP does not accurately report mitochondrial function (Brand and Nicholls, 2011), since there are several mechanisms that can alter the intracellular ATP. If cellular ATP/ADP ratio decreases, the adenylate kinase activity consumes ADP and increases AMP. If the decrease is sustained, AMP degradation by AMP deaminase slows and the pool size may rise (Schwenke *et al.*, 1981). Therefore, it is difficult to understand if an alteration in the ATP content in cells is a reflection of the mitochondrial function or simply an independent change in the metabolism of adenine nucleotides.

Citrate synthase is a crucial pace-making enzyme in the first step of Krebs cycle and a quantitative marker of mitochondrial content (Bayer *et al.*, 1981). Only CZECHII/EiJ showed a decreased citrate synthase activity at passage 3, whereas at passage 10, there was no statistically significant difference. Therefore, this information was important to normalize the data shown in this work, in order to establish a biologically relevant comparison of the mitochondrial function of these four murine cell strains.

We also determined Complex I and Complex IV activities. Generally, rotenone-sensitive complex I activity is very hard to determine in fibroblasts because of the amount of contaminating enzymes which makes the assessment of this enzyme activity very difficult (Janssen *et al.*, 2007). Nevertheless, Complex I deficiencies are a major cause of mitochondrial dysfunction and consequently a decrease in Complex I activity is a strong indicator of a possible mitochondrial dysfunction (Janssen *et al.*, 2007). Our

results showed that PERA/EiJ had increased Complex I activity and CI/CS ratio when compared to C57BL/6J and CZECHII/EiJ at passage 3. Interestingly, both MOLF/EiJ and CZECHII/EiJ, which were also the strains with a higher rate of non-synonymous mutations at subunits of Complex I, showed lower Complex I activity and CI/CS ratio, at passage 10 (Table 3.1.6 and Fig 3.1.10) when compared to the other strains, suggesting a decrease in mitochondrial capacity. In contrast, Complex IV/citrate synthase activity was significantly higher for all of the strains when compared to the reference strain at passage 3, while no differences existed at passage 10 (Table 3.1.6 and Fig 3.1.11). This result might be due to a slightly variable citrate synthase activity/expression among the strains. Despite the fact that the Complex IV/citrate synthase ratio in C57BL/6J was lower than that for the other strains at passage 3, the respiratory measurements did not reveal lower respiratory capacity when compared to the other strains.

An aim of our study was to investigate the role of aging in cell culture. We found that, at passage 10, all four strains of MEFs were less capable of maintaining respiration when glycolysis was inhibited by 2-DG, suggesting that the cells became more glycolytic and/or were less capable of using the OXPHOS to produce ATP at the higher cell passage (Figure 3.1.9). A decrease in the activity of the respiratory chain or a decrease in the ability to shuttle substrates into mitochondria may have been reasons for these observations. We also observed that, in general, intracellular ATP levels decreased with aging in culture for most of the MEF strains (Figure 3.1.4). This may indicate altered ability to produce ATP or increased ATP consumption. We also found that MOLF/EiJ MEFs had a lower maximum respiratory capacity at passage 10 and lower complex I activity *per se* than at passage 3 while PERA/EiJ MEFs had a lower spare respiratory capacity at

passage 10 than at passage 3, showing a lower capacity to respond to an increase of energy demand when aged in culture. The citrate synthase activity decreased for C57BL/6J and increased for CZECHII/EiJ. Since CS is normally used as a mitochondrial marker (Bayer *et al.*, 1981), we can speculate that citrate synthase activity may be a *bona fide* indication of mitochondrial mass. The increased CS activity in CZECHII/EiJ may result from increased mitochondrial proliferation in response to a mitochondrial bioenergetic deficit found in this strain, which is suggested by decreased CIV/CS (Table 3.1.6) and membrane potential (Fig. 3.1.3) at passage 10 when compared with passage 3. For a summary regarding passage 3 versus passage 10 comparison results see Table 3.1.7. Despite the differences in some of the bioenergetic parameters between passage 3 and passage 10, comparison of the mtDNA sequences of each strain of MEFs between the two passages showed that there no differences with C57BL/6J, PERA/EiJ and MOLF/EiJ. Furthermore, the three extra SNPs that we found in CZECHII/EiJ MEFs at passage 10 did not cause amino acid changes. Overall, our mtDNA SNP data showed that the number of passages was not sufficient to create many SNPs. Our data also indicated that mtDNA SNPs cannot be the reason for the differences seen in the functional endpoints between passage 3 and passage 10. This suggests that other factors such as oxidative damage to mitochondrial components, SNPs in nuclear DNA-encoded mitochondrial proteins or decreased nuclear-mitochondrial crosstalk may have to be taken into account (Michikawa *et al.*, 1999; Seibel, 2008; Battersby, 2003 ; Aliev *et al.*, 2011). Nevertheless, it is clear that cell aging in culture should be taken in account before using the present model for bioenergetic or drug safety assessment studies.

One of the challenges in correlating altered mitochondrial bioenergetics with mtDNA SNPs is that confounding factors such as SNPs in nuclear

DNA-encoded genes that encode subunits of Complex I, III, IV and V, are present. For instance, there are 5 SNPs in the nuclear gene that codes for the Complex I subunit NDUFA1 and 3 SNPs in the nuclear gene that codes for the Complex I subunit NDUFA6 in MOLF/EiJ as well as in CZECHII/EiJ when compared to C57BL/6J (http://cgd.jax.org/cgdsnpdb/).

Despite these challenges, our study can be the backbone of an *in vitro* platform to test various drugs and predict toxicity among different sub-populations of cells with variable number of SNPs in both mitochondrial and nuclear DNA. The present study also characterizes, for the first time, the embryonic fibroblast bioenergetic profile of four distinct mouse strains, which can be a reference for further studies and concluded that they present differential mitochondrial fitness.

Unpredictable drug-induced mitochondrial toxicity is currently a major reason for attrition of compounds in the developmental stages and for the post-market drug withdrawals. This study conveys a valuable starting-point to the beginning of a new era where personalized medical care is emerging and individual susceptibilities should be taken into consideration when developing a new drug.

Table 3.1.7 - A summary of the main findings of this study, mtDNA alterations versus bioenergetics profile of fours strains of mouse embryonic fibroblasts at passage 3 and 10.

	Passage 3				Strains	Passage 10			
	C57BL/6J[2]	PERA/EiJ[2]	CZECHII/EiJ[2]	MOLF/EiJ[2]		C57BL/6J[2]	PERA/EiJ[2]	CZECHII/EiJ[2]	MOLF/EiJ[2]
SNPs/Non-synonymous mutations	>		<	<	PERA/EiJ[1]	>		<	<
	>	>		<	CZECHII/EiJ[1]	>	>		>
	>	>	>		MOLF/EiJ[1]	>	>	>	
Mitochondrial membrane potential	>			>	PERA/EiJ[1]	>	#	>	>
	>				CZECHII/EiJ[1]			#	
	>	<			MOLF/EiJ[1]	<			#
Intracellular ATP (24h)			<		PERA/EiJ[1]	<	#		
	>	>		>	CZECHII/EiJ[1]	<		#	
			<		MOLF/EiJ[1]				
Spare respiratory capacity	<		>		PERA/EiJ[1]	<	#		
	<	<		<	CZECHII/EiJ[1]	<		<	
			>		MOLF/EiJ[1]			>	
Maximum respiratory capacity	<			<	PERA/EiJ[1]	<			<
	<			<	CZECHII/EiJ[1]	<			<
		>	>		MOLF/EiJ[1]	<	>	>	
ATP-linked respiration					PERA/EiJ[1]	>	#	>	>
					CZECHII/EiJ[1]		<		
					MOLF/EiJ[1]		<		
Coupling Efficiency					PERA/EiJ[1]	>	#		>
					CZECHII/EiJ[1]				
					MOLF/EiJ[1]		<		
Proton leak					PERA/EiJ[1]		#		<
					CZECHII/EiJ[1]				
					MOLF/EiJ[1]				>
2-DG response*					PERA/EiJ[1]				
	<	<			CZECHII/EiJ[1]			#	
	<	<			MOLF/EiJ[1]				
Complex I activity	>		>		PERA/EiJ[1]				
					CZECHII/EiJ[1]	<	<		
					MOLF/EiJ[1]	<	<		#
Citrate synthase activity*					PERA/EiJ[1]			<	<
	<			<	CZECHII/EiJ[1]		>	#	
			>		MOLF/EiJ[1]		>		
CI/CS	>		>		PERA/EiJ[1]			>	>
		<			CZECHII/EiJ[1]		<		
					MOLF/EiJ[1]	<	<		
CIV/CS	>		<	<	PERA/EiJ[1]				
	>	>			CZECHII/EiJ[1]			#	
	>	>			MOLF/EiJ[1]				
Cell proliferation (48h)					PERA/EiJ[1]	>	#		>
		>			CZECHII/EiJ[1]	>		#	>
					MOLF/EiJ[1]		<	<	

[1] [1] [2] [1] Read the table from Strain to Strain . For instance, PERA/EiJ spare respiratory capacity is higher (>) than CZECHII/EiJ for passage 3 and PERA/EiJ has lower (<) spare respiratory capacity than C57BL/6J at passage 10. Legend: vs- versus, #- passage 10 ≠ passage 3, *- C57BL/6J at passage 10 ≠ from passage 3. In this table, only the major and statistically significant differences are indicated.

Acknowledgments

This part of work had the collaboration of Dr. David Gebhard (Pfizer R&D) for advice on the live cell image analysis, Dr. Sashi Nadanaciva (Pfizer R&D) for general collaboration during the work and Dr. Payal Rana (Pfizer R&D) for general collaboration as well.
This part of work also had the collaboration of Beckman Coulter Genomics Inc. (Danvers, MA) for mtDNA sequencing.

3.2 Drug-induced Toxicity in a Panel of Mouse Embryonic Fibroblasts with Mitochondrial DNA Single Nucleotide Polymorphisms

3.2.1 Introduction

Mitochondria are pivotal players in the development of drug toxicity, due to their critical role in cell bioenergetics (Boelsterli and Lim, 2007). Mitochondrial dysfunction has been implicated in the toxicity of tolcapone, nefazodone and flutamide (Boelsterli and Lim, 2007; Dykens *et al.*, 2008), which are known to also induce drug liver injury (DILI). Mitochondrial DNA (mtDNA) variations including single nucleotide polymorphisms (SNPs) have been proposed to be involved in idiosyncratic drug reactions. Current *in vitro and in vivo* models to evaluate compound toxicity are not suitable to predict mitochondrial off-target toxicity, lacking the genetic diversity in the human population. Previously (see Chapter 3.1), we showed that there were clear differences among the four strains of MEFs at both passages, with CZECHII/EiJ having a lower mitochondrial robustness when compared to C57BL/6J, followed by MOLF/EiJ and PERA/EiJ. Our hypothesis is that different cell strains with distinct mtDNA SNPs and different mitochondrial bioenergetic profiles can be used to investigate drug-induced toxicity. Therefore, we have used the same *in vitro* system composed of four strains of MEFs with mtDNA polymorphisms, previously characterized. Several drugs known to impair mitochondrial function were tested such as rotenone, nefazodone, tolcapone, flutamide, tamoxifen, imipramine, troglitazone and ketoconazole (Fig.3.2.1 and table 3.2.1).

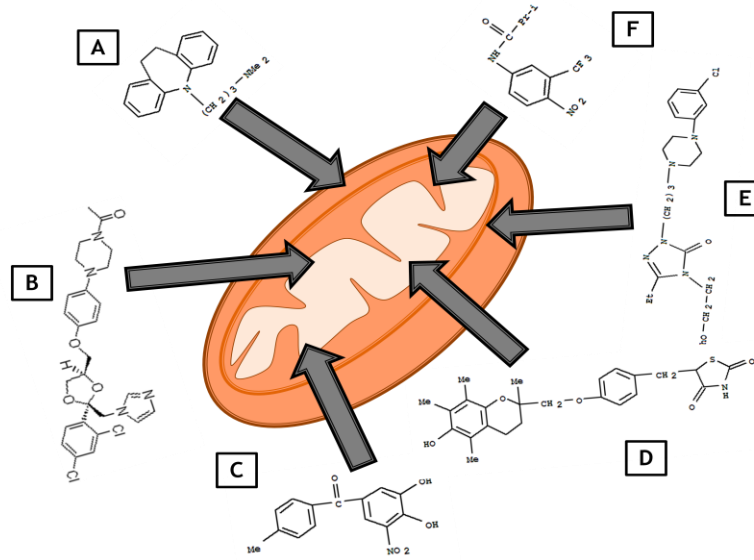


Figure 3.2.1 - Structure of the compounds nefazodone (A), ketoconazole (B), tolcapone (C), flutamide (D), tamoxifen (E), imipramine (F) and troglitazone (H) targeting mitochondria (Chemical structures were taken from http://www.lookchem.com/).

Table 3.2.1 -A summary of the main mitochondrial toxicity mechanisms of tested drugs.

Drug	Classification	Treatment	Mitochondrial Toxicity	References
Flutamide	Nonsteroidal anti-androgen	Metastatic prostate cancer	Decreases aconitase activity Complex I inhibitor Complex I/III inhibitor	(Coe *et al.*, 2007),(Kashimshetty *et al.*, 2009)
Tolcapone	Catechol-O-methyl transferase inhibitor	Adjuvant treatment to L-dopa in Parkinson's disease	Mitochondrial uncoupler, disrupts the mitochondrial membrane potential	(Haasio *et al.*, 2002)

Tamoxifen	Nonsteroidal anti-androgen	Breast cancer	Disturbes mitochondrial calcium accumulation Induces mitochondrial depolarization Inhibits oxidative phosphorylation	(Cardoso *et al.*, 2003; Theodossiou *et al.*, 2012)
Troglitazone	Thiazolidinedione	Type-II diabetes	Mitochondrial uncoupler, disrupts the mitochondrial membrane potential Inhibits oxidative phosphorylation Increases mitochondrial permeability transition pore and calcium efflux Induces apoptosis	(Ong *et al.*, 2007),(Julie *et al.*, 2008)
Nefazodone	Triazolopyiridine	Depression	Mitochondrial Complex I inhibitor	(Dykens *et al.*, 2008)
Imipramine	Tricyclic anti-depressant	Depression	Inhibits mitochondrial respiration; ion channel inhibitor	(Katyare and Rajan, 1995),(Choi *et al.*, 2006)
Ketoconazole	Anti-fungal drug	Fungal infections	Complex I inhibitor	(Rodriguez and Acosta, 1996)

The different cell strains were treated during 24 hours with the mitochondrial toxicants and their effect on the ATP content of the four strains of MEFs was accessed in both glucose- and galactose-containing media. Our results showed that there were strain-dependent differences in the response to some of the drugs. We propose that this model is a useful starting point to study compounds that may cause mitochondrial off-target toxicity in early stages of drug development.

3.2.2 Results

3.2.2.1 Drug-induced Toxicity in the MEF Panel: Role of Primary Energy Metabolism

In order to test our model of genetically diverse MEFs as a potential tool to study drug-induced mitochondrial impairment, we tested the effect of rotenone and seven distinct drugs on the ATP content of the MEFs, as an end-point of cell toxicity. The seven drugs that were chosen are known to display mitochondrial liabilities and were also reported to induce adverse idiosyncratic reactions in several other cell types. Rotenone, complex I inhibitor, was used as a positive control of a mitochondrial poison. The four strains of MEFs, at passage 10, were incubated with the compounds over 24 hours in (a) regular cell culture medium containing glucose and (b) cell culture medium lacking glucose but supplemented with galactose and glutamine as described in Materials and Methods. This latter approach has been used to detect direct mitochondrial effects of different compounds since cells, in general, are almost exclusively reliant on oxidative phosphorylation for their ATP demands in a glucose-free, galactose/glutamine medium (Marroquin *et al.*, 2007). Hence, a compound could potentially cause any of the following four effects when tested in glucose versus galactose medium: (a) it could be non-toxic at the highest drug concentration tested in both glucose medium and galactose medium, (b) it could have similar IC_{50} values in both glucose medium and galactose medium, indicating that it causes multifactorial cytotoxicity, mitochondrial impairment being one of the many potential factors, (c) it could have an IC_{50} value which is at least 3 times lower in galactose medium than in glucose medium, indicating that the primary mechanism of toxicity is mitochondrial impairment, or (d) it could have an IC_{50} value which is lower

in glucose medium than in galactose medium, indicating that it inhibits glycolysis.

Rotenone had an IC_{50} ratio between Glucose:Galactose media that was greater than 3 in all four strains (Table 3.2.2, Figure 3.2.2). The highest ratio was seen in CZECHII/EiJ and the lowest ratio was seen in PERA/EiJ. Nefazodone had an IC_{50} ratio between Glucose:Galactose media > 3 in C57BL/6J and CZECHII/EiJ, indicating that mitochondrial impairment was the primary mechanism of toxicity in these two strains (Table 3.2.2, Figure 3.2.3). The effect was more pronounced in CZECHII/EiJ than in C57BL/6J. In contrast, although nefazodone decreased the ATP content in PERA/EiJ and MOLF/EiJ, both in glucose medium and galactose medium, the IC_{50} ratio between Glucose:Galactose media was < 3, indicating that multifactorial toxicity occurred in these two strains. Ketoconazole had an IC_{50} ratio between Glucose:Galactose media < 3 in C57BL/6J, PERA/EiJ and MOLF/EiJ, and decreased the ATP content in these three strains of MEFs in both glucose and galactose medium. This indicated that the drug caused multifactorial toxicity in these strains. In contrast, ketoconazole had an IC_{50} ratio between Glucose:Galactose media > 3 in CZECHII/EiJ, suggesting that the drug caused mitochondrial impairment as a primary mechanism of toxicity in this strain (Table 3.2.2, Figure 3.2.4). Tolcapone had an IC_{50} ratio between Glucose:Galactose media < 3 in C57BL/6J, PERA/EiJ and MOLF/EiJ, indicating that mitochondrial impairment was not the main mechanism of toxicity (Table 3.2.2, Figure 3.2.5). In contrast, the IC_{50} ratio between Glucose:Galactose media was > 3 in CZECHII/EiJ, indicating that mitochondrial impairment was the primary mechanism of toxicity in this strain (Table 3.2.2., Figure 3.2.5). Flutamide had an IC_{50} ratio between Glucose:Galactose media > 3 in all four strains, indicating that the primary mechanism of toxicity was mitochondrial impairment. The effect

was most pronounced in C57BL/6J where the ratio was 10 (Table 3.2.2, Figure 3.2.6). Tamoxifen and imipramine had an IC_{50} ratio between Glucose:Galactose media < 3 in all four strains (Table 3.2.2, Figures 3.2.7 and 3.2.8). This indicated that these two drugs caused multifactorial toxicity in all the strains. Troglitazone had an IC_{50} ratio between Glucose:Galactose media < 3 in all the strains, indicating that mitochondrial impairment was not the primary mechanism of toxicity (Table 3.2.2, Figure 3.2.9).

In summary, CZECHII/EiJ showed the highest IC_{50} ratio between Glucose:Galactose media with rotenone, nefazodone, ketoconazole and tolcapone when compared with the other strains while C57BL/6J showed the highest IC_{50} ratio between Glucose:Galactose media with flutamide.

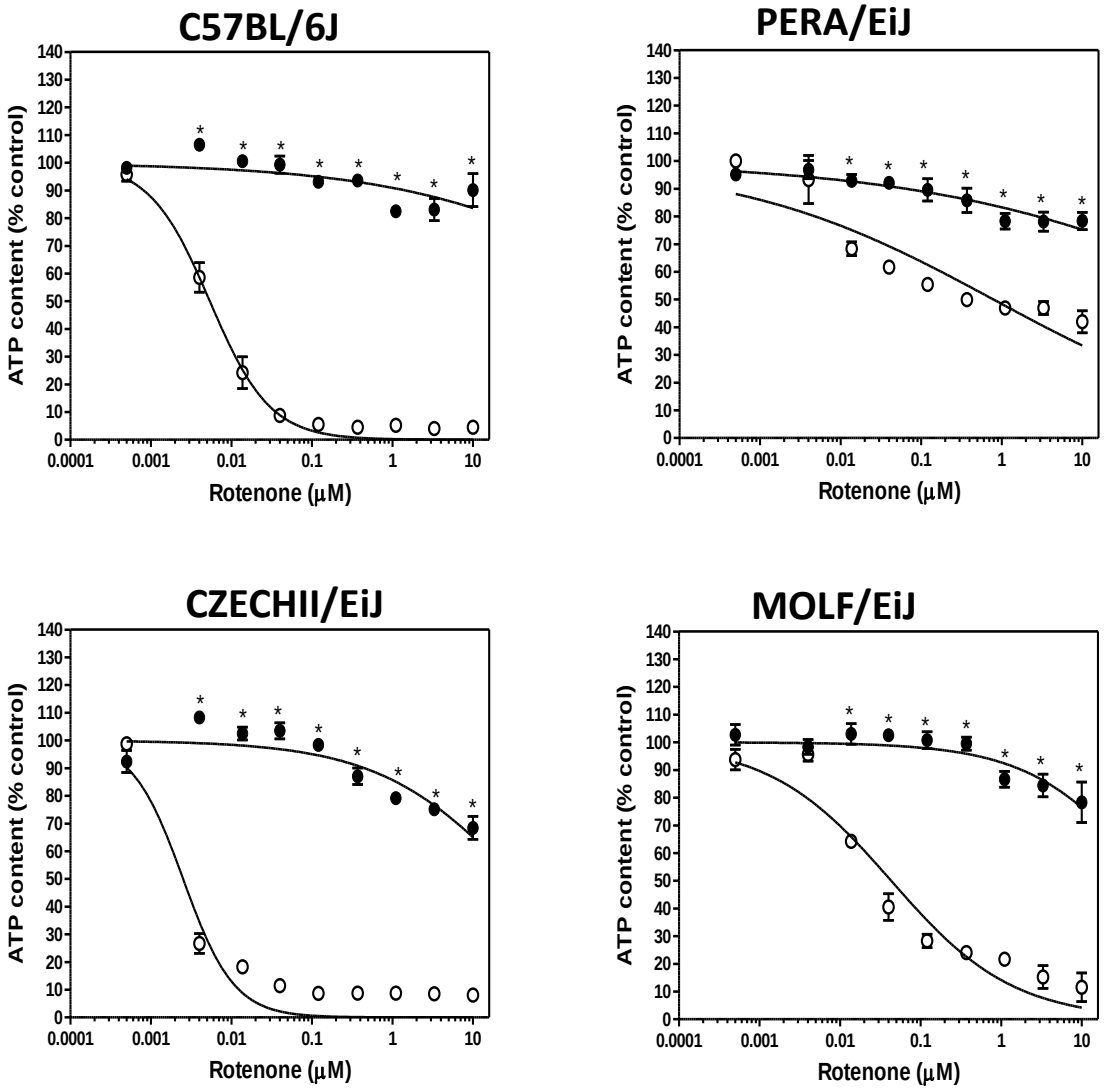


Fig. 3.2.2 - The effect of rotenone on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

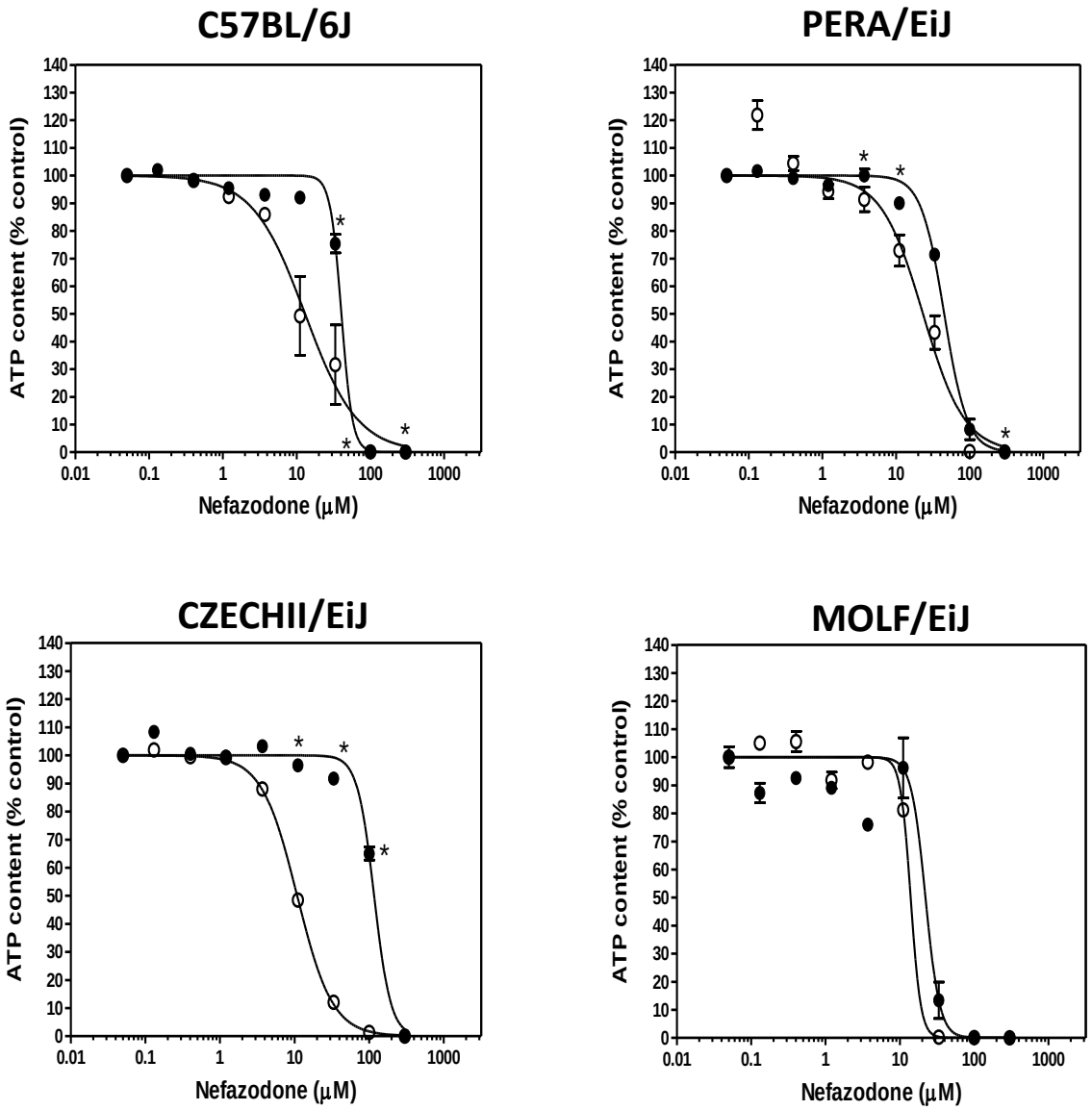


Fig. 3.2.3 - The effect of nefazodone on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

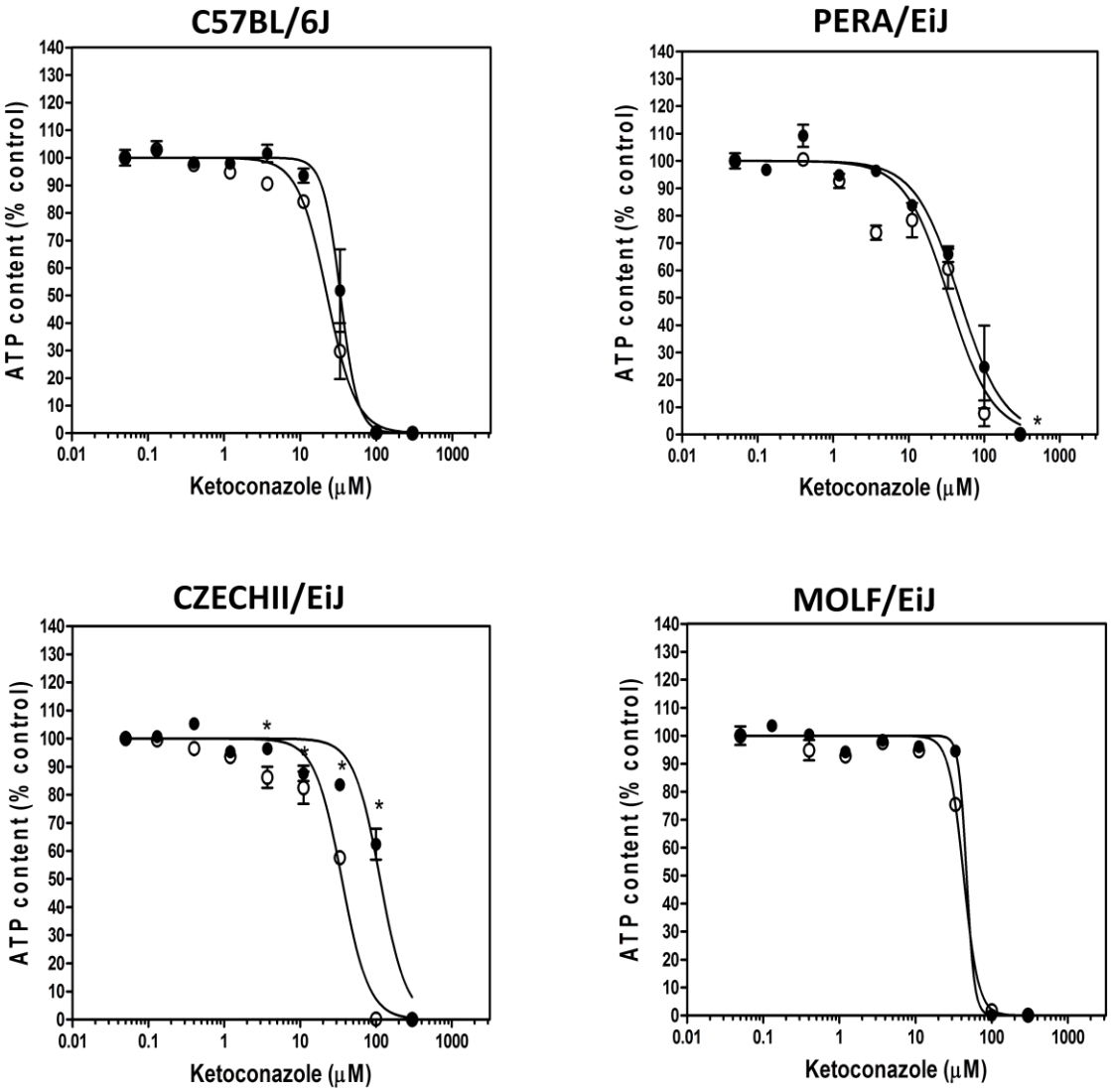


Fig. 3.2.4 - The effect of ketoconazole on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

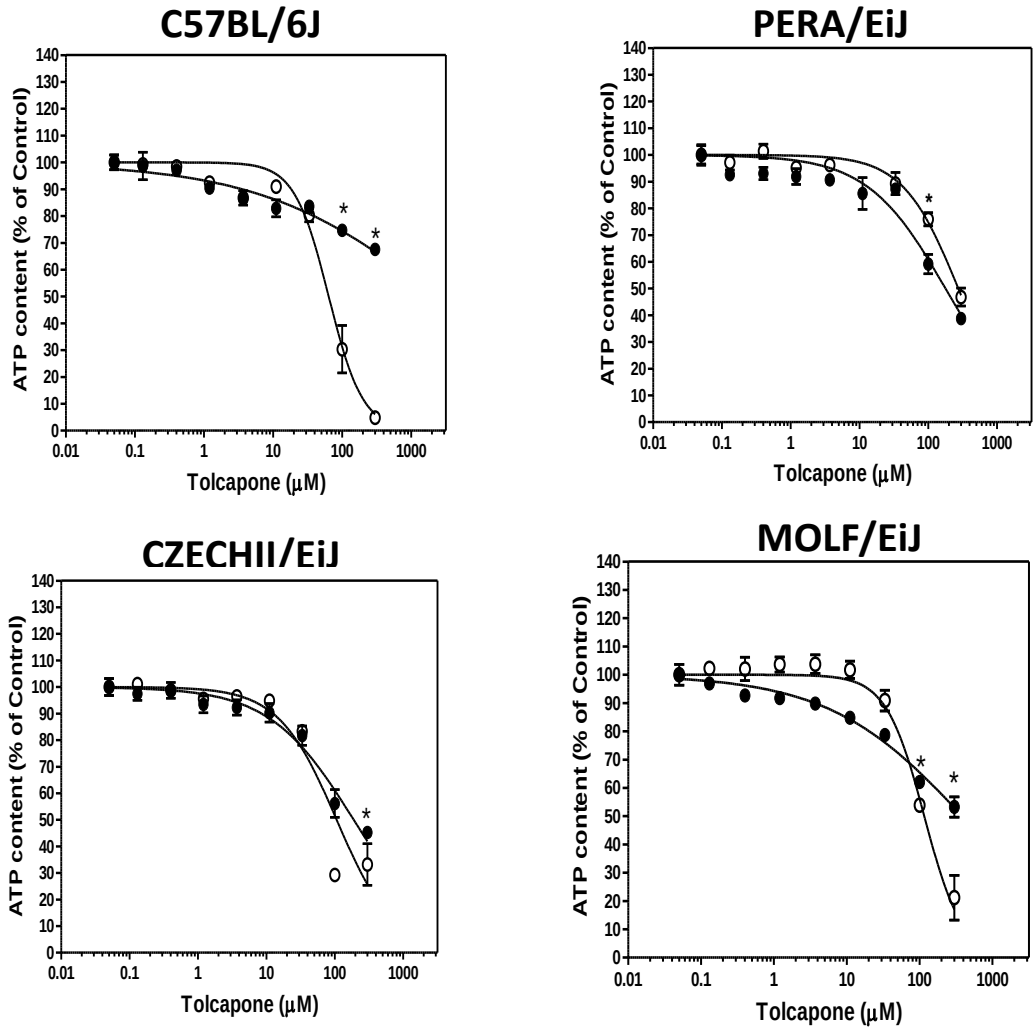


Fig. 3.2.5 - The effect of tolcapone on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

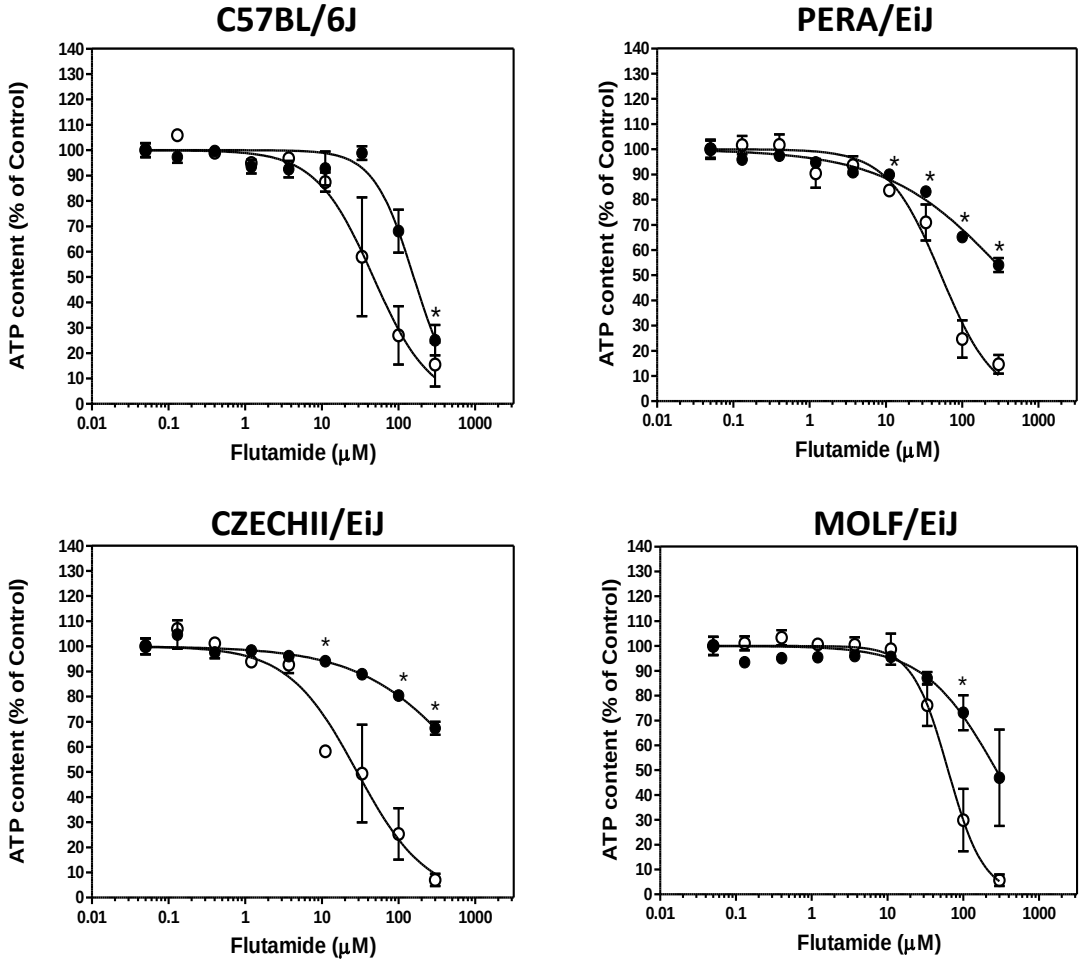


Fig. 3.2.6 - The effect of flutamide on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

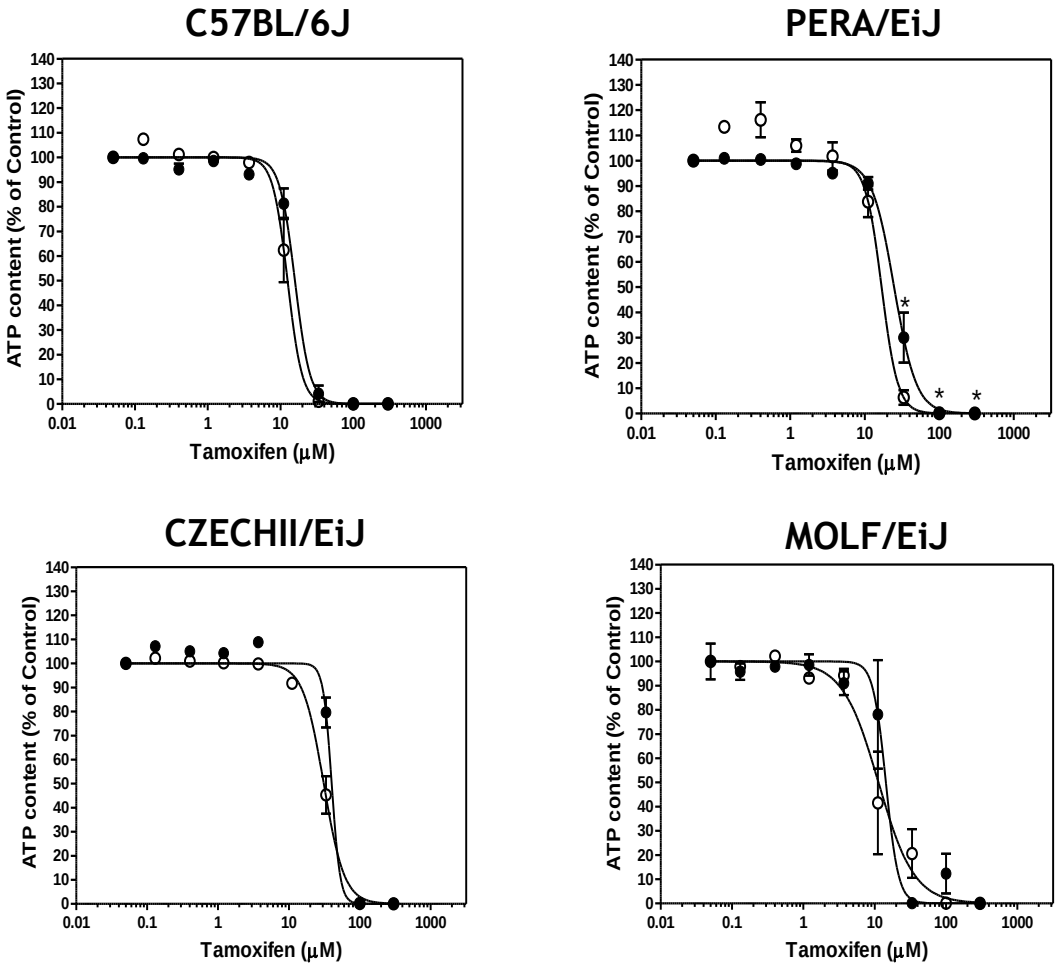


Fig. 3.2.7 - The effect of tamoxifen on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

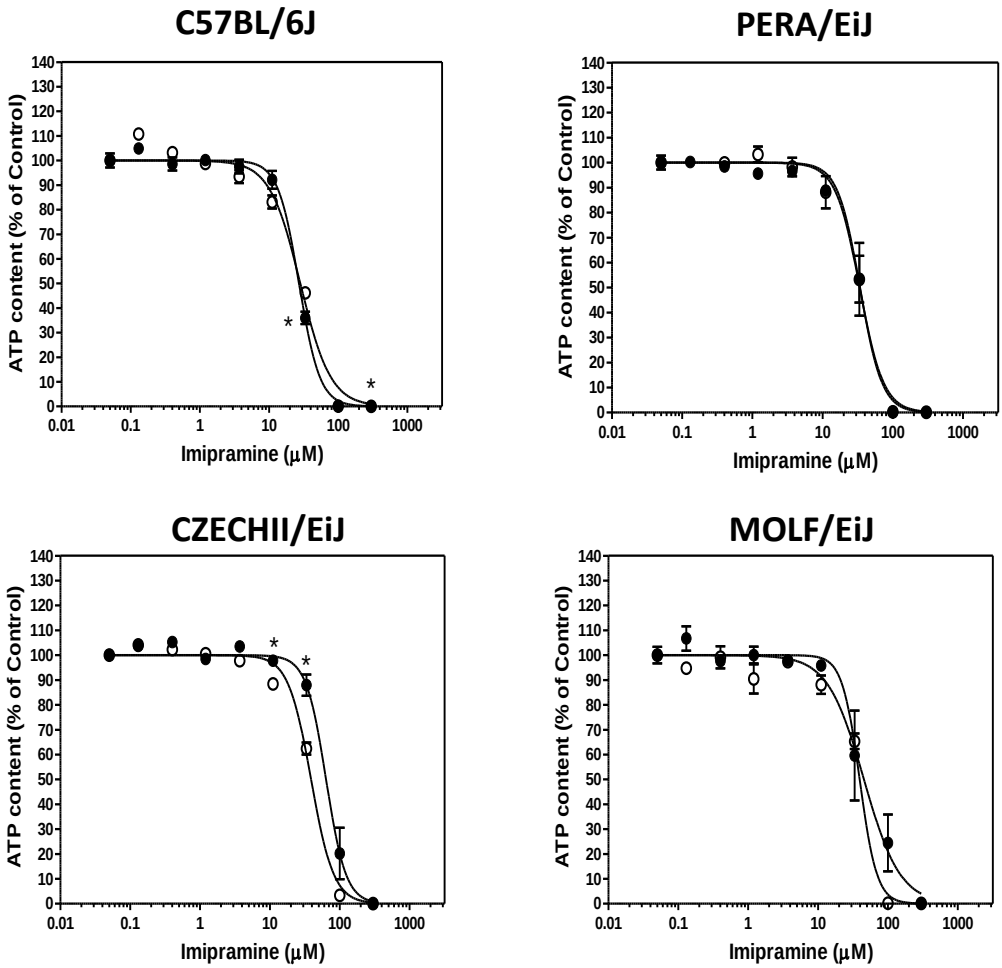


Fig. 3.2.8 - The effect of imipramine on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

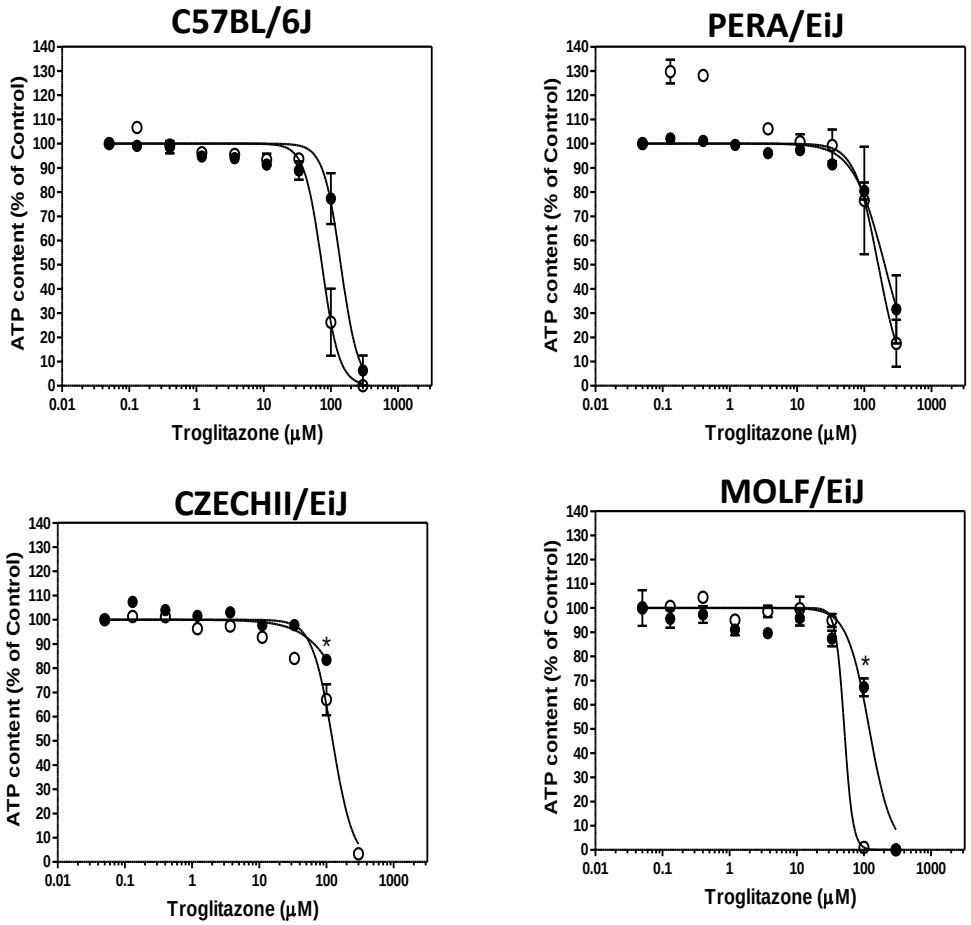


Fig. 3.2.9 - The effect of troglitazone on the ATP content of the four strains of MEFs in cell culture medium containing glucose (filled symbol) and galactose (open symbol) 24 hours after compound addition. Each data point represents the Mean± SD, N = 3 separate experiments. Statistical analysis was performed via Student t-test. $p<0.05$ is considered statistically significant, * vs. galactose-grown.

Table 3.2.2 - The effect of a selection of compounds on the ATP content of the four strains of MEFs in (a) cell culture medium containing glucose and (b) glucose-free cell culture medium supplemented with galactose and glutamine. (Abbreviations: Glu, Glucose; Gal, Galactose).

Compound	Strain	C57BL/6J		PERA/EiJ		CZECHII/EiJ		MOLF/EiJ	
		MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
Rotenone	IC_{50} Glu (µM)	> 10	±4.4	> 10	±2.8	> 10	±2.0	> 10	±6.2
	IC_{50} Gal (µM)	0.005	±0.001	0.8	±0.5	0.002	±0.0001	0.04	±0.02
	IC_{50} Glu/Gal	>2000	± 7	>12.5*	±3.1	>5000*#&	± 5	>250	±7
Nefazodone	IC_{50} Glu (µM)	39.2	±2.4	45.3	±7.1	117.3	±1.5	24.5	±8.3
	IC_{50} Gal (µM)	12.0	±9.2	25.0	±8.5	10.9	±0.8	14.0	±0.5
	IC_{50} Glu/Gal	4.3	±1.8	1.9	±0.5	10.9*&#	±0.9	1.8	±0.6
Ketoconazole	IC50 Glu (µM)	32.9	±10.1	57.1	±26.8	111.6	±12.7	43.0	±7.5
	IC_{50} Gal (µM)	24.1	±7.8	35.3	±17.9	33.8	±3.9	42.1	±4.9
	IC50 Glu/Gal	1.5	±0.8	1.7	±0.6	3.4*&#	±0.8	1.0	±0.3
Tolcapone	IC_{50} Glu (µM)	>300	±18.2	169.8	±24.6	235.6	±6.4	235.6	±4.1
	IC_{50} Gal (µM)	140.5	±29.4	287.4	±34.9	68.6	±2.1	112.7	±1.1
	IC50 Glu/Gal	>2.1@&#	±0.2	0.6*@#	±0.2	3.4*&#	±0.1	2.09*@&	±0.04

Flutamide	**IC_{50} Glu (μM)**	201.2	±12.0	>300	±26.2	>300	±27.7	125.6	±15.9
	IC_{50} Gal (μM)	20.4	±2.4	45.8	±21.0	98.8	±3.2	36.3	±1.7
	IC_{50} Glu/Gal	10.0	±1.5	>6.5	±2.3	>3.0	±4.1	3.5&	±0.5
Tamoxifen	**IC_{50} Glu (μM)**	14.5	±1.6	26.6	±6.3	40.6	±4.3	80.8	±17.4
	IC50 Gal (μM)	11.3	±3.1	16.7	±4.8	30.6	±6.5	28.7	±7.5
	IC_{50} Glu/Gal	1.3	±0.2	1.3	±0.1	1.4	±0.2	2.9*&@	±0.5
Imipramine	**IC_{50} Glu (μM)**	27.8	±3.7	25.0	±3.3	44.1	±3.5	101.9	±8.4
	IC_{50} Gal (μM)	28.0	±3.4	19.2	±2.4	35.9	±1.1	40.6	±1.1
	IC_{50} Glu/Gal	1.0	±0.2	1.3	±0.3	1.2	±0.1	2.5*&@	±0.2
Troglitazone	**IC_{50} Glu (μM)**	111.1	±36.2	>300	±10.1	>300	±27.1	117.8	±8.5
	IC_{50} Gal (μM)	93.0	±32.6	291.5	±13.8	103.6	±19.8	73.6	±12.1
	IC_{50} Glu/Gal	1.2	±0.1	>1.0	±0.1	>2.9*&#	±0.9	1.6	±0.3

Data are Mean ± SD, N=3 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test. $p<0.05$ is considered statistically significant. * vs. reference strain C57BL/6J. & vs. PERA/EiJ. @ vs CZECHII/EiJ. #vs MOLF/EiJ.

3.2.3 Discussion

In an effort to create a new and suitable *in vitro* system to investigate drug-induced mitochondrial toxicity, we previously characterized the mitochondrial bioenergetic parameters of embryonic fibroblasts isolated from four different strains of mice, C57BL/6J, PERA/EiJ, CZECHII/EiJ

and MOLF/EiJ (Section 3.1). These strains of mice have distinct mtDNA polymorphisms that could potentially simulate different sub-populations of mice that could later be extrapolated for different human genetic pools.

It has been suggested that human genetic diversity should be considered when predicting and understanding the mechanisms of idiosyncratic drug-induced toxicity (Boelsterli and Lim, 2007). Multiple enzyme polymorphisms can be involved in rare, but sometimes fatal, episodes of adverse drug reactions. Depending on the activity of the mutated protein, individuals can have a higher predisposition to the development of an adverse reaction or, in other cases, respond with lower efficacy to the treatment (Boelsterli and Lim, 2007).

Recent evidence has shown that mitochondria are off-targets of several drugs, such as nucleoside reverse transcriptase inhibitors (NRTIs) (Feng *et al.*, 2001), diclofenac (Deng *et al.*, 2006), troglitazone (Lee *et al.*, 2008), and doxorubicin (Oliveira and Wallace, 2006), which cause adverse effects. Unpredictable drug-induced mitochondrial toxicity is currently a major reason for attrition of compounds in the developmental stages and for the post-market drug withdrawals. Hence, a goal of this study was to determine whether the four strains of fibroblasts can be used as an *in vitro* platform to identify any differences in the susceptibility towards drugs that are known to cause mitochondrial impairment and further, idiosyncratic drug responses. For this reason, we tested the effect of a selection of drugs described to be involved in mitochondrial impairment, on the ATP content of the MEFs in glucose- and galactose-medium, at passage 10. Rotenone showed the expected response in C57BL/6J, CZECHII/EiJ and MOLF/EiJ in that the cells were significantly more sensitive to rotenone in galactose medium than in glucose medium, with CZECHII/EiJ displaying the most significant sensitivity (Table 3.2.2, Figure 3.2.2). Surprisingly,

PERA/EiJ was much less sensitive to rotenone in galactose than expected and did not show complete ATP depletion even at the highest concentration tested (10 μM). Our results also indicated that, of the four strains, CZECHII/EiJ showed the highest IC_{50} Glucose: Galactose ratio for nefazodone, tolcapone and ketoconazole. Nefazodone is an antidepressant drug and it has been reported to cause mitochondrial impairment namely by causing Complex I inhibition (Dykens *et al.*, 2008). Nefazodone had an IC_{50} ratio between Glucose:Galactose media which was > 10 in CZECHII/EiJ (Table 3.2.2, Figure 3.2.3) indicating that it caused strong mitochondrial impairment in this strain. A slightly less potent mitochondrial effect was seen in C57BL/6J, where the ratio was 4.3 (Table 3.2.2, Figure 3.2.3). Nefazodone has previously been shown to have a mitochondrial effect on HepG2 cells, with a ratio of 3.6 (Dykens *et al.*, 2008). Thus, C57BL/6J resembled HepG2 cells in its response to nefazodone, whereas CZECHII/EiJ was much more sensitive to nefazodone's mitochondrial effects. In contrast, nefazodone had a ratio which was ~2 in PERA/EiJ and MOLF/EiJ, suggesting that although mitochondrial impairment may be occurring in these two strains, nefazodone has other off-targets. This is in accord with a report (Kostrubsky *et al.*, 2006) which showed that nefazodone inhibits the bile salt efflux pump.

Ketoconazole is an antifungal agent that is also known to inhibit Complex I (Rodriguez and Acosta, 1996). Ketoconazole displayed an IC_{50} ratio between Glucose:Galactose media which was > 3 in CZECHII/EiJ, indicating that it caused mitochondrial impairment in this strain (Table 3.2.2, Figure 3.2.4). This drug does not cause mitochondrial toxicity in the HepG2 glucose/galactose model. This suggests that CZECHII/EiJ is more sensitive than HepG2 cells to ketoconazole's mitochondrial effect. In contrast, no mitochondrial impairment was seen in the other strains of

MEFs. This suggests that ketoconazole may have other mechanisms of toxicity in these strains in accord with reports that it depletes glutathione (Rodriguez and Buckholz, 2003), accumulates in lysosomes and causes phospholipidosis (Rodriguez and Acosta, 1995).

Tolcapone, a catechol-O-methyltransferase inhibitor used in the treatment of Parkinson's disease, was reported to be a mitochondrial uncoupler (Haasio *et al.*, 2002). CZECHII/EiJ showed an IC_{50} ratio between Glucose:Galactose media which was > 3 (Table 3.2.2, Figure 3.2.5). This is in contrast to HepG2 cells which do not show a mitochondrial effect in the glucose/galactose model. Hence, CZECHII/EiJ MEFs were more sensitive to tolcapone's mitochondrial effect than HepG2 cells.

Flutamide, a non-steroidal antiandrogen drug, was reported to produce hepatitis, it was also demonstrated to inhibit complex I, decrease the ATP levels in isolated rat hepatocytes (Fau et al, 1994). Flutamide showed an IC50 ratio between Glucose:Galactose media>3 in all four strains, indicating that the primary mechanism of toxicity was mitochondrial impairment. The effect was most pronounced in C57BL/6J where the ratio was 10 (Table 3.2.2, Figure 3.2.6).

Three of the drugs that we tested, tamoxifen, troglitazone and imipramine, had an IC_{50} ratio between Glucose:Galactose media which was < 3 in all the strains, indicating that mitochondrial impairment was not the primary mechanism of toxicity caused by these drugs (Table 3.2.2, Figure 3.2.7, 3.2.8 and 3.2.9). These drugs have been reported to cause multiple mechanisms of toxicity. For example, tamoxifen, an anticancer drug, and imipramine, an antidepressant, are both reported to cause phospholipidosis (Nioi and Nguyen, 2007; Kuroda and Saito, 2010) in addition to mitochondrial impairment. Troglitazone, a thiazolidinedione removed from the market

due to hepatotoxicity, is reported to inhibit the bile salt efflux pump (Funk *et al.*, 2001).

In conclusion, four strains of MEFs which had distinct mtDNA polymorphisms showed different IC50 ratio for some of the tested drugs. Evaluation of rotenone and seven drugs on the ATP content of the MEFs in glucose and galactose media showed that there were strain-dependent differences in the sensitivity towards the tested compounds. Of the four strains tested, CZECHII/EiJ showed the largest IC_{50} ratio between Glucose:Galactose media with rotenone, nefazodone, ketoconazole and tolcapone. In addition, this strain also showed the lowest mitochondrial robustness when compared to the other strains, and this might be one of the reasons why it was easier to show mitochondrial toxicity in this strain, after the treatment with different mitochondrial toxicants.

Our results suggest that high throughput screening of more drugs using this panel of MEFs, particularly the strain CZECHII/EiJ, in glucose/galactose media, may help predicting the toxicity of compounds which cause off-target mitochondrial effects, often missed in early stages of drug development. Moreover, our results suggest that by testing the effect of rotenone on ATP content of fibroblasts from many other strains of genetically diverse mice in glucose/galactose media, one may identify strains that are useful for *in vitro* testing of compounds that have the potential to cause IDRs through mitochondrial impairment.

Highlights of Chapter 3.1 and 3.2:

- mtDNA SNPs are hypothesized to be associated with individual predisposition to drug-induced adverse reactions.

- CZECHII/EiJ and PERA/EiJ mtDNA were sequenced for the first time in this study.

- The bioenergetics profile of CZECHII/EiJ MEFs showed a consistently decreased mitochondrial robustness when compared to that of C57BL/6J, followed by MOLF/EiJ and PERA/EiJ.

- Strain-dependent differences were measured in the toxicity to some of the tested mitochondrial toxicants.

3.3 SIRT3 and regulation of Doxorubicin-induced mitochondrial toxicity

3.3.1 Summary

The mammalian sirtuins are a family of seven protein deacetylases involved in many different cellular functions including calorie restriction and metabolic stress. Sirtuin 3 (SIRT3) has been shown to decrease reactive oxygen species (ROS) production and protect against DNA damage. SIRT3 is a deacetylase protein localized in the mitochondrial matrix, where it regulates the acetylation levels of metabolic enzymes. Doxorubicin (DOX) is a widely used anti-neoplastic agent known for its dose-dependent cardiotoxicity. DOX-induced cardiotoxicity is characterized by increased ROS generation and cardiomyocyte death. The hypothesis for the present study is that DOX-induced toxicity on H9c2 cardiomyoblasts can be regulated by SIRT3 expression levels. Cell mass, intracellular ATP levels, cell death and apoptosis-relevant caspase 3/7 activities were determined in untreated control and DOX-treated human SIRT3 overexpressing cells, hSIRT3-H248Y-HA (mutant SIRT3 lacking deacetylase activity) overexpressing cells and pcDNA control H9c2 cells. We found that hSIRT3 overexpression protected H9c2 cells from DOX-induced cell death and caspase activity when compared with DOX-treated control cells after 24h. Surprisingly, hSIRT3-H248Y-HA overexpression also protected the cells against DOX-induced cell death and caspase activity, while it decrease in ATP levels when compared to the control cells treated with DOX. We concluded that SIRT3 may play a role in preventing cell death in H9c2 cardiomyoblasts, although it may play unique roles in other cell types. It is remarkable that SIRT3 deacetylase activity is not necessary for the cytoprotective effect of SIRT3 in H9c2 cells.

3.3.2 Introduction

As described in Chapter 1 (Section 4), sirtuins (type III histone deacetylases) or Sir2 (silent information regulator 2)-related enzymes have been defined as a family of NAD^+-dependent protein deacetylases, which remove acetyl groups from protein lysine residues, yielding nicotinamide and the metabolite 2'-O-acetyl-ADP-ribose as additional products (Yamamoto *et al.*, 2007). Sirtuins are evolutionarily conserved from archaebacteria to eukaryotes, indicating that these proteins play vital physiological roles. The mitochondrial SIRT3 will be focused in the present study. SIRT3 is ubiquitously expressed, particularly in metabolic active tissues, possessing an NAD^+-dependent deacetylase activity (Onyango *et al.*, 2002; Schwer *et al.*, 2002). SIRT3 is critical in sensing NAD^+ levels in the mitochondria such that increased NAD^+ levels trigger a regulatory pathway that activates SIRT3 leading to the deacetylation of specific protein targets. Furthermore, recent findings that SIRT3 deacetylates cyclophilin-D, resulting in either enhanced apoptosis in HeLa cells or increased cell survival and protection against age-related cardiac hypertrophy, clearly showing that SIRT3 might function differently based on cell type (Hafner *et al.*, 2010b). In the presence of conditions leading to a decrease in mitochondrial NAD^+, the activity of human SIRT3 (hSIRT3) significantly decreases leading to relative hyperacetylation of its substrates and to a decrease in O-acetyl-ADP ribose production. The disruption of the $mt\Delta\Psi$ also inhibits mitochondrial import of hSIRT3, thus, reducing its levels in mitochondria (Schwer *et al.*, 2002). Accordingly, constitutive hSIRT3 activity may play an important role in protection against apoptosis and drug-induced mitochondrial damage, and its inhibition may lead to increased acetylation of factors directly involved in apoptotic pathways.

In these studies, we aimed to explore the possible protective role of SIRT3 during mitochondrial drug-induced toxicity initiated by a cardiotoxic drug, doxorubicin (DOX). Doxorubicin (DOX, also known as Adriamycin) is an antineoplastic drug widely used for the treatment of various types of cancer, but the treatment often causes cardiac toxicity limiting its clinical application (Sardao *et al.*, 2009a). The toxic effects of DOX include mitochondrial damage, specific and non-specific membrane interactions, lipid peroxidation in mitochondrial membranes, oxidation of mitochondrial DNA, and the activation of calcium-dependent, cyclosporine-A sensitive mitochondrial permeability transition pore (MPTP)(Lebrecht *et al.*, 2010). Although the full role of SIRT3 in the cell remains largely unknown; the localization of this enzyme to the mitochondrial matrix gives important insight to its potential substrates and functions. The aim of this study was to understand the role of SIRT3 in DOX-induced toxicity in H9c2 cells, a myoblastic cell line widely used as an *in vitro* model for cardiac muscle DOX-induced toxicity. For the present work, hSIRT3 and a mutant form of hSIRT3 (hSIRT3-H248Y-HA) lacking deacetylation activity were over-expressed in H9c2 cells.

3.3.3 Results

3.3.3.1 Basal SIRT3 gene and protein expression levels in H9c2 cells

In order to analyse SIRT3 expression levels in H9c2 cells, cell pellets were harvested 48 hours after transfection and lysed to be subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. With the commercial antibody from Cell Signaling (catalog number 2627) used in this study, no endogenous SIRT3 protein content was detected. SIRT3 gene expression quantified by real-time PCR was also very low. hSIRT3 overexpression in H9c2 cells lead to a significant increase in both gene

expression as detected by RT-qPCR analysis (with 18S as a reference gene), and protein expression as determined by using the antibody described above and Western-blot analysis. The two bands observed represent the long (≈44KDa) and the short forms (≈28 KDa) of hSIRT3, and hSIRT3 mutant overexpression also showed a significant increase in H9c2 cells, indicating an efficient transfection (Figure 3.3.1).

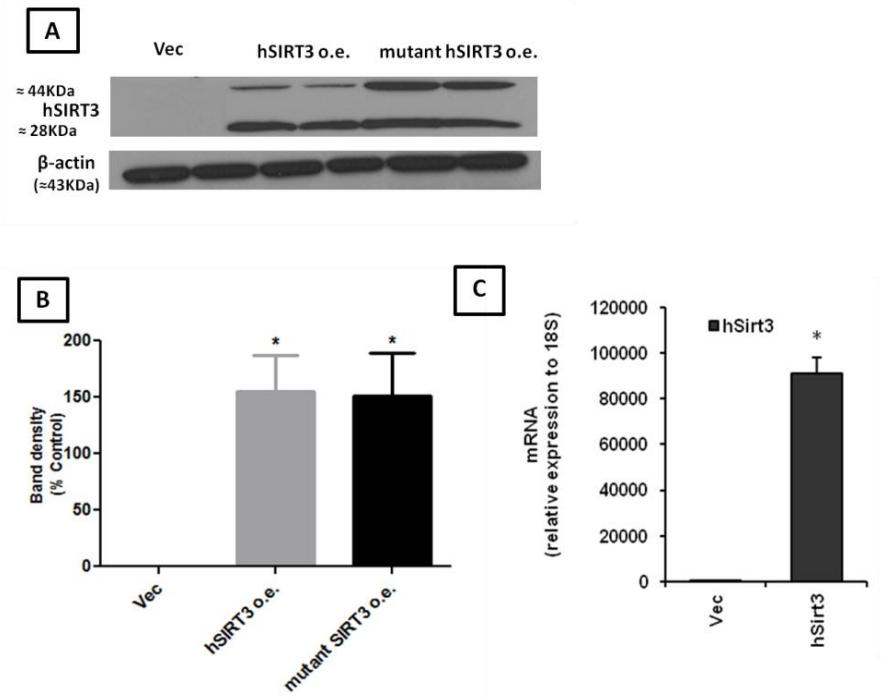


Fig. 3.3.1- SIRT3 is not endogenously expressed in H9c2 cells. Representative Western Blot of SIRT3 (≈44 and 28 KDa) protein expression in the control vector, hSIRT3 o.e. and mutant hSIRT3 o.e. in H9c2 cell extracts (A) and graphical representation of SIRT3 protein content in H9c2 cells normalized to β-actin (≈43 KDa) (n=5). Western blotting was performed by using a commercial antibody from Cell Signaling (#2627). (B) SIRT3 mRNA expression levels were quantified by qRT-PCR in pc-DNA control and hSIRT3 overexpressing cells (n=3) (C). Data represents Mean ±SEM, n=3-5 separate experiments. Statistical analysis was performed by using one-way ANOVA followed by the Bonferroni Multiple Comparison. For mRNA expression experiment, a two-tail Student t-test was performed. A p value of <0.05 was considered significant. *versus Vec. Legend: Vec, vector; hSirt3, human SIRT3; o.e., overexpression.

3.3.3.2 SIRT3 overexpression does not protect against DOX-induced decreased H9c2 cell mass

Rat H9c2 cardiomyoblasts were incubated during 24h with 1μM of DOX and cell mass was analysed by the SRB assay. The results showed that both hSIRT3-overexpressing cells and mutant hSIRT3-overexpressing cells were not dissimilar in terms of DOX toxicity, shown as a decrease in cell mass. Neverthless, the present method does not distinguish between DOX-induced inhibition of cell division and induction of cell death (Figure 3.3.2).

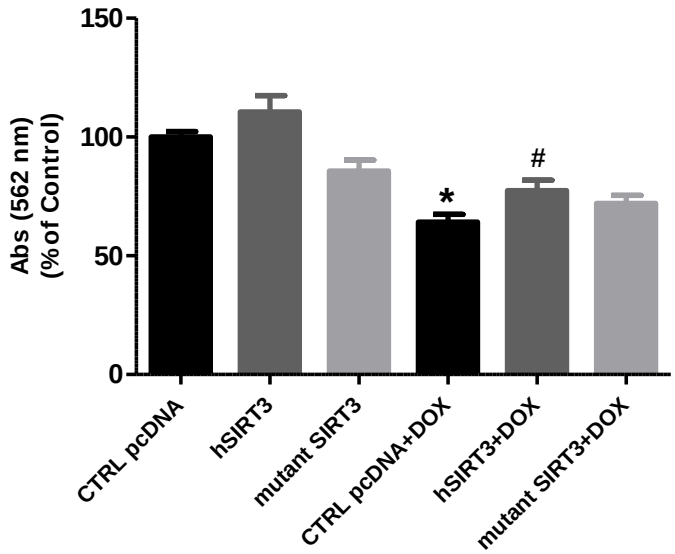


Fig. 3.3.2 - hSIRT3 overexpression does not protect cells against DOX-induced decreased cell mass. The experiment was performed 24 hours after doxorubicin (1 μM) treatment in control cells (CTRL pcDNA), hSIRT3 overexpressing cells (hSIRT3) and in hSIRT3 deacetylase mutant cells (hSIRT3-H248Y-HA). Data represents Mean±SEM of 5 independent experiments. Statistical analysis was performed by using one-way ANOVA followed by the Bonferroni Multiple Comparison test. $p<0.05$ is considered statistically significant. * versus CTRL pcDNA, # versus hSIRT3 overexpression.

3.3.3.3 hSIRT3 overexpressing H9c2 cells does not protect against DOX-induced decreased intracellular ATP

In order to analyse cell viability, intracellular ATP levels were measured 24 h after DOX treatment. Cells were treated with 1μM DOX after 48h of transfection with an empty vector (pc DNA), hSIRT3 or a mutant deacetylase form of hSIRT3 (mutant hSIRT3). Overall, all treated cells in the three different conditions exhibited statistical differences from untreated cells. The mutant hSIRT3 ATP content was also statistically different from pcDNA+DOX. The ATP levels decreased around 40-50% for both pcDNA+DOX and hSIRT3 overexpression+DOX and around 60% for the mutant hSIRT3+DOX (figure 3.3.3).

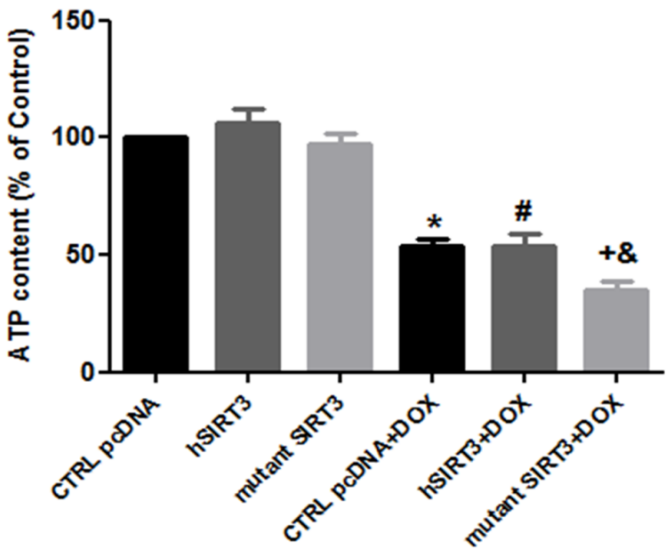


Fig. 3.3.3 - Intracellular ATP content after 24h of DOX treatment (1 μM). Statistical analysis was performed by using one-way ANOVA followed by Bonferroni Multiple Comparison test. $p<0.05$ is considered statistically significant. * versus CTRL pcDNA, # versus hSIRT3, & versus mutant SIRT3, + versus CTRL pcDNA+DOX. Data represents Mean± SEM of 3 independent experiments.

3.3.3.4 hSIRT3 overexpression and mutant hSIRT3 overexpression decrease DOX-induced H9c2 cell death

H9c2 cells showed a 6-fold increased in cell death after 1 μM DOX treatment in Control pcDNA transfected cells, while toxicity of DOX on hSIRT3 overexpressing cells and mutant hSIRT3 overexpressing cells resulted in an approximately 2-fold increase in cell death. These results were statistically different from the untreated cells and both the hSIRT3 and mutant hSIRT3 overexpressing cell death results were also statistically different from the Control pcDNA+DOX, demonstrating an approximately 2-fold decrease in cell death (figure 3.3.4).

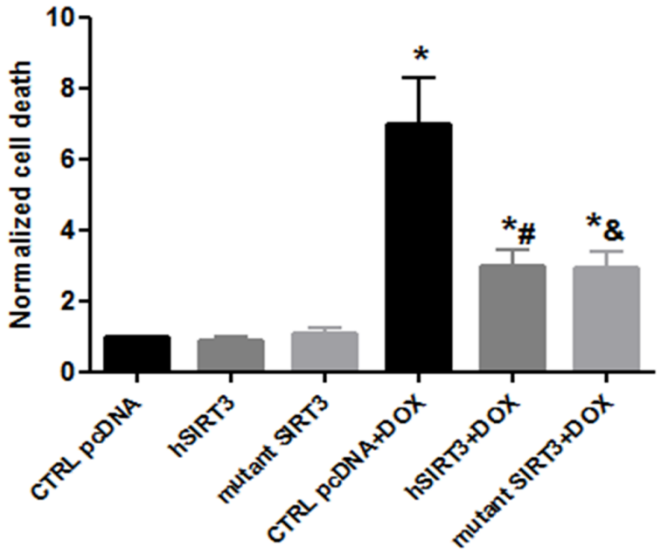


Fig. 3.3.4 - hSIRT3 overexpression protects H9c2 cells against DOX-induced cell death. Cell death was determined with the MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI), according to the manufacturer's instruction and normalized with the SRB results (Cell death (%)/Cell mass (%) ratio) and determined 24 h of DOX treatment with 1 μM in H9c2 cells. Data represents Mean±SEM of 3 independent experiments. Statistical analysis was performed by using one-way ANOVA followed by Bonferroni Multiple Comparison test. * versus CTRL pcDNA, # versus hSIRT3 O.E., & versus mutant SIRT3. Data represents Mean±SEM of 3 independent experiments.

3.3.3.5 hSIRT3 and mutant hSIRT3 overexpression DOX-induced decrease caspase 3/7-like activity in H9c2 cells when compared with the treated control

Doxorubicin treatment of control pcDNA-treated H9c2 cells resulted in increased caspase activity of approximately 7-fold while hSIRT3 overexpressing and mutant hSIRT3 overexpressing cells treated with the same DOX concentration had an increased caspase 3/7-like activity of about 3 and 4 fold, respectively. Control pcDNA+DOX cells were statistically different from untreated control cells, while hSIRT3+DOX overexpressing cells were statistically different from untreated hSIRT3-overexpressing cells. A statistical difference was also observed between CTRL pcDNA+DOX and hSIRT3+DOX-overexpressing cells. Mutant hSIRT3+DOX overexpressing cells were similarly statistically different from mutant hSIRT3 and also, from the CTRL pcDNA+DOX (Figure 3.3.5).

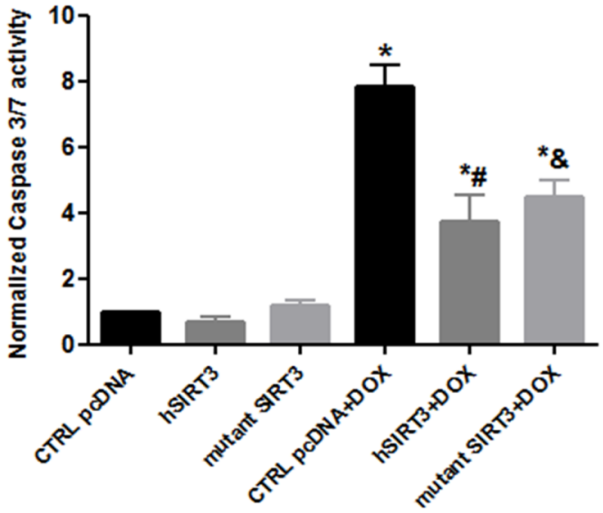


Fig. 3.3.5 - hSIRT3 overexpression decreased caspase 3/7 activity in H9c2 cells. Caspase-like activity was determined by using the Caspase-Glo® 3/7 Assay (Promega, Madison, WI), according to the manufacturer's instruction and normalized with the SRB results (%Caspase 3/7 activity/%cell mass ratio), calculated 24 h after DOX (1 μM) treatment. Data represents Mean± SEM of 3 independent experiments. Statistical analysis was performed by using one-way ANOVA followed by Bonferroni Multiple Comparison test. $p<0.05$ is considered statistically significant. * versus CTRL pcDNA, # versus hSIRT3 O.E., & versus mutant SIRT3, + versus CTRL pcDNA+DOX.

3.3.4 Discussion

Doxorubicin is an anthracycline antibiotic with a wide spectrum of clinical antineoplastic activity, however, it leads to cardiotoxicity characterized by increased cardiomyocytes apoptosis. Doxorubicin depresses left ventricular function and increases apoptotic myocardial cells, besides results in increasing oxidative stress (Zhang *et al.*, 2011). Previous studies have demonstrated that DOX is primarily accumulated in the nucleus of H9c2 cells (Sardao *et al.*, 2009a), which is explained by the fact that DOX intercalates in the DNA double helix (Goto *et al.*, 2001). Thus, DNA

synthesis can be suppressed by DOX, which explains DOX-induced cell cycle arrest in H9c2 cells. Doxorubicin also induces p53 activation and downstream apoptotic events involving BAX over-expression and translocation to mitochondria (Sardao *et al.*, 2009a). Oxidative stress, mitochondrial dysfunction and cytosolic calcium deregulation are also consequences of DOX on cardiac mitochondria and on H9c2 cells (Sardao *et al.*, 2009a). Doxorubicin interacts with mitochondria and undergoes redox cycling in complex I resulting in increased oxidative stress, decreased mitochondrial membrane potential and increasing cytosolic calcium levels (Wallace, 2003; Wallace, 2007b). Sirtuin 3 (SIRT3) is described as protecting mitochondria against oxidative stress-induced damage, contributing to enhanced cell survival. SIRT3 protects cardiomyocytes and HeLa cells from genotoxic and oxidative stress-mediated cell death, preventing BAX translocation to mitochondria (Sundaresan *et al.*, 2008). SIRT3 binds to Ku70 and deacetylates it, promoting the interaction of Ku70 with the proapoptotic protein Bax (Sundaresan *et al.*, 2008). This mechanism may prevent apoptosis during stressful conditions. SIRT3 directly interacts with and deacetylates p53, thereby abrogating its activity to execute growth arrest and senescence in bladder carcinoma cells (Li *et al.*, 2010). SIRT3 also regulates the mitochondrial permeability transition pore via deacetylating cyclophilin-D (Hafner *et al.*, 2010b). Additionally SIRT3 deacetylates and activates the superoxide dismutase 2 (SOD2) (Chen *et al.*, 2011), protecting cells against ROS-mediated cell damage. In contrast to such studies including our own, other data have supported the proapototic role of SIRT3 (Allison and Milner, 2007).

The present study aimed to understand the role of SIRT3 on H9c2 cardiomyoblasts treated with DOX. Our initial hypothesis was that SIRT3 overexpression protects cells from DOX-induced toxicity. With the

antibody used in this study (Cell Signaling, catalog number 2627) we were not able to detect SIRT3 endogenous expression levels in H9c2 cells, unless a human SIRT3-flag or a catalytically inactive HY mutant form of hSIRT3 in which a single amino acid residue has been modified (histidine-to-tyrosine at amino acid residue 248 rendering SIRT3 with defective deacetylase activity), was expressed (Fig. 3.3.1A). In addition, SIRT3 transcripts in H9c2 cells were also almost undetectable, although over-expression with hSIRT3 resulted in a significant increase in those transcripts (Fig. 3.3.1B). The reason why the particular antibody used in this study was not able to detect SIRT3 in the studied cell line remains to be elucidated, but recently, our group showed detectable endogenous amounts of SIRT3 protein expression levels in H9c2 cells with another Cell Signaling antibody (catalog number 5490), refuting the previous findings. This new finding might be due to the fact that the #5490 monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Val130 residue in the mouse SIRT3 protein, while the previously tested antibody (# 2627) was produced by immunizing animals with a synthetic peptide corresponding to the sequence of human SIRT3 protein. This may explain also why a detectable increase in SIRT3 was observed after over-expression with hSIRT3 and why several human cell lines had detectable SIRT3 content using the same antibody (Fig. 3.3.6).

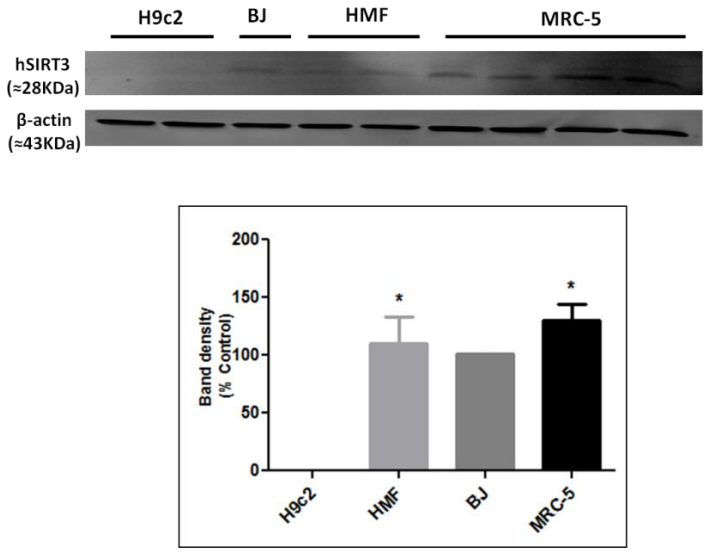


Fig. 3.3.6 - Differential SIRT3 expression levels in different cell lines. SIRT3 basal expression levels by Western-blot analysis of H9c2 myoblast cell line (n=4), BJ fibroblasts cell line (n=1), HMF mammary fibroblasts cell line (n=2) and MRC-5 human lung fibroblasts (n=4), by using an antibody from Cell Signaling (#2627). Data represents Mean±SEM. Statistical analysis was performed by using one-way ANOVA followed by Bonferroni Multiple Comparison test. $p<0.05$ is considered statistically significant. * versus H9c2 cells.

H9c2 cells overexpressing the inactive catalytic form of SIRT3 showed a statistically significant decrease of the intracellular ATP levels after DOX treatment, when compared with the control pcDNA cells treated with DOX (Figure 3.3.3). This result was unexpected, since the mutant form of SIRT3 lacks deacetylase activity, then one would expect that ATP intracellular levels would be similar to Control pcDNA+DOX and not significantly lower, unless the mutant overexpression has a detrimental effect on cell viability, which is not in accordance with the SRB results. The next set of experiments aimed to determine cell death and caspase 3 and 7-like activities, in non-treated and DOX-treated cells (Figures 3.3.4 and

3.3.5). The overexpression of hSIRT3 after DOX treatment significantly decreased cell death in comparison to the control after DOX treatment. Surprisingly, the overexpression of mutant SIRT3 also resulted in decreased cell death when compared with the control. The same outcome is true for DOX-induced caspase-like activity, which significantly decreased after hSIRT3 and mutant SIRT3 overexpression, when compared with the control pcDNA+DOX. These unexpected results once again made us suspect that the mutant overexpression is also rescuing cells from DOX-induced apoptosis. The reason for this result is unknown and to the best of our knowledge there is no study which may explain this. Although just in theory, one can predict that SIRT3 might have another enzymatic activity independent from deacetylation, this and the fact that the localization of SIRT3 might not be only in mitochondria, but also in the nucleus, might possibly explain our findings (Hallows *et al.*, 2008). Further studies need to be developed to understand the role of the mutant form of SIRT3 in DOX-induced toxicity in H9c2 cells. Perhaps different plasmid concentrations, as well as different time-points should be tested with the mutant SIRT3 overexpression, in order to achieve the experimental optimal conditions.

As a side note, it is interesting that native and mutant SIRT3 over-expression inhibited apoptosis without having an effect on DOX-decrease cell mass, as measured by the SRB technique. The fact that this technique also assesses decrease in cell number caused by DOX-induced arrest in proliferation may be a good explanation.

In conclusion, the present study results in more questions than answers. Although over-expression of hSIRT3 appears to result in increased protection against DOX toxicity, there are questions regarding the mechanisms involved. These questions are increased facing the conflicting results obtained when using different antibodies and which must be

clarified. Still, whether SIRT3 has a physiological effect against drug-induced toxicity is still an open discussion. Future studies are necessary with different cell media, such as glucose-free, galactose-containing medium, which forces the cells to rely on mitochondria to produce ATP, since DOX is known to be a mitochondrial toxicant. In this manner, we would be able to better understand whether functional differences of SIRT3 exist dependent on the metabolic status of the cells. Also, oxidative damage should be accessed, in addition to p53 and BAX protein expression after overexpressing cells with hSIRT3 and DOX treatment. Furthermore, alterations of mitochondrial membrane potential and impact of SIRT3 activity must also be determined. Future work will provide a better understanding of the role of SIRT3 in distinct cell lines with various levels of SIRT3, specially once antibody-based antibody detection is clarified. Such studies will uncover the actual role of SIRT3 in cardioprotection against different cardiotoxic stimuli, including drug-induced alterations. In fact, one of the facts behind IDR may be intrinsic mitochondrial defenses which are host-dependent. It is not totally unlikely that specific tissue content/activity of different sirtuins, can impact the response of an organ to a certain drug.

Acknowledgements

This part of work had the collaboration of:

- Mark Stevens (PhD, NHLBI): general collaboration
- Bradley Webster (PhD/MD student, NHLBI): general collaboration
- Kye-Young Kim (PhD, NHLBI): qRT-PCR experiments.

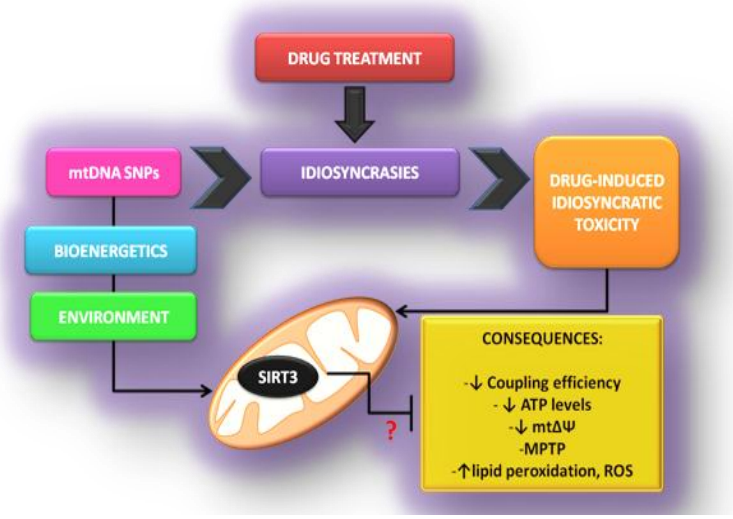
Highlights of Chapter 3.3:

- H9C2 cells have low SIRT3 mRNA content under the experimental conditions of this study.

- Conflicting results exist when using two different commercial antibodies against SIRT3, which may indicate that previous results from the literature may have been erroneous.

- hSIRT3 and mutant hSIRT3 overexpression in H9c2 cardiomyoblasts decreased cell death and caspase 3 and 7-like activities induced by DOX treatment, when compared with the control pcDNA.

- SIRT3 may have a dual role depending on the cell type and tissue, so the possible use of SIRT3 activators, in the treatment of different pathologies, warrant caution.



4- Final Conclusions and Future perspectives

Adverse drug reactions (ADRs) are a major problem for pharmaceutical companies and can also be fatal to patients. The major problem is that these reactions occur in a minority of patients but still can lead to drug withdrawal from the market. This is a serious complication since idiosyncratic drug reactions (IDRs) can worsen a disease condition and at the moment, the only way to avoid such reactions and to prevent a heavy financial burden to the pharmaceutical company is to withdrawn the drug from the market.

As it was debated in sections 1 and 3 of Chapter 1, the lack of *in vitro* and *in vivo* models to predict drug toxicity and thus later avoid these type of reactions, is still very scarce and is limited. The time course of the experiments and the lack of appropriate models which can simulate the disease conditions and drug-induced idiosyncratic reactions is extremely difficult to achieve. Despite most used animals are normal, non-diseased and not being representative of the genetic diversity found in humans, there are still some animal models, such as the $SOD2^{+/-}$ mouse, which can be used to demonstrate the role of mitochondria in IDRs. This type of models, with increased mitochondrial alterations, can now be used to investigate the potential of a certain molecule to cause mitochondrial-mediated injury and organ toxicity. Having this in mind, we decided to develop a new *in vitro* model which can mimic the genetical diversity which is known to contribute, in part, for the mechanisms of IDRs. In this case, we sum possible nuclear DNA variation with single nucleotide polymorphisms (SNPs) found in mtDNA. A panel of mouse embryonic fibroblasts, which contained different mtDNA SNPs was characterized in terms of mitochondrial bioenergetics and further investigated for drug-induced toxicity. The results showed that all the four strains (C57BL/6J, MOLF/EiJ, PERA/EiJ and CZECHII/EiJ) had different mitochondrial

fitnesses (Chapter 3.1). Single nucleotide polymorphisms in the four strains were mainly found in complex I and IV, which is not surprising since the majority of mutations occurs in these two complexes. Complex I is particularly sensitive to damage and therefore, to mitochondrial drug-induced toxicity (Pitkanen and Robinson, 1996).

We also aimed to understand if cell culture aging could be important and would increase the number of SNPs in the mtDNA of the tested strains. The results showed that from passage 3 to passage 10, there was no significant increase in the number of SNPs, with the exception of CZECHII/EiJ, although it did not result in amino acid changes. Perhaps we should have tested a higher passage number, since MEFs do not immortalize before passage 15 (Bao *et al.*, 2010), and so this would be a better passage number to test our hypothesis. Importantly, we found differences in the spare respiratory capacity and maximum respiratory capacity of the strains (see chapter 3.1). PERA/EiJ and CZECHII/EiJ showed lower spare and maximum respiratory capacity when compared with the other cell lines. Nevertheless, the SNPs data did not correlate with these findings since MOLF/EiJ was found to be more philogenetically closed to CZECHII/EiJ while PERA/EiJ was more philogenetically closer to the reference strain, C57BL/6J.

Overall, complex activity data and mitochondrial membrane potential analysis showed that CZECHII/EiJ would be a better candidate for increased susceptibility to drug-induced toxicity. Next, we decided to test different mitochondrial toxicants, which have been described to cause idiosyncratic reactions, in the four cell strains. Not surprisingly, of the four strains tested, CZECHII/EiJ showed the largest IC_{50} ratio between Glucose:Galactose media with rotenone, nefazodone, ketoconazole and tolcapone (see Chapter 3.2). This is in accordance with the idea that if we

have a cell model with lower mitochondrial robustness, high predisposition to drug-induced toxicity can be found.

Even though we attempted to create a new *in vitro* model to better predict drug-induced mitochondrial toxicity and IDRs, further studies need to be done in order to validate this model. The next step would be to measure oxygen consumption and determine the spare respiratory capacity as well as the maximum respiratory capacity, after treating the cells with the previously described mitochondrial toxicants. Since Complex I is a source of ROS (Kushnareva *et al.*, 2002), an attractive experiment would be to determine superoxide anion content in mitochondria by using MitoSox Red testing whether a higher number of SNPs affecting complex I would translate in altered superoxide anion production under basal conditions and after treatment with a mitochondrial toxicant. In addition, the mtDNA copy number of the four strains of MEFs could also be determined. An interesting study would be to use human fibroblasts with the same mtDNA SNPs and compare with the results obtained in the mouse embryonic fibroblasts. It would be important to extrapolate the data that was obtained in our *in vitro* studies to sets of human cells, and therefore, increase the predictive potential of the experimental method. Still, it is not clearly defined what is the role of each SNP in altered mitochondrial bioenergetics, since nuclear DNA SNPs also play a role. In order to circumvent this, a possibility would be by using chimeras (Yamaoka *et al.*, 2000), meaning by fusing cells with the same mtDNA content with different nuclei. Although difficult and time-consuming, this could really shed some light on the role each mtDNA SNP plays in regulating mitochondrial bioenergetics and drug-induced toxicity.

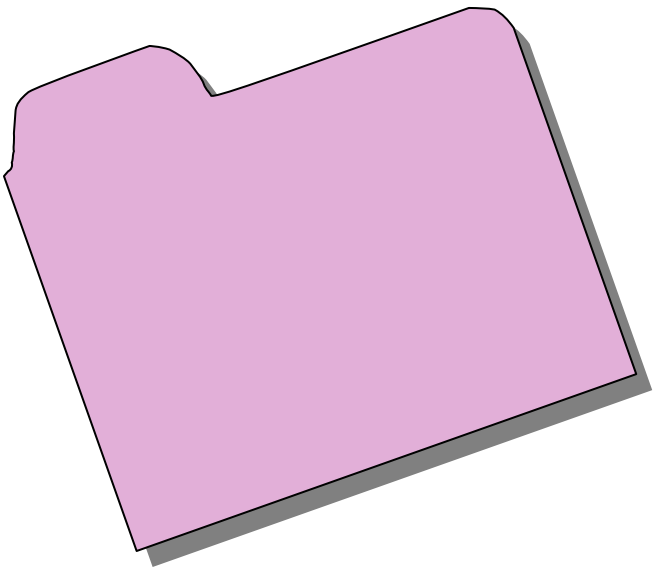
The second part of this thesis focused on the role of SIRT3 in the regulation of drug-induced toxicity, trying to predict its importance for

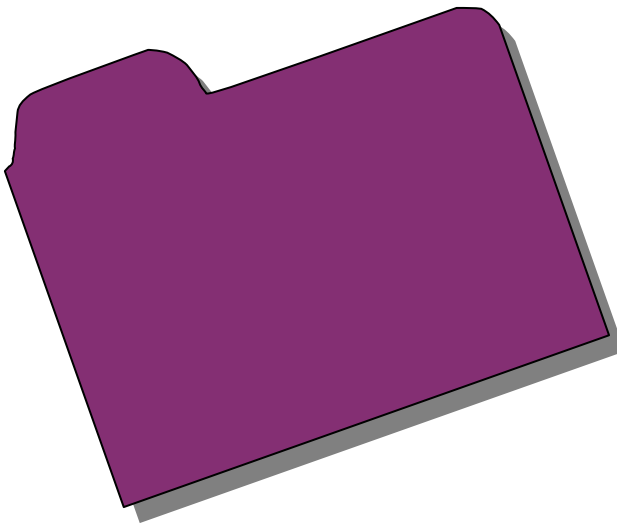
IDRs. We hypothesized that if we overexpressed SIRT3 in H9c2 cells, the toxicity of some cardiotoxic agents including DOX, could be decreased. It is well known that DOX presents a dose-dependent cumulative cardiotoxicity, although technically DOX toxicity is not considered idiosyncratic for humans. Still, DOX is widely used to treat a variety of cancers. It has been proposed that DOX suffers redox cycle on mitochondrial complex I (Wallace, 2003), increasing ROS production and cell death, when the damage surpasses a certain threshold. SIRT3 has been pointed to be involved in the protection against oxidative damage, and it is known to deacetylate complex I subunit NDFU9. The fact that SIRT3 regulates p53 activity (Li *et al.*, 2010) led us to hypothesize that SIRT3 may decrease DOX toxic effects. Importantly, we found that hSIRT3 overexpressing cells treated with DOX showed decreased cell death suggesting that SIRT3 may protect cells against DOX-induced apoptosis, in H9c2 cells. Interestingly, the inactive deacetylase form of SIRT3 also protected cells in a similar manner. This result was hard to explain since it goes against what is described in the literature. The catalytically inactive form of hSIRT3 is a negative control of the effects of the active hSIRT3. Further experiments need to be conducted in order to understand this controversial result. Measurements of cell mass (Fig. 3.3.2, Chapter 3.3) demonstrated that cell mass is lower after transfecting the cells with the mutant (although not statistically significant), with the cell viability (Fig. 3.3.3, Chapter 3.3) also showing a slight decrease. In this experimental set-up, different measurements should be further performed such as measurement of oxidative stress and antioxidant defenses, caspase activity, cell death and deacetylase activity. Besides, the mitochondrial membrane potential and cellular respiration measurements should also be performed in order to explore this topic. In addition, protein expression levels of proteins involved in cell death mechanisms activation such as p53 and BAX, would

be interesting to determine. The same should be performed in the H9c2 cells. Also, we have not done any assay to investigate total and SIRT3-dependent protein deacetylation. Similar protein content in distinct cell lines may present different activity. The different expression of SIRT3 in distinct tissues may predict distinct downstream effects and metabolic consequences; for example SIRT3 can act as a tumor promoter in certain conditions and as a tumor suppressor in others (for further reading on this topic see Alhazzazi *et al.*, 2011b comprehensive review).

We report here a very recent study which aimed to investigate whether inherited mitochondrial DNA variants modulate the expression profiles of mammalian sirtuins, under oxidative stress conditions. This study comprised three components of the cellular stress response, mitochondrial DNA variability, mitochondrial functionality and SIRT3 (D'Aquila *et al.*, 2012). Patrizia et al (2012), performed the experiments in cybrid cell lines, obtained by repopulating 143B.TK$^-$ osteosarcoma Rho^0 cells with foreign mitochondria presenting different mtDNA, classified within H and J haplogroups. It was shown that under cellular stress, mtDNA affected SIRT3 gene expression and shed a light in the importance of mtDNA variations in the response of SIRT3, under oxidative stress conditions. Importantly, future studies with SIRT3 should be performed in a genetically diverse panel of cells which can account with the variations of SIRT3 expression due to genetic factors, and not only due to oxidative stress and metabolic status of the cells. Other experiments could be performed in order to link Chapter 3.1 and 3.3, since the panel of mouse embryonic fibroblasts could be used also to screen SIRT3 expression and activity levels and therefore, understand the role of SIRT3 in the mechanisms of protection or toxicity during drug-induced liabilities.

To conclude and from the results of this thesis, we can conclude that mtDNA variants and SIRT3 activity may impact drug responses and thus have an important role in IDRs. Finally, in the future, the use of molecules which can target SIRT3 to modulate its activity should be seen with caution since SIRT3 might have a dual role in cell survival and in different metabolic processes, including those leading to carcinogenesis.





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