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Chromosome instability in Fanconi Anemia: searching for therapeutic drugs to prevent the progression of the disease

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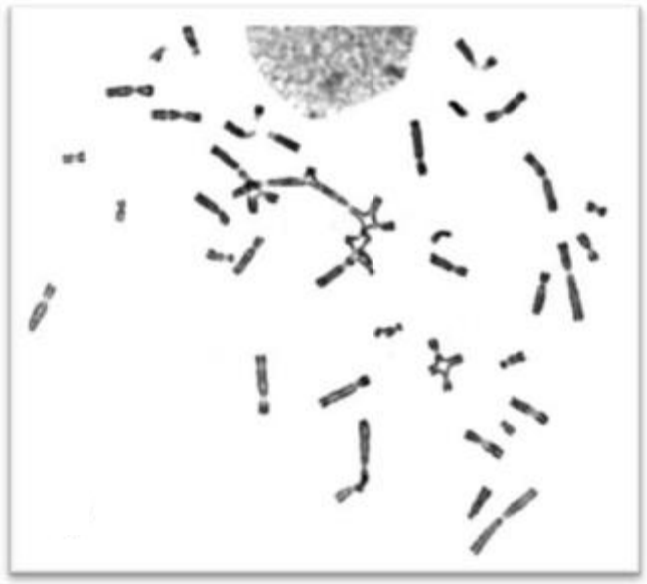

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The author states to have afforded a major contribution to the conceptual design, technical execution of the work, interpretation of the results and manuscript preparation of the published or under publication articles included in this thesis.

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Sousa R, Ponte F, Porto B (2011) Accuracy in the cytogenetic diagnosis of Fanconi Anemia and other chromosome instability syndromes. *Chromosome Research*, 19 (1) S36.

Ponte F, Carvalho F, Porto B (2010) Acute toxicity of diepoxybutane to mononuclear leukocytes. Multifactorial mechanisms? *Toxicology Letters,* 196 Suppl, S86.

Ponte F, Carvalho F, Porto B (2009) Acute toxicity of diepoxybutane to human mononuclear lymphocytes. *Toxicology Letters* 189, Suppl, S121.

Sinto-te como um rio
na exata medida
que desvendas caminhos
misteriosos.
Da nascente até ao mar
cruzas-te com obstáculos
na tentativa vã
de pararem a torrente
mas teu rio corre inteiro
cálido, rebelde, impetuoso
sem se perder na paisagem
que desperta outras correntes.

Quando o objetivo é seguir
o caminho é sempre em frente.

Sentes a limpidez das águas
que correm nas tuas veias
e teu caudal majestoso
quando em ti confluem rios
que com o mesmo fulgor
têm pressa na chegada.
Ficas contente e sorris
mas segues o teu traçado
e quando estás já tão perto
quase a desaguar
olhas para ti surpresa
dizes com voz sublime:

- afinal sou uma gota
majestoso
é o mar!

Sinto-te navegar...

Teresa Brinco

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Preface

Four years ago it was straightforward to choose Fanconi anemia (FA) as a model for studying chromosome instability (CI) disorders and relate them with oxidative stress (OS). In the Laboratory of Cytogenetics of Institute of Biomedical Sciences (ICBAS) a special focus has long been given to FA. The blood samples that arrived there were frequently to confirm the diagnosis of FA. Thus, the interest for this disease slowly “grew” inside me and the will to learn more about it became natural. The relation with OS came later, after reading several papers about this disease, in which the main theory to explain the phenotype and the molecular aspects of the disease was impairment in DNA repair system. Notwithstanding this postulate, in my perspective, many aspects were not explained based on it and a conjunction of the two theories would make much more sense. The works of Pagano and co-workers were very important for establishing my perspective. So, the idea about antioxidant treatments simply arose. I even remember the first discussion about this with Professor Beatriz and since the beginning we believed that we could aim to solve some of the FA problems, finding the right antioxidant! And the work began! But I didn't know back then how difficult it was to study such a rare disease...

The works in the Laboratory of Toxicology of Faculty of Pharmacy of University of Porto (FFUP) were the beginning of a long and not always happy journey. It took almost one year to establish the right techniques and procedures. The next step was to test a variety of drugs in healthy donors, in the hope of finding a successful one to test in the blood of FA patients. One after another and the results were always negative…One year later, after testing 8 or 9 compounds, I got the first positive result and a big smile in my face! "Maybe I will achieve something", I thought. Working in FFUP, although it was very difficult, due to the scarcity of space and available material (we were 20 colleagues working at the same time in a class laboratory!!!), it was funny, even when everything went wrong and I wanted to do something and I wasn’t able to! Working in FFUP taught me to deal with stress and to work as a team, a very important lesson to a future scientist.

In the beginning of the third year of my PhD I came back to laboratory of Cytogenetics. The work with FA patients was finally about to start! First we tested one compound with good results! All the good results were celebrated with lots of smiles, because getting blood samples from FA patients was always so difficult… I remember all the phone calls to all the clinicians of North of Portugal to remember them that we needed blood samples of all their patients… The work was hard and the phone calls were almost daily! We had to move clinicians to help us. And the work went on… We waited many weeks for blood samples, we spent many hours on the microscope and in the end, I was tired but happy. Then we tried a combination of compounds and the work gained another shape. The results were simply unbelievable! We wanted to jump but at the same time we

were afraid. Was this real?? More blood please! More calls and the confirmation arrived! It was true, we found something. And the recognition started. First, with the European Cytogenetic Conference where I was selected to present our work and 3 months later the unthinkable happened. I sent an abstract for Fanconi Anemia Research Fund Scientific Symposium, with our results, but to be selected for an oral presentation seemed out of range…I didn't know the real importance of our results. And one day, when I opened my e-mail I saw an answer. I read once, twice, three times until I believe in those words! I had been selected for an oral communication! My colleague Rosa and I screamed, laughed, jumped, cried…and we ran off the lab to go tell Professor Beatriz! After this day I started to believe in me… In October we went to Barcelona for the congress. It was one of the most satisfying experiences in my life. To speak for 300 FA researchers and to listen "congratulations!" from everyone got me with a huge smile on my face and made me feel like I was living a dream. And there, in Barcelona, I saw that my work was valid and more important, that I wanted to do more to save the life of these children, that born fated to an early death. The FA children and parents were there, and seeing them was very exciting and gave us another strength to go on. And of course, getting to know Professor Pagano was another unthinkable happening! Our guru, the one that always associated FA with OS, found me to personally say that he shares the same idea about the work I was developing and that he also has proposed this in his new paper! Could this be real?! And, with this meeting, arose the collaboration for an European Project…

Working with rare disease is not easy. We are a small country and the scarcity of patients is a big challenge. It needs dedication, patience and, above all, to know how to wait! We don´t work with cell lineages, we have to wait for the right patient! We don't put solutions in a machine, click the button and make the results show up! We have to pass many hours and many days counting chromosomes on the microscope, like an ant collecting its food. We don´t have a true government support. We don't have projects accepted for founding, with the justification that it is not a priority to study rare diseases. Once it was even stated that our work was esoteric! We do almost everything by our own! And, sometimes, I feel misunderstood. So, this journey was hard, but now I can smile and think that it is so much better than I thought it would be! Four years ago this was a dream, now I know it's true, and this fills my heart. I feel very close, but I know I won't stop until my work can significantly contribute to save these children.

I devote this thesis to all FA patients and parents, especially to those that continue their hard life battle.

Filipa Ponte

Abbreviations

$^1O_2$ – Singlet oxygen
3-AT – 3-amino-1,2,4-triazole
8-OHdG – 8-hydroxy-2'-deoxyguanosine
ALC – Acetyl-L-carnitine
AML – Acute myeloid leukemia
ANC – Absolute neutrophil count
ATM – Ataxia telangiectasia mutated protein
AIDS – Acquired immune deficiency syndrome
BMF – Bone marrow failure
BMT – Bone marrow transplant
CDU – Cyclohexyl-3-dodecyl urea
CHX – Cycloheximide
CI – Chromosome instability
CYP – Cytochrome
DC – Dendritic cells
DEB – 1,2:3,4-diepoxybutane
DHPLC – Denaturing high performance liquid chromatography
DHLA – Dihydrolipoic acid
EBV – Epstein-Barr virus
EH – Epoxide hydrolase
EPO – Erythropoietin
FA – Fanconi anemia
FARF – Fanconi Anemia Research Fund
FOXO 3a – Forkhead box 3a
G-CSF – Granulocyte colony-stimulating factor
GM-CFS – Granulocyte macrophage colony-stimulating factor
GR – Glutathione reductase
GSH – Reduced glutathione
GPx – Glutathione peroxidase
GST – Glutathione S-transferase
GSTP1 – Glutathione S-transferase P1
GSTT1 – Glutathione S-transferase T1
H_2O_2 – Hydrogen peroxide
HClO – Hypochlorous acid
Hb – Hemoglobin

HbF – Fetal hemoglobin

HD – Healthy donor

HO-1 – Heme oxygenase 1

HSTC – Hematopoietic stem cell transplantation

ICL – Interstrand cross-link

IFAR – International Fanconi Anemia Registry

IFN-γ – Interferon gamma

IL – Interleukin

IR – Ionizing radiation

LPS – Lipopolysaccharides

MDS – Myelodysplasic syndrome

mEH – Microsomal EH

MLPA – Multiplex ligation-dependent probe amplification

MMC – Mitomycin C

mtDNA – Mitochondrial DNA

NAC – N-acetylcysteine

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

NK – Natural killer

$O_2^{\bullet-}$ – Superoxide anion

•HO – Hydroxyl radical

OS – Oxidative stress

PCR – Polymerase chain reaction

PRDX3 – Peroxyredoxin 3

RBC – Red blood cells

ROS – Reactive oxygen species

SCC – Squamous cell carcinoma

sEH – Soluble EH

SH – Sulfhydryl

SOD – Superoxide dismutase

TNF-α – Tumor necrosis factor alfa

TNF-β – Tumor necrosis factor beta

UVA – Ultraviolet A

Zn^{2+} – Zinc

α-LA – α-Lipoic acid

Resumo

A anemia de Fanconi (AF) é uma doença genética rara, caracterizada pela falha progressiva da medula óssea e elevada predisposição para o cancro, o que implica uma esperança média de vida reduzida. A instabilidade cromossómica (IC) é um dos principais defeitos celulares, e a hipersensibilidade das células AF a agentes "crosslinking", tais como o diepoxibutano (DEB), constitui um marcador único para o diagnóstico. Geneticamente a AF é uma doença muito heterogénea, com 15 genes até agora caracterizados (FANC-A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P). A função destes genes está ligada principalmente à reparação de "DNA crosslinks". O aumento da IC, e consequente aumento do risco para a malignidade, mostram a importância de compreender onde e como falham os mecanismos envolvidos na defesa celular. Além do conhecido envolvimento dos genes AF na reparação do DNA, vários estudos relacionam o fenótipo AF com desequilíbrio redox, demonstrando que o stress oxidativo (SO) é uma das principais causas para os defeitos celulares. Estudos recentes demonstram também defeitos ao nível das mitocondrias das células AF, o que está intimamente relacionado com o aumento da produção de espécies reativas de oxigénio e pela concomitante depleção das defesas antioxidantes, das quais a glutationa (GSH) é um conhecido biomarcador. Além disso, está bem documentada na literatura a relação entre a toxicidade do DEB e o SO, bem como a relação destes com disfunção mitocondrial. Daí que a caracterização das defesas antioxidantes contra a toxicidade induzida pelo DEB possa ser útil para uma melhor compreensão do que está a falhar nas células AF.

Na presente tese o grande objectivo foi conseguir uma abordagem profilática com recurso a antioxidantes, com o intuito de atrasar a progressão dos sintomas clínicos causados pela IC em doentes AF. O plano de trabalhos foi dividido em duas partes distintas mas com objetivos complementares. Na parte I o propósito global foi contribuir para uma melhor compreensão da toxicidade induzida pelo DEB em linfócitos humanos, através do estudo da contribuição de alguns parâmetros bioquímicos potencialmente envolvidos na reatividade deste composto. Este estudo foi realizado em suspensões de linfócitos humanos isolados de dadores saudáveis que foram expostos ao DEB e a uma variedade de compostos, como a ciclohexil-3-dodecil ureia (CDU), a elaidamida, o zinco (Zn^{2+}), a N-acetilcisteína (NAC), a acetil-L-carnitina (ALC), o ácido lipóico (α-LA), o ácido tânico, a 3-amino-1,2,4-traizole (3-AT) e a cicloheximida (CHX). Na parte II o propósito global foi selecionar os melhores compostos testados na parte I e que poderiam ser utilizados para diminuir a IC espontânea e induzida pelo DEB em linfócitos de doentes AF. Dos compostos inicialmente testados somente a ALC, o NAC, o α-LA e o Zn^{2+} foram selecionados para testar, numa primeira fase, em culturas de linfócitos primários de

dadores saudáveis e, numa segunda fase, em culturas de linfócitos primários de doentes AF.

Os ensaios efetuados demonstraram alguns novos e importantes resultados. Relativamente à toxicidade induzida pelo DEB foi claramente evidenciado, pela primeira vez, que a exposição aguda de suspensões de linfócitos humanos a este agente resulta na depleção severa da GSH e na perda de ATP, seguida de morte celular. Além disso, foi demonstrado que a ALC contribui para um significativo efeito protetor da toxicidade induzida pelo DEB, efeito esse potenciado pelo α-LA. Coletivamente, estes resultados contribuíram para aumentar o nosso conhecimento sobre a toxicidade induzida pelo DEB e que foi bastante útil para aplicar nos estudos subsequentes. Os resultados obtidos considerando o objetivo final revelaram que um cocktail com α-LA e NAC melhora drasticamente a estabilidade genética em culturas de linfócitos de doentes AF *in vitro*, diminuindo a IC em 60% e 80% em culturas primárias de doentes AF e doentes AF com mosaicismo e/ou quimerismo, respetivamente. Os resultados finais apresentados nesta tese sugerem que o cocktail estudado deve ser usado como uma medida profilática para atrasar a progressão dos sintomas clínicos da doença causados pela IC, e portanto atrasar a falha progressiva da medula óssea e diminuir a suscetibilidade ao desenvolvimento de cancro. Desta forma, este estudo revela uma elevada importância clinica, pelo que é esperado que inspire os clínicos a começar ensaios clínicos do tipo III, especialmente porque tanto o α-LA como o NAC são compostos pouco dispendiosos e que apresentam um perfil farmacocinético bom e seguro, tendo sido já aprovados para uso humano.

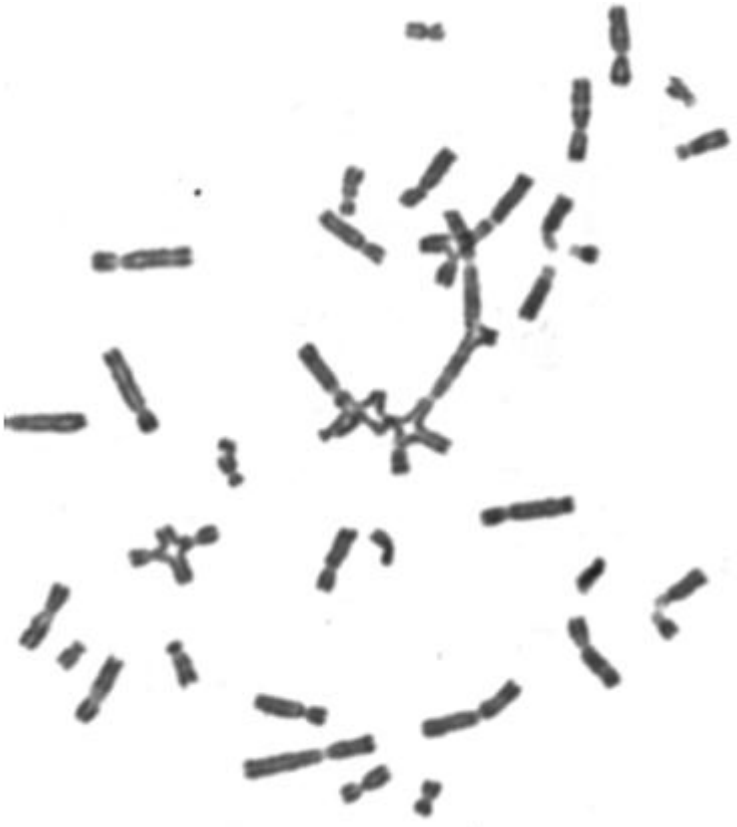
Abstract

Fanconi anemia (FA) is a rare genetic disorder, characterized by progressive bone marrow failure and increased predisposition to cancer, which accounts for a reduced life expectancy. Chromosome instability (CI) is a major cellular defect and the unique hypersensitivity of FA cells to interstrand crosslinking agents, such as diepoxybutane (DEB), is an exclusive marker for the diagnosis. Genetically FA is a very heterogeneous disease, with 15 genes so far characterized (FANC-A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P). Functions of these genes are mostly attributed to DNA interstrand crosslinks repair. The increased CI with consequent increased risk of malignancy highlights the importance of understanding where and how the mechanisms involved in cellular defense fail. Apart from the known involvement of FA genes in DNA repair, several studies have related redox imbalances with FA phenotype, showing that oxidative stress (OS) is a major feature in FA's cellular defect. Recent studies point to defective mitochondria in FA cells, which is closely related with increased production of reactive oxygen species and concomitant depletion of antioxidant defenses, of which glutathione (GSH) is a well-known biomarker. Besides, established literature also relates DEB toxicity to OS and mitochondrial dysfunction, and so characterization of oxidant defenses against this toxicity can also help to understand what is failing in FA cells.

In the present thesis the big aim was to get an antioxidant prophylactic approach to delay progressive clinical symptoms in FA patients caused by CI. The work plan was divided in two distinct parts, with complementary objectives. In part I the global purpose was to contribute for a better understanding of DEB-induced toxicity to human lymphocytes, by studying the putative contribution of biochemical pathways postulated to be involved in the reactivity of this compound. This study was done in freshly isolated human lymphocyte suspensions from healthy donors (HD) exposed to DEB and to a variety of compounds, such as cyclohexyl-3-dodecyl urea (CDU), elaidamide, zinc (Zn^{2+}), N-acetylcysteine (NAC), acetyl-L-carnitine (ALC), α-lipoic acid (α-LA), tannic acid, 3-amino-1,2,4-triazole (3-AT) and cycloheximide (CHX). In part II the global purpose was to select the best compounds found in part I that could turn out to be useful to prevent spontaneous and DEB-induced CI in lymphocytes from FA patients. From the compounds firstly tested only ALC, NAC, α-LA and Zn^{2+} were selected to test in primary lymphocyte cultures from HD and FA patients, to study the spontaneous and DEB-induced CI.

The results showed some novel and important findings. Relatively to DEB induced toxicity it was clearly evidenced, for the first time, that acute exposure of freshly isolated human lymphocytes to DEB results in severe GSH depletion and loss of ATP, followed by cell death. Moreover, it was demonstrated that ALC elicits a significant protective effect on

DEB induced toxicity, which was potentiated by α-LA. Collectively, these findings contribute to increase our knowledge of DEB-induced toxicity and they were very useful to apply in the subsequent studies. The obtained results considering the ultimate goal revealed that a cocktail of α-LA and NAC can drastically improve the genetic stability in FA lymphocytes *in vitro*, decreasing CI by 60% and 80% in cultures from FA patients and FA mosaic/chimera patients, respectively. The final results presented in this thesis suggest that the studied cocktail can be used as a prophylactic approach to delay progressive clinical symptoms in FA patients caused by CI, which can culminate in the delay of the progressive bone marrow failure and decrease in the predisposition to cancer development. Therefore this study is of great clinical importance and it is expected that it will hopefully inspire clinicians to begin phase III clinical trials, especially because α-LA and NAC are inexpensive drugs that present a good and safety profile, being already approved for human use.



PART I – GENERAL INTRODUCTION

Introduction: Fanconi anemia, from the past towards present

Fanconi anemia (FA) was first described in 1927 by the Swiss pediatrician Guido Fanconi (Fanconi, 1927), who described a familial form of aplastic anemia in three brothers between the ages of 5 and 7 with short stature, hypogonadism, skin pigmentation and pancytopenia. Fanconi's observations formed the basis for the diagnosis of FA for many years and included hyperpigmentation, pancytopenia, small stature, skeletal malformations, urogenital malformations and familial occurrence. In earlier times, FA children had the inevitable outcome of death, due to progressive aplastic anemia, with no supportive care available. In the first part of the twentieth century, the advent of modern blood banking allowed the clinician to stem the immediacy of anemia with transfusions. Another major problem became infection, even with the development of antibiotics. Neutropenic infections were generally not well tolerated and typically not cured with antibiotics alone, and many FA children succumbed to bacterial and fungal infections. Finally, even when a child could be supported through the huge problem of aplastic anemia, the looming problem of acute myelogenous leukemia (AML) inevitably and inexorably presented itself. It was the exceptional rare patients who survived to adulthood (Green and Kupfer, 2009).

During many years the diagnosis of FA was only based on clinical observed features and most cases remained unknown. In 1981 the diagnosis gained accuracy through the work of Auerbach and co-workers (Auerbach et al., 1981). It was well evidenced, for the first time, that FA lymphocytes were hypersensitive to the clastogenic effect of DNA crosslinking agents, such as mitomycin C (MMC) and diepoxybutane (DEB). Since that time, the chromosome fragility test with MMC, and especially with DEB, provides the unique marker for the diagnosis of FA. According to these findings, this cellular characteristic can be used to identify pre-anemic cases, as well as patients with aplastic anemia, who do not have characteristic physical signs. Besides, it is now known that FA patients may also present a phenotype similar to Seckel syndrome, Nijmegen breakage syndrome, Dubowitz syndrome, Holt-Oram syndrome, thrombocytopenia absent radius syndrome, Townes-Brocks syndrome, Saethre-Chotzen syndrome (TWIST1 mutation), velocardiofacial syndrome, Diamond-Blackfan anemia, and dyskeratosis congenita (Auerbach, 2009). Therefore, the clinician must recognize the considerable overlap of FA phenotype with these other syndromes and not be misled by preexisting 'diagnostic labels'. The issues of misdiagnosis and therefore mismanagement have decreased with the applicability of chromosome fragility test, as a gold standard for the diagnosis.

FA is found in all populations and ethnic groups. It has been widely reported to have a carrier frequency of 1 in 300 in Europe and United States, the incidence being

approximately three per million births. The true gene frequency is likely to be considerably higher than this; a low estimate can result from an incomplete ascertainment of positive cases before the widespread application of chromosomal breakage tests for FA diagnosis. In 1982, in order to study a large number of patients with a so rare genetic disorder, and find the full spectrum of diverse features of the syndrome, including chromosome instability (CI), the International Fanconi Anemia Registry (IFAR) was established at The Rockefeller University. The registry serves as a centralized repository for clinical and genetic information on patients with FA, as well as biological samples from patients. Patients with one or more clinical features associated with FA are referred to the IFAR by their physicians. Patients in the IFAR have the diagnosis confirmed by chromosomal breakage studies, mostly using the DNA crosslinking agent DEB. In agreement with IFAR, in 1989 was founded the Fanconi Anemia Research Fund (FARF), Inc., to provide support to FA families and to raise money for scientific research.

With the support of IFAR and FARF and with the progressive advances of technology and science, particularly in the field of molecular biology, FA is now a well-known genetic disease. So far 15 genes have been characterized and cloned, which are responsible for the known FA complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P) (Kim et al., 2011). The discovery of such genes gave rise to new knowledge about the pathogenesis of the disease and also provided new methodologies for the accurate diagnosis. Approximately 85% of all IFAR patients with known complementation group are defective in one of the three most common disease-causing genes *FANCA*, *FANCC*, *FANCG.* The FA proteins have been extensively studied and divided according to their function. It is consensual that FA genes and proteins function as a FA pathway in the repair of DNA damage, most probably caused by excess of oxidative damage. Although this field has undergone a long way within the past decades, there is still much to learn. Elucidation of the complexities of the FA pathway and its relation with oxidative pathways will ultimately allow for more individualized and efficacious treatment of FA patients.

Recent years have revolutionized the medical care of FA patients. Blood counts can be improved with androgens administration and hematopoietic growth factors can be used in cases of neutropenic infections (Guinan et al., 1994; Rackoff et al., 1996). However, hematopoietic stem cell transplantation (HSCT) is at present, the only curative therapy for the hematologic manifestations. Although HSCT is being performed for almost 30 years, and it is, till now, the truly hope for FA patients, it was only in recent years that such approach has been done more safely and successfully. Even with greater survival of children into adulthood as a result of HSCT the potential development of solid tumors, such as squamous cell carcinomas (SCC) of the head, neck and genitourinary track, remains a serious problem.

We are now in the era of genomics, proteomics, gene therapy, embryonic stem cells and induced pluripotent cells, and all these new fields are being used for future therapies and for a better understanding of FA. Despite all improvements in therapy, FA patients continue, nowadays, to die very early due to progressive bone marrow failure (BMF) and cancer development. It is time to do something more, to avoid such inexorable fate and increase the hope. And the way must pass through prevention, although that hasn't been properly addressed in FA. Sometimes, it is with simple thoughts, simple methods and simple ways that science makes great advances, as we hope to contribute with the present thesis.

1. Clinical features of Fanconi anemia

1.1. Fanconi anemia phenotype

FA is a rare genetic disorder with an estimated incidence of 1:360,000 births, based on an assumed carrier frequency of 1:300 and an autosomal recessive model (Swift, 1971). In some populations, like Ashkenazi Jewish, Spanish Gypsy and black South African, the carrier frequency of FA is estimated at around 1:100 (Callén et al., 2005; Kutler and Auerbach, 2004; Morgan et al., 2005). This disorder is characterized by several congenital malformations, progressive BMF and higher predisposition to cancer. However, the clinical phenotype is not always conclusive for the diagnosis, since 25% of FA patients are phenotypically normal (Shimamura and Alter, 2010). With a so complex clinical condition the average life expectancy of FA patients is around 20 years, and patients reaching the age of 50 are extremely rare (Kalb et al., 2006).

1.1.1. Congenital malformations

The likelihood of physical abnormalities is approximately 75%, though generally these are not a cause of mortality in individuals with FA (Alter and Kupfer, 2002 (update 2011)). The most commonly reported abnormalities and their frequencies are described in Table 1.

Table 1. Congenital malformations in patients with Fanconi anemia (adapted from Shimamura and Alter, 2010).

Low birth weight		5%*
Microsomia	Short stature	40%
Skin	Generalized hyperpigmentation; café au lait spots, hypopigmented areas	40%
Skeletal		
Upper limb, unilateral and bilateral		35%
Thumbs	Absent or hypoplast, bifid, duplicated, rudimentary, attached by thread, triphalangeal, long, low set	35%
Radii	Absent or hypoplastic (only with abnormal thumbs), absent or weak pulse	7%
Hands	Flat thenar eminence, absent first metacarpal, clinodactyly, polydactyly	5%
Ulnae	Dysplastic, short	1%
Lower limbs		5%
Feet	Toe syndactyly, abnormal toes, club feet	5%
Legs	Congenital hip dislocation	5%
Neck	Sprengel deformity, Kippel-Fiel anomaly, short, low hairline, webbed	1%
Spine	Spina bifida, scoliosis, hemivertebrae, abnormal ribs, coccygeal aplasia	2%
Craniofacial		
Head	Microcephaly	20%
Face	Triangular, birdlike, dysmorphic, micrognathia, mid-face hypoplasia	20%
Eyes	Small, cataracts, astigmatism; strabismus, epicanthal folds, hypotelorism, hypertelorism, ptosis	20%

Renal	Kidneys: horseshoe, ectopic or pelvic, abnormal, hypoplastic or dysplastic, absent; hydronephrosis or hydroureter	20%
Gonads		
Males	Hypospadias, micropenis; undescended testes, absent testes	25%
Females	Bicornuate uterus, malposition, small ovaries	2%
Developmental delay	Intellectual disability, developmental delay	10%
Ears	Hearing loss, abnormal shape	10%
Cardiopulmonary	Congenital heart defect	6%
Gastrointestinal	Esophageal, duodenal, jejunal atresia; imperforate anus; tracheoesophageal fistula; annular pancreas; malrotation of the gut	5%
Central nervous system	Small pituitary, pituitary stalk, interruption syndrome, absent corpus callossum, cerebellar hypoplasia, hydrocephalus, dilated ventriculus	3%

* Percentages are calculated from 2000 cases reported in the literature from 1927 to 2009. Frequencies are approximate, since many reports did not mention physical description.

The clinical manifestations can be highly variable. Some patients can have many congenital malformations at the same time, while in others none of such findings are observed at the time of diagnosis. Furthermore, other non-FA disorders can also have many of the clinical findings associated with FA. In conclusion, the presence or absence of physical malformations does not fully establish, neither rule out the diagnosis of FA.

1.1.2. Hematologic manifestations

Hematologic abnormalities in FA patients typically occur within the first decade of life, at a median age of 7 years, but are highly variable. Thrombocytopenia is often associated with elevated levels of fetal hemoglobin (HbF) and macrocytosis, and usually precedes onset of anemia or neutropenia. Pancytopenia generally worsens over time, and can be present as early as in the newborn period. Sweet syndrome (neutrophilic skin infiltrations) has been reported in a few individuals with FA and myelodysplasic syndrome (MDS).

BMF is clinically manifested by blood counts that are below age-appropriate values due to decreased effective marrow hematopoiesis. While many patients progress to frank aplastic anemia, others may remain at mild abnormal levels indefinitely. Clinical surveillance and therapeutic management are guided by the severity of the cytopenias, the stability of the blood counts, the presence of morphologic and cytogenetic marrow abnormalities, and potentially high-risk genotypes such as *FANCC, FANCD1/BRCA2* or *FANCN* mutations (Shimamura, 2008). BMF was classified into three broad categories, depending upon the degree of cytopenia(s) (Table 2). These definitions are more than semantic; they also define points at which different clinical management options should be considered.

Table 2. Severity of bone marrow failure (adapted from Shimamura, 2008).

	Mild	**Moderate**	**Severe**
ANC	<1,500/mm^3	<1,000/mm^3	<500/mm^3
Platelets	150,000-50,000/mm^3	<50,000/mm^3	<30/mm^3
Hb	≥8g/dl	<8g/dl	<8g/dl

ANC, absolute neutrophil count; Hb, hemoglobin.

Severe BMF may lead to death and requires HSCT. It has a peak hazard rate of about 5% per year at age of 10 years (Alter and Kupfer, 2002 (update 2011); Auerbach, 2009). Data from the IFAR suggest that BMF develops in up to 80% of patients (Seif, 2011).

1.1.3. Impairment in immunological function

The human immune system recognizes, eliminates, and protects the body from viral and bacterial infections, as well as from transformed cells (pre-cancer cell) (Masserot et al., 2008). A variety of cells are actively involved in this process of immune surveillance, including B and T-lymphocytes, natural killer (NK) cells, dendritic cells (DC), macrophages, and polymorphonuclear leucocytes. FA, as mentioned before, is characterized by pancytopenia, which means lower levels of leucocytes, platelets and hemoglobin (Hb), and is a cancer prone disease. One of the first studies that characterized impairment in the immune system of FA patients was reported in 1982.

Hersey and co-workers, (Hersey et al., 1982) found that the immune function of one FA patient revealed selective defects in NK cell activity, which is known to be important in surveillance against tumors. This case report suggested that the absence of NK activity was secondary to a defect in interferon release from lymphocytes on exposure to tumor antigens. They considered that these defects may be an important predisposing factor in the development of malignancy, not only in this patient but possibly other patients with FA. Lebbé and co-workers (Lebbé et al., 1993) reported the same results in another patient, where NK activity was undetectable even with a developing carcinoma.

Another study revealed that both lymphoblasts and fibroblasts from FA patients demonstrated a reduction in interleukin (IL) 6 production. IL-6 is an interleukin that acts as both a proinflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response. This study showed that this lymphokine is not induced by tumor necrosis factors α and β (TNF-α and TNF-β) in FA cells, as is the case in normal cells. It was suggested that the observed deficiency in IL-6 production may account for one of the major characteristics of FA disease, the defect in differentiation of the hematopoietic system (Rosselli et al., 1992). The same group also showed that, in comparison to normal cells, TNF-α is overproduced by FA lymphoblasts from the four genetic complementation groups A, B, C and D. Indeed, up to an eight-fold increase in TNF-α is observed in the growth medium of FA cells. Moreover, addition of anti-TNF-α antibodies partially corrects the FA hypersensitivity to MMC. Treatment of FA cells with IL-6, which partially restored an almost normal sensitivity to MMC, also reduced the TNF-α overproduction in FA lymphoblasts (Rosselli et al., 1994). With this study, the authors concluded that abnormal TNF-α production seems to be associated with the FA genetic background, which was later confirmed by authors of the same group (Briot et al., 2008). One important activity of this cytokine is its cytotoxic/cytostatic effect on normal and cancer cells (Sugarman et al., 1985). Moreover, TNF-α enhances intracellular and extracellular superoxide anion ($O_2^{\bullet-}$), production, and can induce DNA breakage and cell death (Rubin et al., 1988; Yamauchi et al., 1989). This protein is of interest in the context of the FA phenotype, not only for its relation with IL-6 expression, but also for the large spectrum of its biologic activities (Schindler et al., 1990), which are completely deregulated in FA cells. Further investigations using FA mouse models revealed that Fancc(-/-) mice underwent excess inflammatory response, as a result of hematopoietic suppression, that was corrected by wild-type Fancc gene, suggesting a potential role of the FANCC protein in innate immunity (Sejas et al., 2007). Fancc(-/-) mice challenged *in vivo* with lipopolysaccharides (LPS) at doses that induce septic shock have increased peripheral blood levels of inflammatory mediators (Sejas et al., 2007), although it remains unknown what cell type(s) is responsible for this response.

As mentioned above, a number of clinical studies indicate that FA patients have altered levels of circulating cytokines. In addition, it has been suggested that FA patients may have an increased susceptibility to a variety of pathogens (Fagerlie and Bagby, 2006), although it is unclear whether this observation is a result of a subtle immunodeficiency or secondary to leukopenia from evolving BMF. A study of Liu and co-workers, (Liu et al., 2012) provides compelling evidence for a cell-autonomous defect in Fancc(-/-) macrophages. Specifically, functions requiring dynamic cytoskeletal changes are impaired, including adhesion, migration, and phagocytosis, as well as in vivo inflammatory monocyte mobilization and recruitment. Macrophages are a primary line of defense in the innate immune system (Rees, 2010). The biologic functions of macrophages are complex, including elimination of pathogens via phagocytosis and cytokine/chemokine production and repairing damaged tissues during inflammation (Gordon and Taylor, 2005; Rees, 2010). Most of these functions require macrophages to migrate to an inflammatory site, and these dysfunctions could explain the cytokine deregulation and increased susceptibility to pathogens.

Recently it was performed a study that reported a cross-sectional immunological assessment in 10 children with FA, representing the first attempt at a comprehensive quantitative and functional evaluation of immune function (Myers et al., 2011). The study reported a significant, novel, and previously unappreciated abnormality in cytotoxic T cell function, despite normal quantitative evaluation, and significantly reduced NK cell number and function in the majority of these children, this last result being confirmed by previous studies. Additionally, Castello and co-workers (Castello et al., 1998) demonstrated a decrease in CD4+ T lymphocytes, which was not detected in Myers' study. Moreover, Myers's cohort of patients demonstrated a quantitative abnormality in the B cell compartment, with significantly lower absolute number of B cells relative to age-based normal. The phenotype of the remaining B-cells was nearly identical in these patients, showing reduced percentage of CD5 and CD10 expressing B cells. This particular B-cell phenotype would be expected in adults, especially at advanced age (Bleesing, 2004). As B and NK cells mature in the bone marrow, they may probably be most affected as BMF develops (Myers et al., 2011). Figure 1 resumes the information known till now about impairment in immunological system in FA patients and possible relation with FA phenotype.

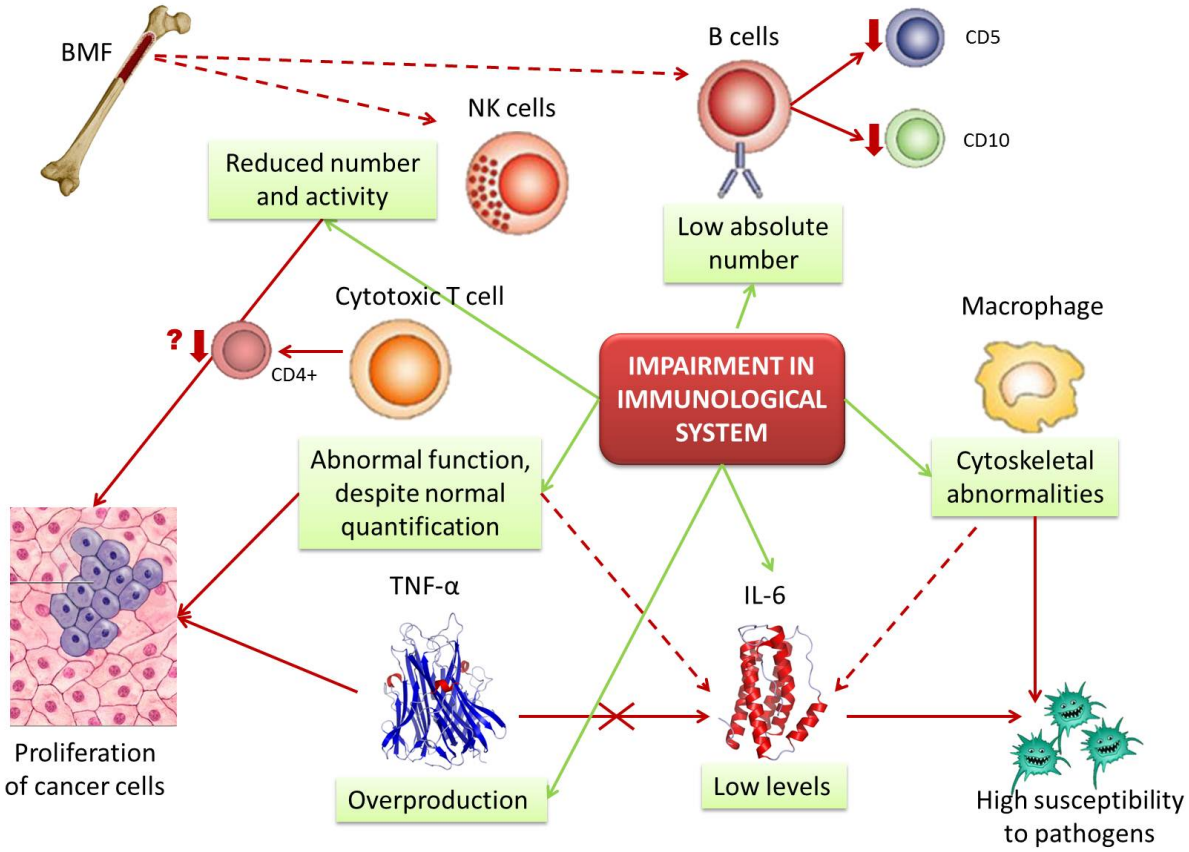


Figure 1. Impairment in immunological function of Fanconi anemia patients. Possible interactions among immunological cells, cytokines and Fanconi anemia phenotype (original figure). Fanconi anemia patients show an impairment in immunological system, characterized by low absolute number of B lymphocytes, abnormal function of T lymphocytes, reduced number of NK cells, cytoskeletal abnormalities in macrophages, low levels of IL-6 and overproduction of TNF-α. In turn, abnormal function of T lymphocytes, reduced number of NK cells and overproduction of TNF-α increase the probability of cancer cells proliferation. The typical deregulation of IL-6 can be aggravated by the abnormal function of T lymphocyte and macrophages, the cells that produce this cytokine. Additionally, overproduction of TNF-α could be regulated by IL-6 activity, but this event apparently doesn't occur. Therefore, with abnormal macrophages and IL-6 production, FA patients have high susceptibility to pathogens and, thus, to infections. In BMF conditions, B cells and NK cells are more affected, as they mature in bone marrow, so their number and activity keep impaired. BMF, bone marrow failure; IL-6, interleukin 6; NK, natural killer; TNF-α, tumor necrosis factor alpha.

1.1.4. Cancer susceptibility

In addition to the early onset of BMF, FA is a cancer prone disorder, being the one with higher risk of cancer development among BMF syndromes. Hematologic malignancies are the first to appear and approximately 10% of patients develop leukemia (Kutler et al., 2003). Over 90% of these hematologic malignancies and pre-malignancies are myeloid in origin, representing a 600-fold increase in risk of AML and more than a

5,000-fold risk increase for MDS compared to the general population (Shimamura and Alter, 2010). In a review of the literature reporting clinical cases of patients with FA, 9% developed AML and 7% developed MDS (Alter et al., 2003). The median age of leukemia diagnosis in FA patients is between 11 and 14 years, with almost all cases arising before 25 years old (Alter et al., 2003; Rosenberg et al., 2003).

Table 3. Malignancies in FA cases cited in the literature between 1927 and 2001 (adapted from Alter, 2003).

Characteristics	All	Leukemia	MDS*	Solid tumors	Liver tumors
No. of cases	1301	116	89	68	37
% of total	100	8.9	6.8	5.3	2.8
Male:female	711:578	70:46	46:42	23:45†	22:15
Ratio	1.2	1.5	1.1	0.5†	1.5
FA diagnosis age (yrs)					
Mean	8.3	10.1†	11.3†	12.7†	9.2
Median	7	8.6	9.3	9	7
Range	0-48	0.13-28	0.2-43	0-44	3-48
Complications age (yrs)					
Mean	–	14.5	15.7	22.6†	15.7
Median	–	14	14	25.5	13
Range	–	0.13-29	1.8-43	0.2-45	6-48
No. of report deceased	488	84	44	41	30
% of report deceased	38	72	51	61	81
Estimate median survival age (yrs)	20	16	21	31†	16
Age on general population (yrs)	68	68	–	47-68	68

* Includes 13 patients who subsequently developed leukemia; † P<0.01 compared with patients without that complication.

Hematologic malignancies are not the only cause of early death in patients with FA. Nonhematologic malignancies are especially striking in these patients. They are unusually young when they develop cancer and the incidence of the malignancy probably would be considerably higher if patients had a longer life expectancy. Most of the nonhematologic tumors in FA patients are solid tumors, especially SCC of the head and neck and anogenital regions. The risk of head and neck SCC is 1000-fold greater in patients with FA than that of the general population, and also occurs at an earlier age (Myers et al., 2011). Epidemiologic analyses strongly suggest that solid tumors will become the predominant clinical problem of post-transplanted FA patients. HSCT is becoming available for a growing number of patients, because of an increased pool of alternative donor options and new transplant protocols. The result is an improved probability of survival to an age when the incidence of solid tumors begins to increase (Auerbach, 2009). There is also a probably of development of liver tumors, especially in patients receiving androgen treatment for BMF. This information is summarized in Table 3.

Interestingly, patients with no congenital anomalies have the highest risk of AML (cumulative incidence 23.7%) (Rosenberg et al., 2008). Significant dysmorphia is more closely associated with BMF and with a much lower total cancer cumulative risk (1.4%), although more seriously affected children may die of BMF before attaining their full cancer risk (Rosenberg et al., 2008). Importantly, in approximately 25% of patients, FA is identified only after the leukemia diagnosis as a result of associated complex cancer-related cytogenetic aberrations or excessive therapy-related toxicity (Alter, 2007; Gyger et al., 1989).

1.2. Fanconi anemia genotype

Based on somatic cell fusion studies, at least 15 complementation FA groups have been identified each one with a corresponding gene. Each of these genes, when biallelically mutated, causes FA. Gene denomination and chromosome location, as well as their proportion among FA patients are summarized in Figure 2 and Table 4 based on the work of Alter and Kupfer (Alter and Kupfer, 2002 (update 2011)) and Green and Kupfer (Green and Kupfer, 2009).

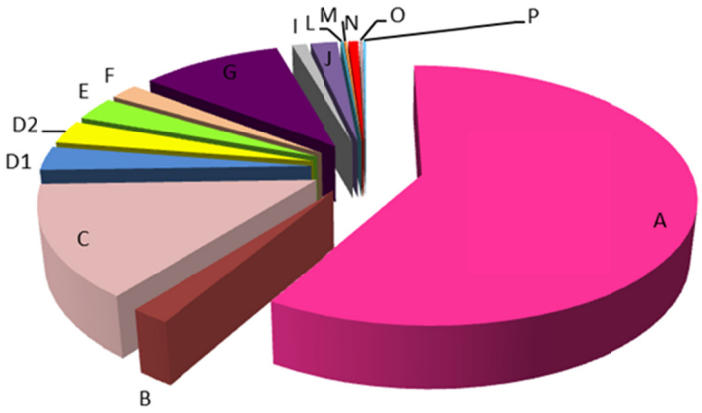


Figure 2. Relative frequency of the FA complementation groups (genes). FANCA, FANCC and FANCG mutations are the most common in general population: however, the frequency can be variable considering the race and ethnic group.

Table 4. The 15 FA genes and their location (adapted from Alter and Kupfer, 2011 and Green and Kupfer, 2009).

Complementation group	Responsible gene	Chromosome location	% of FA attributable to mutation in this gene	References
FA-A	*FANCA*	16q24.3	60% - 70%	(Apostolou et al., 1996; Lo Ten Foe et al., 1996)
FA-B	*FANCB*	Xp22.31	~2%	(Meetei et al., 2004)
FA-C	*FANCC*	9q22.3	~14%	(Strathdee et al., 1992)
FA-D1	*BRCA2*	13q12.13	~3%	(Howlett et al., 2002)
FA-D2	*FANCD2*	3p25.3	~3%	(Timmers et al., 2001)
FA-E	*FANCE*	6p21-22	~3%	(de Winter et al., 2000)
FA-F	*FANCF*	11p15	~2%	(de Winter et al., 2000)
FA-G	*XRCC9*	9q13	~10%	(de Winter et al., 2000)

FA-I	*KIAA1794*	15q25-26	~1%	(Dorsman et al., 2007; Sims et al., 2007; Smogorzewska et al., 2007)
FA-J	*BRIP1/BACH1*	17q22-24	~2%	(Levitus et al., 2005; Levran et al., 2005; Litman et al., 2005)
FA-L	*PHF9*	2p16.1	~0.2%	(Meetei et al., 2003)
FA-M	*FANCM*	14q21.3	~0.2%	(Meetei et al., 2005)
FA-N	*PALB2*	16p12	~0.7%	(Reid et al., 2007; Xia et al., 2007)
FA-O	*RAD51C*	17q22	~0.2%	(Vaz et al., 2010)
FA-P	*SLX4*	16p13.3	~0.2%	(Kim et al., 2011; Stoepker et al., 2011)

These genes account for over 95% of all known FA patients. Some patients do not appear to have mutations in any of these 15 genes, so it can be anticipated that additional FA genes will be discovered in the future.

1.2.1. The FA pathway

FA genes and proteins function together in a common pathway, involved in DNA repair and in the maintenance of genomic stability (Bogliolo et al., 2002b). The encoded proteins for each gene can be subdivided into three groups: (1) eight proteins that make up the core complex of the upstream FA proteins, FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM; (2) the FANCD2 and FANCI proteins, which compose the ID complex; and (3) the downstream effector proteins, FANCD1, FANCJ, FANCN, and presumably FANCO and FANCP (Kim et al., 2011; Moldovan and D'Andrea, 2009; Vaz et al., 2010).

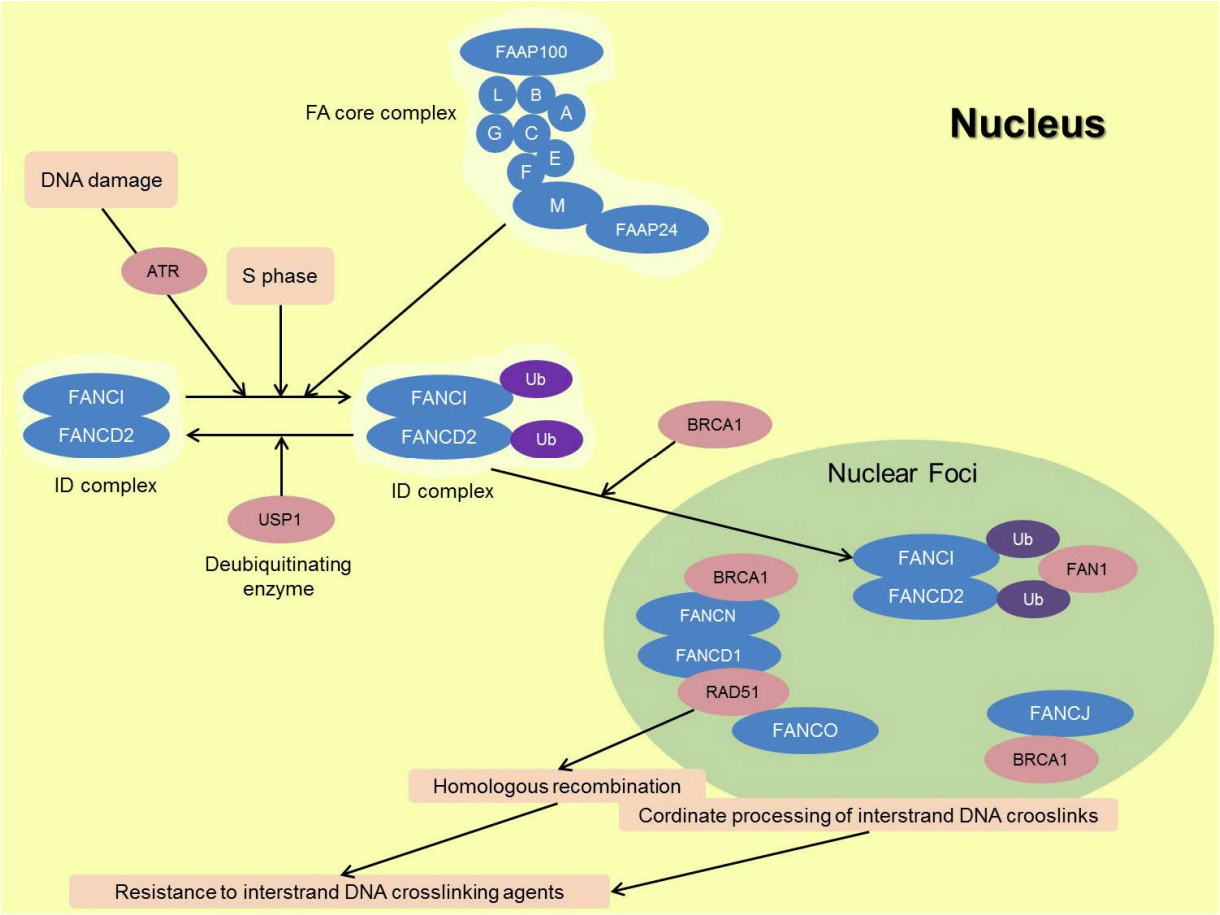


Figure 3. Schematic representation of the FA pathway and associated proteins in response to DNA damage (original figure). A cascade of events starts with DNA damage, being activated the FA core complex, which in turn activates de monoubiquitination of ID complex. These events activate other FA proteins, culminating in the rearrangement of the damage through different repair processes.

In a simple manner and summarizing all this information (Figure 3) the eight upstream FA proteins together with FAAP100 and FAAP24 form a complex with ubiquitin ligase activity, termed the FA core complex. This complex is required for monoubiquitination of FANCD2 and FANCI in response to DNA interstrand crosslink (ICL) lesions during replication in S phase. FANCD2 and FANCI thereafter localize to DNA repair foci together with FA effector proteins and other proteins. BRCA1 is required for FANCD2 foci formation in response to DNA damage. FANCC, FANCE, and FANCG also form nuclear foci and co-localize with FANCD2. All these factors are required for cellular resistance to interstrand DNA crosslinking agents, through homologous recombination, trans-lesion synthesis and some other unknown mechanisms. A few other gene products were found to be associated with the FA protein complexes and are required for FA activation. Thus, it is presumed that their inactivation would lead to FA; however, patients

with such mutations have not yet been described (Hucl and Gallmeier, 2011; Moldovan and D'Andrea, 2009).

Interestingly, many of the FA proteins contain no recognizable motifs. Therefore, discovering their contributions to the FA pathway and the main function of the FA pathway will be an important challenging in the future (Green and Kupfer, 2009).

1.3. Diagnostic tests for Fanconi anemia

Considering the high genetic FA variability and the vast clinical phenotype, with a great overlap with other BMF syndromes, and the particularity that 25% of FA patients are phenotypically normal, a rapid and correct clinical diagnosis is difficult and may be delayed or even missed. Fortunately more accurate diagnostic methods were developed, the most important being the chromosomal fragility test.

1.3.1. The chromosomal fragility test

Schroeder and co-workers were the first to report that FA is a CI syndrome (Schroeder et al., 1964). They first suggested the use of spontaneous chromosomal breakage as a cellular marker for FA, but subsequent studies of CI in more FA patients showed these findings to be inconsistent. Years later, Auerbach and co-workers evidenced that FA cells have an unique hypersensitivity to the clastogenic (chromosome breaking) effect of cross-linking agents and so this characteristic became a reliable cellular marker for the diagnosis of FA (Auerbach et al., 1981). DEB and MMC are the most widely used agents for the diagnosis, but DEB demonstrated to be the one that proportionated more accuracy.

The chromosomal fragility test with DEB is the cytogenetic method for excellence of FA diagnosis (Auerbach et al., 1989). Peripheral blood lymphocytes are cultured in the presence and absence of DEB and a minimum of 50 cells arrested in metaphase are scored and analyzed for chromosomal breakage (the most used parameter is the number of breaks per cell) and formation of tri- and tetraradial figures. These figures are the hallmark of FA diagnosis, since this parameter is the only one that can differentiate FA from the other CI syndromes. Results are compared with those of normal control cells. Normal cells are able to correct most of the damage and are not severely affected by DEB, whereas FA cells show marked chromosome breakage, as can be seen in Figure 4. This test can also be performed prenatally on cells from chorionic villi or amniotic fluid (Auerbach et al., 1981).

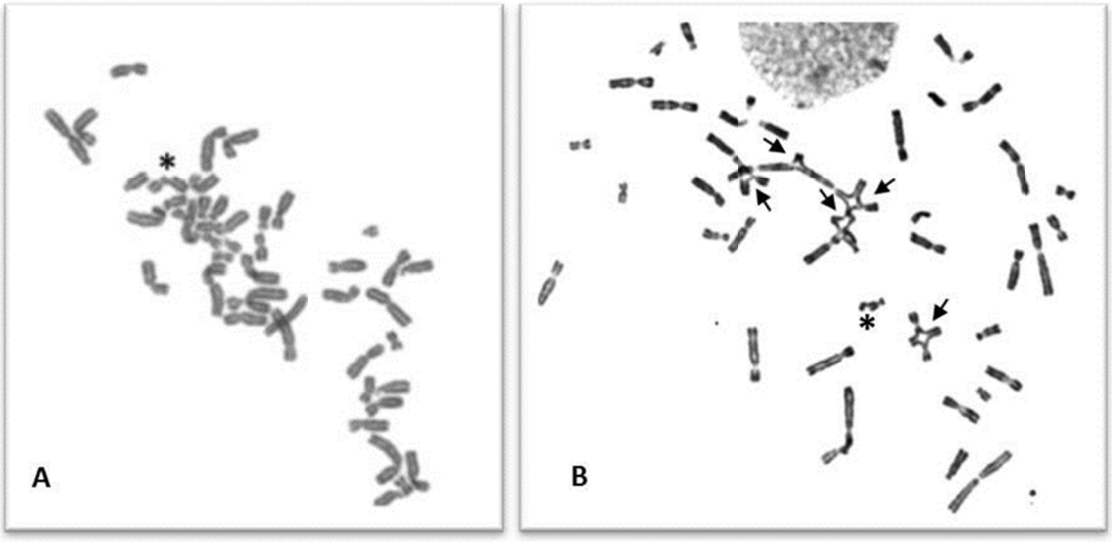


Figure 4. Chromosome instability pattern in two metaphases selected from DEB-induced lymphocyte cultures from a healthy donor (HD) and a FA patient. Chromosomes were stained in a 4% Giemsa solution. The images were observed with an optical microscope. **(A)** Selected metaphase from a HD lymphocyte culture exposed to 0.2 µg/ml of DEB for 48 h. One chromatid break can be seen (asterisk). **(B)** Selected metaphase from a FA patient lymphocyte culture exposed to 0.05 µg/ml of DEB for 48 h. High level of CI can be visualized, the tri- and tetraradial figures (arrow) being especially important, since that represents the hallmark for the diagnosis of FA. It can also be seen a chromatid break (asterisk).

The existence of mosaicism may complicate the FA diagnosis by chromosome breakage tests. Somatic mosaicism is defined as the presence of genetically distinct populations of somatic cells in a given organism and is relatively common in FA (25% of the cases), which can present a cell line showing increased sensitivity to cross-linking agents and the other showing normal levels of chromosome breakage in response to the same agents (Auerbach, 2009). In that case the percentage of cells with aberrations may be more useful than the number of breaks per cell. Somatic mosaicism in FA patients has been shown to be caused by new DNA mutations or the spontaneous reversion of inherited mutations (Gregory et al., 2001; Lo Ten Foe et al., 1997). When there is a suspicious of mosaicism, chromosome fragility test in fibroblast is more accurate than in T-lymphocytes. Recently, a new chromosome instability index (CFI) was established to accurately differentiate mosaic FA patients (Castella et al., 2011).

1.3.2. Other diagnostic methods

Cell cycle arrest. Arrest in G2 is another characteristic of FA cells. Flow cytometry examines cell cycle kinetics and can detect the proportion of cells that are arrested at G2/M after culture with a clastogen such as nitrogen mustard or MMC. In contrast with the

100 cells examined microscopically for chromosomal aberrations, flow cytometry can have the advantage of examining thousands of cells and being less labor-intensive and subjective. However, it requires sophisticated instrumentation. This test is usually done in a specialized laboratory and is not used as widely as the chromosome breakage assay. Flow cytometry may give a false negative result in FA patients with MDS or AML (Alter, 2008).

Immunoblot assay of FANCD2 protein monoubiquitination. Following DNA damage, the complex of upstream FA gene products (A, B, C, E, F, G, L, M) leads to ubiquitination of the product of *FANCD2*, forming a longer protein (D2-L), which can be distinguished from the shorter non-ubiquitinated form (D2-S) on a Western blot with a D2-specific antibody. This relatively inexpensive assay may be useful for screening patients for whom FA is in the differential diagnosis, such as those with radial ray anomalies, short stature, and hypogonadism or *café au lait* spots or for population-based FA incidence studies; however, it is usually a limited tool. FA patients whose gene defect is downstream of *FANCD2 (FANCD1, FANCI, FANCJ, FANCN, FANCP* and *FANCO)* will not be detected with a D2 Western blot, as well as mosaic individuals (Alter, 2008).

Complementation analysis. Patient lymphocytes, EBV (Epstein-Barr virus)-lymphoblasts or fibroblasts can be cultured with retroviruses that introduce known normal FANC genes into the patient's cells, leading to correction of the FA cellular phenotype (Alter, 2008).

Mutation testing. Determination of the specific mutation in FA genes is complicated and is done in laboratories with specific expertise. It requires sophisticated methods and involves DNA amplification, sequencing and detection of large deletions. Many laboratories rely on knowing the complementation group before sequencing, while in some contexts targeted sequencing of candidate genes is more appropriate. One center goes directly to gene sequencing for patients with a positive DEB test: *FANCA* by multiplex ligation-dependent probe amplification (MLPA) for large deletions and full sequencing; *FANCB* by MLPA and full sequencing, if indicated; *FANCC, E, F, G* by denaturing high performance liquid chromatography (DHPLC) and sequencing; *FANCD2* by Western blot; *FANCD2* sequencing if D2 bands are absent; *FANCL* and *FANCM* sequencing if only D2-S is seen; *FANCD1/BRCA2* sequencing, if indicated; *FANCJ/BRIP1* and *FANCN/PALB2* sequencing; and finally *NBS1* and *ESCO2* sequencing for Nijmegen breakage and Roberts syndromes. Mutation testing is used to confirm known cases and for family studies to determine affected or carrier status. Genetic counseling should be included in these processes (Alter, 2008).

1.4. Fanconi anemia treatments

Hematologic complications represent a major problem to FA patients and are a major cause of death. Additionally, SCC is another dramatic problem, especially in adult patients. Some therapies are available, but no one can avoid the drastic problem of CI, which is a common feature to all FA complications.

Hematopoietic stem cells transplantation (HSCT). Also known as BMT is the only curative therapy for the hematologic manifestations of FA, including aplastic anemia, MDS and AML, although it does not cure other non-hematopoietic complications. Donor stem cells may be obtained from bone marrow, peripheral blood or cord blood. Ideally the HSTC is performed prior the onset of MDS/AML and before multiple transfusions are given for hematopoietic support (MacMillan and Wagner, 2010). However, issues regarding timing of transplant are complicated by the up-front risk of transplant-related mortality and the unknown long-term side effects of transplant. Since individuals with FA are extremely sensitive to the toxicity of the usual chemotherapy and radiation regimens used in preparation for BMT, reduced doses are typically used. Unfortunately, individuals whose hematologic manifestations have been successfully treated with HSCT appear to be at an increased risk for solid tumors, particular tongue SCC. In a study of Rosenberg and co-workers, (Rosenberg et al., 2005) the risk of developed SCC was increased fourfold and the median age of onset was 16 years younger than in persons with FA who were not transplanted.

Androgen administration. Androgens have been widely used for the treatment of cytopenias in FA. The effects of androgens are most pronounced in the red cells and platelets, but neutrophil counts may also improve (Diamond and Shahidi, 1967). The major effect of androgen therapy is to increase Hb levels, though it can also improve the platelet count. Since there is no evidence that androgens can forestall BMF, treatment is initiated when cytopenias drop to clinically significant levels but before the marrow becomes completely devoid of hematopoietic stem cells for androgens to stimulate. The mechanism(s) whereby androgens raise blood counts is currently unclear. The advantages of androgens include the low risk of therapy-related mortality and the long history of experience with their use. The major potential side effects associated with androgen therapy include liver toxicity reflected as elevated liver enzymes, cholestasis, peliosis hepatis and hepatic tumors (Shimamura and Alter, 2010). The standard recommended androgen is oxymetholone, although danazol and oxandrolone have also been used. About half of all treated patients will respond to androgen therapy, and a

subset of those who initially respond may become refractory over time. An additional significant risk is that androgens do not prevent progression to AML that, once developed, creates a significantly higher transplant risk (Shimamura, 2008).

Cytokines. Studies have demonstrated that granulocyte colony-stimulating factor (G-CSF) or granulocyte macrophage colony-stimulating factor (GM-CSF) can improve the neutrophil counts in FA patients (Guinan et al., 1994; Rackoff et al., 1996). Treatment with G-CSF or GM-CSF should be considered if the neutropenia is associated with recurrent or serious infections, particularly if the neutrophil counts persistently fall below 500/mm^3 or fail to rise in response to infection. A few patients have also shown improvements in Hb or platelet counts during G-CSF or GM-CSF therapy. No comparative trials of G-versus GM-CSF are available in FA patients. Hematopoietic growth factors should be administered cautiously or not at all in the setting of a clonal cytogenetic bone marrow abnormality (Alter and Kupfer, 2002 (update 2011); Shimamura, 2008), in order to prevent proliferation of the abnormal clone that may lead to cancer development.

2. Diepoxybutane as a golden standard test for Fanconi anemia diagnosis and as an oxidative stress inducer – Implications on Fanconi anemia research

2.1. DEB: why is it a golden standard test for Fanconi anemia diagnosis?

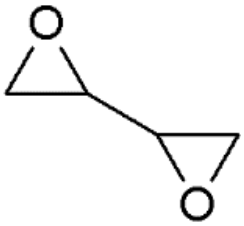


Figure 5. Chemical structure of DEB, showing the typical epoxide rings.

DEB (1,2:3,4 diepoxybutane) is a genotoxic metabolite of 1,3-butadiene produced by further epoxidation of 1,2-epoxybutane (Vlachodimitropoulos et al., 1997). It is also known to be a carcinogenic agent (Boogaard and Bond, 1996) and a highly reactive specie due to its chemical structure (Figure 5).

DEB is a bifunctional alkylating agent that can drastically damage DNA, specially causing DNA ICL (Moldovan and D'Andrea, 2009). ICLs are formed when the bifunctional alkylating molecule reacts with bases on opposing strands of the DNA double helix forming covalent links (Figure 6).

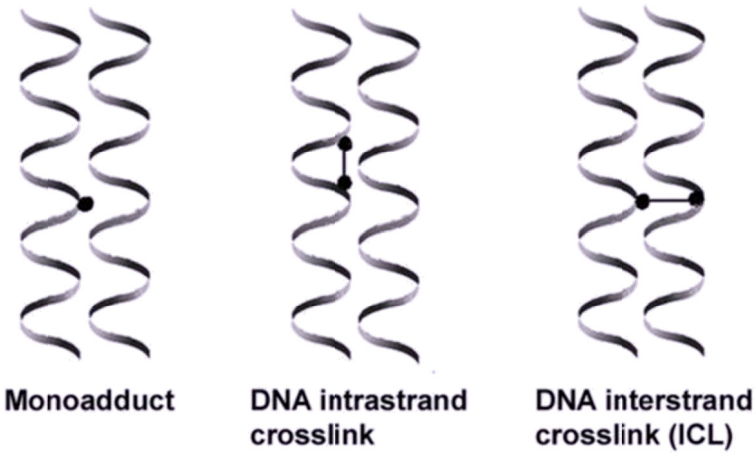


Figure 6. DNA adducts formed by interstrand cross-linking agents. Bifunctional compounds can form mono-adducts that affect a single nucleotide. Or these compounds can form adducts affect two nucleotides in the same strand or two paired strands to generate DNA intrastrand and interstrand crosslinks, respectively (from Pang and Anderson, 2009).

These links prevent DNA unwinding, thereby blocking DNA replication, transcription and recombination (Pang and Andreassen, 2009; Shen and Li, 2010). Cellular toxicity and quantification of chromosomal abnormalities induced by this chemical have been used for clinical diagnosis of the FA, being the most striking cellular hallmark of the disease. Healthy cells are capable to repair ICL, whereas in FA cells the two opposing bases affected by the crosslinking moiety renders error-free repair unfeasible in the absence of undamaged homologous sequence (Shen and Li, 2010).

2.2. DEB cytotoxicity: biotransformation as a potential cause

Cellular and molecular mechanisms mediating DEB-induced cytotoxicity are not completely understood. As it was mentioned previously, DEB has a bifunctional alkylating activity exhibiting, especially, ICLs, although it was suggested that it has also capability to produce intrastrand DNA cross-links (Millard et al., 2005). This suggestion remains to be clarified. Besides, DEB-induced cytotoxicity has been related to its ability to react with protein sulfhydryl (SH) groups (Loecken and Guengerich, 2007), which explains its capability to produce DNA-protein cross-links (Michaelson-Richie et al., 2010), accounting for its higher toxicity too. DEB can form glutathione (GSH) conjugates in reactions driven by glutathione S-transferase (GST) (Boogaard et al., 1996), this being another important mechanism of toxicity (Ahmed et al., 2010; Bogliolo et al., 2002b; Pagano et al., 2005). It is widely acknowledged that GSH-based pathways are involved in the metabolism and detoxification of DEB and its metabolites. Furthermore, it is also recognized that expression of glutathione S-transferase T1 (*GSTT1*) gene has been related to resistance to DEB toxicity (Kligerman et al., 1999; Vlachodimitropoulos et al., 1997). Pioneer studies performed by Boogaard and co-workers contributed for this assumption, demonstrating

that human, rat and mouse liver cytosolic fractions were able to conjugate [3H]glutathione with DEB, in a concentration-dependent manner and further confirmed this pathway in rat and mouse lung cytosolic fractions (Boogaard et al., 1996). Corroborating this assumption, GSH depletion was later reported to increase DEB-induced cytotoxicity on mouse germ cells (Spanò et al., 1998).

DEB was also shown to deplete GSH in sea urchin embryos (Korkina et al., 2000). However, the depletion of GSH after exposure to DEB, in mammalian cells, hadn't been demonstrated.

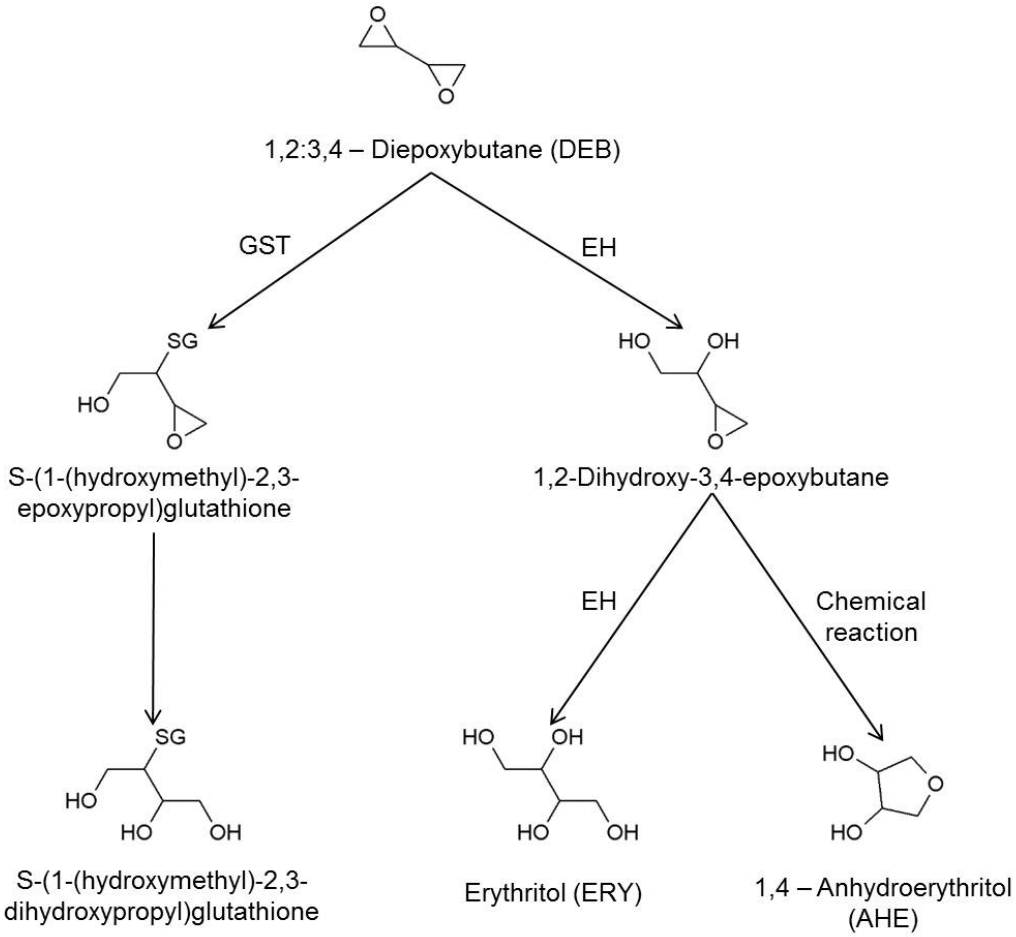


Figure 7. **Proposed scheme for the detoxification reaction of DEB** (Boogaard and Bond 1996). DEB can be conjugated with glutathione through glutathione S-transferase (GST) or can be subject to hydrolysis via epoxide hydrolase (EH).

Since DEB has a particular structure, 2 epoxide rings, its metabolism may be also related with epoxide hydrolase (EH), mainly microsomal EH (mEH), which is implicated in the metabolism of the majority of xenobiotics where epoxidation is involved (Boogaard and Bond, 1996; Morisseau and Hammock, 2005). Although mEH and soluble EH (sEH)

are highly concentrated in the liver, they are also found in nearly all tissues that were assayed for EH activity (Morisseau and Hammock, 2005), including lymphocytes. Boogaard and Bond (Boogaard and Bond, 1996) suggested that DEB metabolism and detoxification in humans are, in its majority, effectuated by EH enzymes in liver and lungs, mainly by mEH, and that metabolites formed in this process are less toxic than metabolites formed by DEB conjugation with GSH. The relative contribution of these EH and the GSH to the toxic mechanism of DEB remains to be clarified (Figure 7).

Knowing that biotransformation can contribute to DEB-induced cytotoxicity a good approach to better clarify this matter is to study the contribution of several biochemical pathways for DEB-induced acute toxicity in human lymphocytes, using inhibitors of EHs, inhibitors of protective enzymes as GST and determine the level of GSH depletion and compounds that can counteract it, as performed in the present thesis.

2.3. DEB cytotoxicity: generation of reactive oxygen species and oxidative stress

It has already been postulated that FA cell sensitivity to DEB should also reflect some deficiency or deficiencies in coping with oxidative stress (OS) (Mukhopadhyay et al., 2006). In fact, DEB toxicity has been associated with OS. First, the epoxide structure of DEB implies redox-mediated catalysis in the rearrangement of oxygen bonds (Bartók and Láng, 1980). Secondly, DEB-induced cytogenetic damage can be partially corrected by low molecular weight antioxidants in lymphocytes from FA patients and FA heterozygotes, namely all antioxidants that are known to be donors of SH groups) (Dallapiccola et al., 1985) and by overexpression of thioredoxin gene (thioredoxin is an important intracellular antioxidant and an effective regulator of redox sensitive gene expression) in FA fibroblasts (Ruppitsch et al., 1998). These evidences indirectly show that DEB toxicity is, in some way, associated with OS and that the GSH system has an important role in this toxicity, as explained in the previous chapter.

From a more direct approach, the work of Korkina and co-workers (Korkina et al., 2000) demonstrated that DEB induces high mortality and modulates catalase activity, affecting hydrogen peroxide (H_2O_2) balance in sea-urchin embryos. Moreover, they found a highly significant correlation of DEB-induced toxicity and oxygen levels. In the same study model, Pagano and co-workers (Pagano et al., 2001) demonstrated that DEB induces DNA oxidative damage, measured as 8-hydroxy-2'-deoxyguanosine (8-OHdG). They observed a dose-dependent increase in 8-OHdG levels, which was significantly correlated with developmental defects in sea urchin embryos. In addition, 8-OHdG is

recognized as an outcome of pro-oxidant state. Erexson and Tindall (Erexson and Tindall, 2000b) in a study using fibroblast of big blue mouse and rat showed that glutathione peroxidase (GPx) (which primary role is the protection of red blood cells – RBC - against damage from reactive oxygen species - ROS) and RBC (that are known to be a major source of GSH) may be important in the detoxification of DEB-induced DNA damage. This suggests that DEB can generate ROS that, in turn, can either damage DNA or produce H_2O_2. Altogether, these results gave further support to the body of evidence associating DEB toxicity with OS, including DNA oxidative damage.

2.4. DEB cytotoxicity: relation with mitochondrial dysfunction

Gille and Joenje (Gille and Joenje, 1992) have shown that OS affects key mitochondrial enzymes and lead to a decline in ATP production in mammalian cell cultures. Carcinogens, and in particular alkylating agents, have long been known to preferentially target mitochondrial DNA (mtDNA) (Backer and Weinstein, 1980; Backer and Weinstein, 1982; Clarke et al., 1998; Wunderlich et al., 1972). The mtDNA can bind 20–600 times the amount of alkylating agent compared to genomic DNA. This difference may in part be due to mtDNA not being protected by histone binding. Thus, it is reasonable to suggest that DEB also damages mitochondria. A recent study reported that DEB-induced cell death in human TK6 lymphoblasts exposed to low concentrations of DEB was due to the occurrence of apoptosis, and not necrosis (Yadavilli and Muganda, 2004). Apoptosis in response to DEB exposure has also been observed in the big blue rat cultured cells, mouse L929 cultured cells, as well as in human CD34+ bone marrow cells (Brockmann et al., 2006; Erexson and Tindall, 2000a; Irons et al., 2000). The molecular mechanisms by which DEB induces apoptosis was resolved by the study of Yadavilli and co-workers (Yadavilli et al., 2007). This study reported that DEB-induced apoptosis in TK6 lymphoblasts involves activation and up-regulation of pro-apoptotic Bcl-2 proteins (Bax, Bak), dissipation of mitochondrial membrane potential, depletion of mitochondrial cytochrome c levels, and activation of initiator caspase 9, accompanied by activation of executioner caspase 3 by caspase 9. The occurrence of these rate-limiting steps implicates the activation and involvement of the mitochondrial apoptotic pathway in mediating DEB-induced apoptosis in human lymphoblasts. These findings also point to the involvement of an OS-mediated mechanism in the execution of DEB-induced apoptosis in human lymphoblasts. Since the DEB-dependent generation of ROS is an early event and since the activation of DEB-induced apoptotic signaling pathways (MAP kinases and p53) is prevented by the ROS scavenger N-acetylcysteine (NAC), then the

DEB-dependent OS is likely to exert its effect on the apoptotic pathway at early times, upstream of mitochondria (Yadavilli et al., 2007). Through this point of view is also reasonable to suppose that DEB can induce ATP depletion in human lymphocytes, but this information is still lacking.

The knowledge of DEB induced cytotoxicity is an important route to understand what is falling in FA cells, principally related to OS induction. Studies with mitochondrial protective agents may be very useful to clarify mitochondrial damage as well as studies with compounds that can inhibit the activity of enzymes related with ROS production and OS and studies of cell signaling pathways.

3. Oxidative stress in Fanconi anemia: from cells and molecules towards prevention against chromosome instability

3.1. Pro-oxidant state in Fanconi anemia cells and patients

OS causes severe damage to membranes, proteins, and DNA and is regarded as an important factor in the development of many chronic and degenerative aging diseases (Packer, 1995). The role of OS in FA cells damage was described for the first time by Nordenson (Nordenson, 1977) followed by Joenje and co-workers (Joenje et al., 1981). Two years later, Joenje and Oostra (Joenje and Oostra, 1983) established a direct correlation between high tensions of oxygen and increased damage in FA lymphocytes. After these early discoveries, oxygen levels were found to modulate cell growth and cycle in FA fibroblasts, pointing to toxicity at high oxygen tension, with accumulation in S and G2/M phases compared to control cells (Schindler and Hoehn, 1988). Korkina and co-workers (Korkina et al., 1992) also showed the overproduction of ROS by FA leukocytes, resulting from activation of NADPH oxidase, decrease in the cellular antioxidant defense systems, increase in cellular free iron levels and a deficiency in erythrocyte superoxide dismutase (SOD). Another work suggested that the high susceptibility of FA lymphoblastoid cells to oxidative DNA damage is possibly due to a decrease in catalase activity (Takeuchi and Morimoto, 1993). In addition, a study reported by Ruppitsch and co-workers (Ruppitsch et al., 1997) indicated that cytochrome P-450 enzymes, especially CYP1A2, play a crucial role in ROS metabolism in FA cells that showed reduced oxygen consumption and increased activity of the antioxidant phospholipid-hydroperoxide-glutathione-peroxidase. This enzyme plays a key role in protecting cells from lipid peroxidation. It is the only enzyme known to metabolize phospholipid hydroperoxides and,

together with α-tocopherol, is the only known protection against ROS within the cell membrane. In fact, malondialdehyde, a product of lipid peroxide degradation, is elevated in FA cells (Ruppitsch et al., 1997). Elevated levels of ROS in FA fibroblasts may result in a slight induction of this enzyme, like other antioxidant enzymes. The resultant enzymatic activity is still not enough to counteract the increased production of ROS.

It has been reported that plasma from FA patients and, in a lesser extent, from FA heterozygotes contains increased levels of clastogenic factors (lipid peroxidation products and cytokines such as TNF-α) (Emerit, 2007; Petrovic et al., 2011). Excess TNF-α was found in bone marrow plasma and peripheral blood plasma from patients with aplastic anemia (Schultz and Shahidi, 1994) and the same group also found higher levels of this cytokine in plasma of FA patients, about 4 times higher than in controls (Schultz and Shahidi, 1993). Another work reported that TNF-α level in FA fibroblasts is 8-fold higher than in normal cells (Rosselli et al., 1994). These reports opened the way of a series of investigations ascertaining a major role for TNF-α and another cytokines in FA cells, since it is known that they can significantly enhance ROS production by phagocytic cells (Figure 8) (Du et al., 2008).

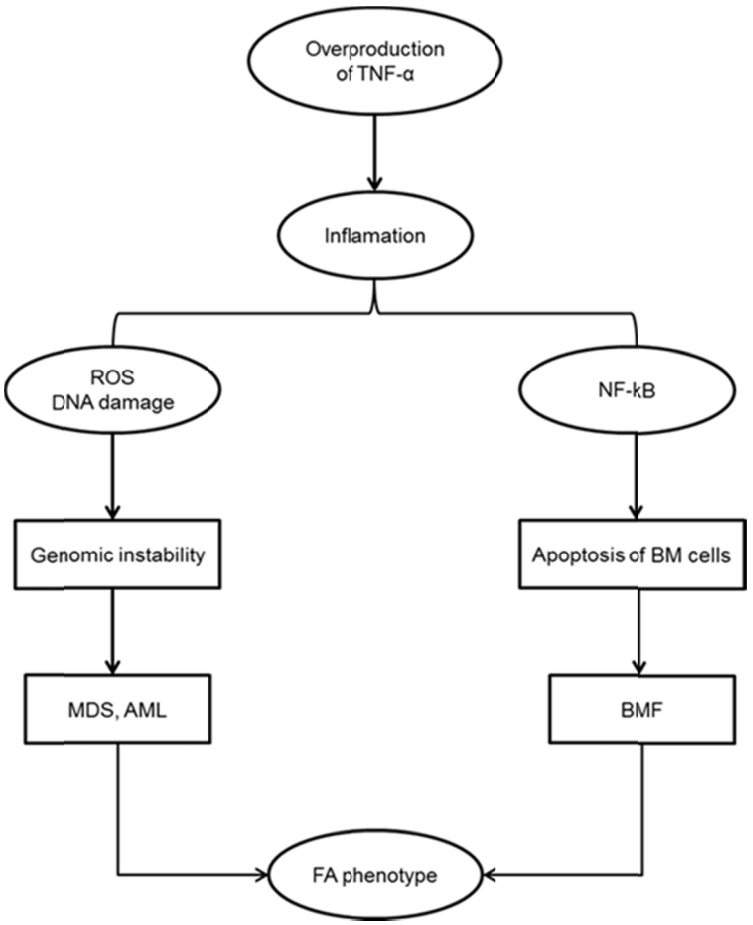


Figure 8. The proinflammatory cytokine TNF-α and its potential role in FA pathophysiology. Overproduced TNF-α plays a role not only in pro-apoptotic signal suppressing FA hematopoietic progenitor activity, but also promoting leukemic transformation of FA hematopoietic stem/ progenitor cells, which lead to typical phenotype of FA patients. BM, bone marrow; BMF, bone marrow failure; ROS, reactive oxygen species; MDS, myelodysplasic syndrome; AML, acute myeloid leukemia; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells (Adapted from Du et al, 2008).

The interactions between TNF-α and its receptors, in particular, enhance intracellular events as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation

and transcriptional activity, which are enhanced in FA cells compared to normal cells. NF-kB is a potent indicator for stress factors such as ROS and is capable to activate the transcription of various genes that are involved in stress defense. Therefore it can be postulated that a defect in the NF-kB system would thus limit the ability of the cell to respond to OS (Ruppitsch et al., 1997). Moreover, TNF-α treatment induces FANCA and FANCG via NF-kB (Macé et al., 2007).

Another marker of pro-oxidant state in FA cells is the excess accumulation 8-OHdG. This excess was correlated with spontaneous chromosomal breaks (Degan et al., 1995; Pagano et al., 2004). Another work suggested that FA cells, at least FA complementation group A cells, have increased susceptibility to oxidative DNA damage possibly due to decreased catalase activity, which indicates that catalase plays an important role in protecting DNA from oxidative damage (Takeuchi and Morimoto, 1993). Zunino and co-workers (Zunino et al., 2001), also showed an increased basal level of 8-OHdG in FA patients from the complementation group C and E. Besides, the same group also demonstrated that H_2O_2-induced 8-OHdG formation is higher in FA than in normal cells. On the other hand, more recently, Castillo and co-workers (Castillo et al., 2011) concluded that the FA pathway is redundant for the repair of this oxidative damage. Therefore, the accumulation of 8-OHdG in FA cells reflects sustained overproduction of ROS rather than defective processing of oxidized bases.

Beyond the direct information about an *in vivo* pro-oxidant state, it should be remembered that the FA phenotype is characterized by some clinical features, or complications, that find recognized mechanistic explanations on the grounds of redox imbalances. Together, the available literature provides both direct and indirect evidence that OS is at least a component of FA cellular and clinical phenotype.

3.2. Redox-related functions of FANC proteins

3.2.1. FANCA, FANCC and FANCG: links with xenobiotic-processing pathway

The FANCC protein was found to be associated with redox-related activities, namely interacting with enzymes involved with bioreductive action of xenobiotics. Kruyt and co-workers (Kruyt et al., 1998) reported that FANCC functions in the cytoplasm, at a step before DNA repair, and binds to NADPH cytochrome-P450 reductase, a microsomal membrane protein involved in transferring electrons from NADPH to an isoenzyme of the cytochrome P450 family, as well as to cytochrome c, in both transfected COS-1 and normal murine liver cells. Cumming and co-workers (Cumming et al., 2001) also showed that FANCC interacts with glutathione S-transferase P1-1 (GSTP1) and that

overexpression of both proteins in a myeloid progenitor cell line prevents apoptosis following factor deprivation. GSTP1 is an enzyme that catalyzes the detoxification of xenobiotics and by-products of OS by conjugation with GSH, and it is frequently upregulated in neoplastic cells. Studies in animal models also showed a relation between FANCC and OS. Hadjur and co-workers (Hadjur et al., 2001) found defective hematopoiesis and hepatic steatosis in double knock-out mice with *Fancc(-/-)* and *SOD1(-/-)*. The authors suggested that the altered redox state likely present in *Fancc(-/-)* and *sod1(-/-)* hematopoietic progenitors was responsible for an impairment of cell proliferation and survival.

FANCG protein is localized both in the nucleus and cytoplasm. After treatment with clastogenic agents it increases in cells, with predominant expression in the cytoplasm (Futaki et al., 2002). FANCG was found to interact with cytochrome P450 2E1 (CYP2E1), which is associated with the production of ROS and the bioactivation of carcinogens. These results suggest that FANCG has an additional role in protection against oxidative DNA damage. Additionally, Park and co-workers (Park et al., 2004) showed that FANCA and FANCG are redox-sensitive proteins that are multimerized and/or form a nuclear complex in response to OS/damage. Both FANCA and FANCG proteins exist as monomers under non-oxidizing conditions, whereas they become multimers following H_2O_2 treatment. Treatment of cells with oxidizing agent not only triggers the multimeric complex of FANCA and FANCG *in vivo* but also induces the interaction between FANCA and FANCG. Finally, in another study (Noll et al., 2002) mice were genetically modified with a targeted mutation in FANCA and were crossed with FANCC disrupted animals, revealing that FANCC and FANCA are part of a multi-protein nuclear FA complex with identical function in cellular responses to DNA damage and germ cell survival. This group showed that fibroblast cells and hematopoietic precursors from FANCA/FANCC double-mutant mice were not more sensitive to MMC than those of both single mutants and that FANCA/FANCC double mutants had no evidence for an additive phenotype at the cellular or organism level.

A report by Reuter and co-workers (Reuter et al., 2003) identified 69 proteins which have not previously been linked to the FA pathway as direct interactors of FANCA, FANCC, or FANCG. Most of these proteins are associated with four functional classes including transcription regulation (21 proteins), signaling (13 proteins), oxidative metabolism (10 proteins), and intracellular transport (11 proteins). The potential interactors found in this study point towards an additional involvement of the FA pathway in four other major functional purposes, namely transcription, cell signaling, oxidative metabolism, and cellular transport.

3.2.2. FANCD2 and FANCJ: multiple identities and redox-related roles

The FANCD2 protein features an unique role in the FA pathway, due to its interactions in the activation of the FA core complex, in cooperation with FANCI and following monoubiquitination (Smogorzewska et al., 2007). Mammalian forkhead members of the class O (FOXO) transcription factors, are implicated in the regulation of diverse physiologic processes, including cell-cycle arrest, apoptosis, DNA repair, stress resistance, and metabolism (Brunet et al., 2004). Among these FOXO proteins, forkhead transcription factor forkhead box O 3a (FOXO3a) functions as a major regulator of OS (Huang and Tindall, 2011). It is known that FANCD2 interacts with the FOXO3a in response to OS (Li et al., 2010). In a very recent study presented at the American Society of Hematology Annual Meeting, in December 2011, it was shown that expression of FOXO3a is associated with HbF levels in hereditary persistence of HbF. Thus, study FANCD2-FOXO3a pathway in cells from FA patients, which have an increased level of HbF, may be an important question to address and probably FOXO3a is over expressed in FA cells. Another recognized interaction of FANCD2 is with ataxia telangiectasia mutated protein (ATM) (Castillo et al., 2011). ATM has been suggested to function, at least in part, in the cellular response to oxidative damage (Barzilai et al., 2002). Support for this hypothesis comes from observations that ATM-deficient cells are unusually sensitive to the toxic effects of H_2O_2, nitric oxide, and superoxide treatments as determined by colony-forming efficiency assays (Barlow et al., 1999).

The FANCJ protein was recognized to coincide with two previously known BRCA1-interaction entities, the DNA helicase BRIP1 (Levitus et al., 2005; Levran et al., 2005) and the transcription factor BACH1 (Litman et al., 2005). This protein features a helicase activity and ATPase activity (Cantor et al., 2004; Cantor et al., 2001). It may be noted that ATPases are frequently involved in OS related mechanisms (Bigelow and Squier, 2005). Thus, it might be suggested that the ATPase component of helicase activity plays a direct role in redox-active functions of these DNA repair proteins. Before the recognition of its identity with FANCJ, BACH1 was termed as a transcription repressor and found to regulate redox-related activities, as heme oxygenase-1 (HO-1) while, in turn, increased levels of HO-1 resulted in BACH1 inactivation (Kitamuro et al., 2003; Okada et al., 2010; Reichard et al., 2007). Moreover, BACH1 was reported to form a p53-containing complex, and was regulated by OS and HO-1; thus BACH1 was related to OS as a negative regulator of p53 (Dohi et al., 2008).

3.3. Mitochondrial dysfunction in Fanconi anemia cells

Mitochondria contain about 1% of the cell's DNA. These organelles account for more than 90% of cellular oxygen consumption and are prone to DNA oxidative mutation (Richter et al., 1988). Indeed, mitochondrial dysfunction, as a result of accumulative oxidative lesions has been correlated with ageing (Shigenaga et al., 1994). It is also known that mitochondrial dysfunctions ultimately lead to a defect in energy transduction, with increased formation of ROS, which, by itself, can lead to lower cellular energy charge, oxidative modification of DNA, protein and lipids. In addition, there is also a possibility of a positive feedback cycle between ROS formation and mitochondrial damage, exacerbating the processes of cellular dysfunction (Tarnopolsky, 2008). In a preliminary study Liebetrau and co-workers (Liebetrau et al., 1997) have examined the mtDNA profile in tissues of FA patients, using polymerase chain reaction (PCR) primer pairs to encompass a 5 kb 'hot spot' region for deletion mutations seen in ageing and in some mitochondrial diseases. Two of the seven studied patients showed evidence of the common 5 kb deletion, another indication that mtDNA is involved in the mechanism of oxidative sensitivity in FA cells.

More recently, direct evidence for mitochondrial dysfunction in FA cells was provided by Mukhopadhyay and co-workers (Mukhopadhyay et al., 2006) who reported that FANCG protein is found in mitochondria and interacts with the mitochondrial peroxidase, peroxyredoxin 3 (PRDX3). Mitochondrial PRDX3 is an important cellular antioxidant that regulates physiological levels of H_2O_2, leading to decreased cell growth while protecting cells from the apoptosis-inducing effects of high H_2O_2 levels (Nonn et al., 2003). PRDX3 depletion results in the acceleration of apoptosis, with increased rates of mitochondrial membrane potential collapse, cytochrome c release, and caspase activation (Chang et al., 2004). PRDX3 was found to be deregulated in FA-G cells, which displayed distorted mitochondrial structures, and mitochondrial extracts had a significant decrease in thioredoxin-dependent peroxidase activity. In addition, overexpression of PRDX3 suppressed the sensitivity of FA-G cells to H_2O_2, and a decreased PRDX3 expression increased the sensitivity to alkylating agents (Chang et al., 2004). Moreover, cells from FA-A and FA-C subtypes also had PRDX3 cleavage and decrease peroxidase activity (Mukhopadhyay et al., 2006).

Previous reports had provided evidence for mitochondrial dysfunction in FA cells (Afanas'ev, 2006; Bogliolo et al., 2002a; Rousset et al., 2002). Paulin-Levasseur and co-workers (Paulin-Levasseur et al., 1998) reported that mitochondria in FA cells have a filamentous shape and form an interconnected cytoplasmic reticulum running in parallel with both vimentin filaments and microtubules. This was unlike control (HeLa) cells, where

mitochondria appears as individual tubular compartments of variable length and are closely associated with vimentin filaments. Bogliolo and co-workers (Bogliolo et al., 2002a), tried to understand the intriguing relationships between apoptotic susceptibility and alterations in redox state and energy metabolism, studying the response of FA cells to a number of metabolic inhibitors known to interfere with ATP production and the consequences of these treatments on proliferation and survival of FANCA and FANCC-defective lymphoblastoid cells. They reported that FA cells, unlike control cells, were not affected by treatments with rhodamine-1,2,3 (induces membrane depolarization, affects the efficiency of electron transport and impairs the respiratory chain and oxidative phosphorylation) and doxycycline (inhibits synthesis of mitochondrially encoded proteins by 50% at every cell division), which result in acute ATP depletion and in a significant enhancement of the fraction of cells undergoing apoptotic death. Additionally, apoptosis is particularly sensitive to the redox state of the cells and is strictly associated with alterations in mitochondrial functionality (Kannan and Jain, 2000). On the other hand, FA cells were very sensitive to 2-deoxy-D-glucose and iodetic acid, two inhibitors of the glycolytic metabolism. These findings suggested that FA cells are adapted to withstand mitochondrial stress, while relying on glycolytic metabolism. Rousset and co-workers (Rousset et al., 2002) reported an oxygen-dependent sensitivity of mitochondria in FA-A fibroblasts. FA cells, following 8-methoxypsoralen photoreaction or ultraviolet A (UVA) irradiation, underwent mitochondrial matrix densification, unlike control cells, which was accompanied by some modifications in transmembrane potential. This effect was oxygen dependent because it was more enhanced at 20% than at 5% oxygen tension. It is important to note that UVA exposure leads to the formation of free radicals and that psolaren acts through photobinding, by means of UVA excitation, to pyrimidine bases of nucleic acids, to amino acids and proteins and to membrane constituents (Schmitt et al., 1995). Moreover, psolaren photoreaction and UVA radiation are also responsible for the induction of lipid peroxidation, which can damage membranes by preventing the methylation of phospholipids (Caffieri et al., 1996), known to be implicated in the adjustment of membrane fluidity and in enzyme activities associated with the cell membranes (Kaneko et al., 1990).

The evidence for mitochondrial dysfunctions in FA-G, FA-A and FA-C cells are well documented. Thus, it might be questioned if FA-J cells are involved in mitochondrial dysfunction too. Since FANCJ is related with ATPase activity it is linked by definition to the availability of ATP that is the major product of mitochondrial function. However, this is a currently open question that needs to be addressed. Figure 9 summarizes the possible interactions between FANC proteins, mitochondria and oxygen metabolism.

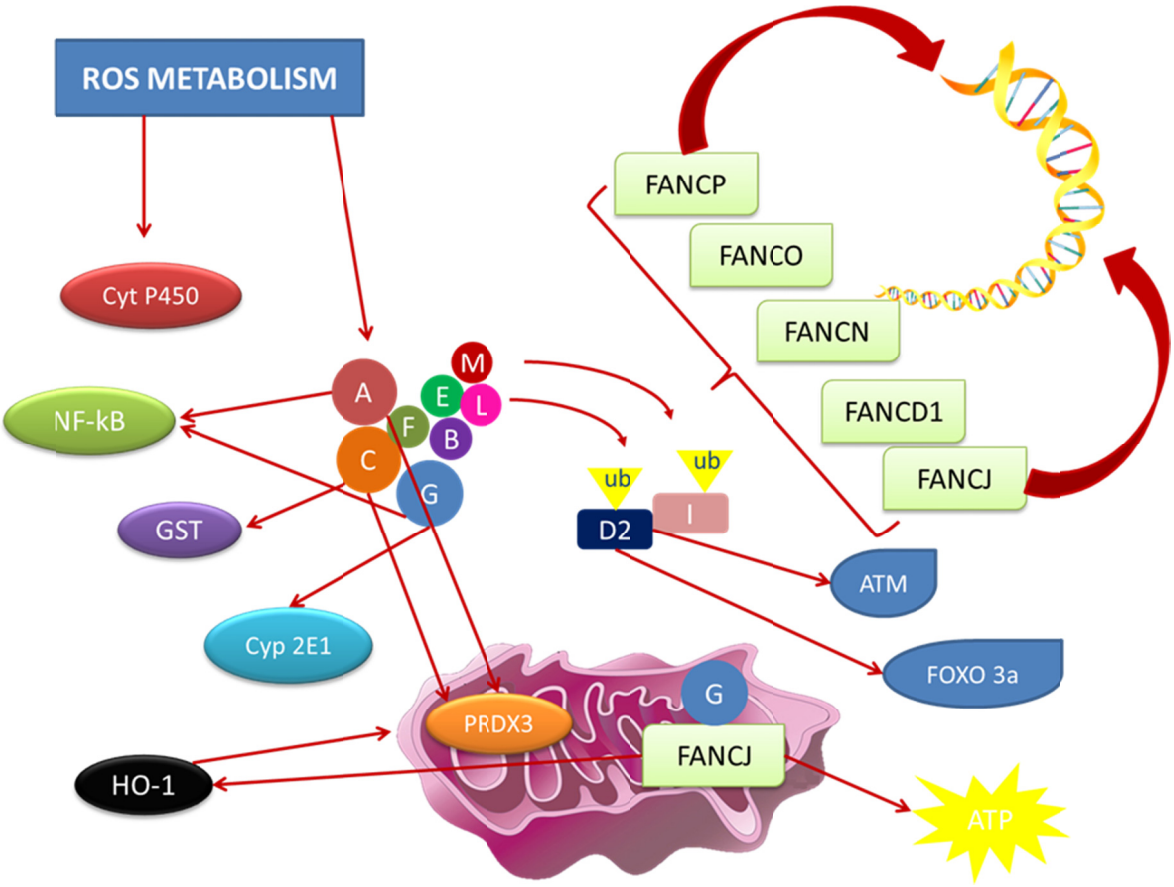


Figure 9. ROS metabolism in Fanconi anemia cells. Possible interactions between FANC proteins, mitochondria, DNA damage, redox stress signaling proteins and antioxidant enzymes (adapted and modified from Pagano et al, 2011). ATM, ataxia telangiectasia mutated; Cyp 2E1, cytochrome P450 2E1; Cyt P450, cytochrome P450; FOXO 3a, forkhead box 3a; GST, glutathione S-transferase; HO-1,heme oxygenase 1; NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PRDX3, peroxyredoxin 3; ROS, reactive oxygen species.

The use of "mitochondrial nutrients" as antioxidants to counteract the ROS production can be a good approach to evaluate mitochondrial dysfunction in FA cells. FA pathogenesis can also be a part of a set of diseases and conditions associated to mitochondrial cytopathies, such as aging (Pallardó et al., 2010; Tarnopolsky, 2008). The preexisting clinical experience in managing patients with mitochondrial cytopathies by means of therapeutic mitochondrial cocktails may provide useful background in planning measures to avoid the pro-oxidant state and disease progression in FA patients.

3.4. *In vitro* and *in vivo* experimental therapies – Antioxidant agents as protectors of chromosomal instability

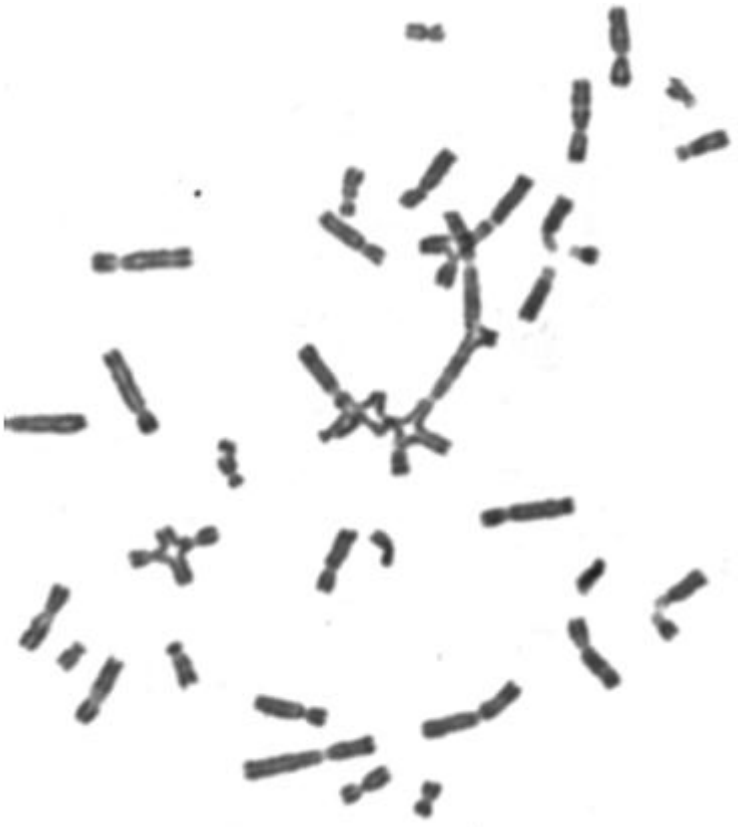
Some *in vitro* studies have been performed testing the use of antioxidants for decreasing chromosome damage in FA cells. Dallapiccola and co-workers (Dallapiccola et al., 1985) obtained a partial correction of CI in FA lymphocytes treated with several antioxidants, namely L-cysteine, 2-mercaptoethanol, α-mercaptopropionylglycine and GSH, all chemicals which are known to be donors of the SH group. Later, the same group (Porfirio et al., 1989) extended this study to desferoxamine, an iron chelator, with 50% reduction of the spontaneous chromosome breakage. Korkina and co-workers (Korkina et al., 1992) suggested that hydroxyl radical (•OH) scavengers and chelators can be used for the treatment of FA patients. Rutin, a nontoxic natural bioflavonoid (vitamin P), was found to be an efficient inhibitor of luminol-dependent chemiluminescence (a technique that measures the production of oxygen radicals) produced by FA leukocytes. After administration of rutin to three FA patients they observed that it substantially decreased oxygen radical production by leukocytes, diminished to some degree the amount of chromosomal aberrations, and improved hematologic characteristics of patients. Pincheira and co-workers (Pincheira et al., 2001) demonstrated that Vitamin E (α-tocopherol) decreases the frequency of chromosomal damage in 50.8% and the duration of G2 in FA lymphocytes. More recently, it was reported that honey can have a cytoprotective effect against CI in FA cells, due to the antioxidant capacity of flavonoids and other polyphenols, which are common constituents of honey (Mogib El-Dahtory and Yahia, 2011). 2 ml of a solution of 10% of honey can abolish the induced chromosomal aberrations in FA cells. The effect of these studies once more agrees with the assumption of oxygen sensitivity in FA cells and the putative beneficial effect of antioxidant treatments.

Some *in vivo* studies were also performed in animal models. Zhang and co-workers (Zhang et al., 2008) showed that dietary supplements with the antioxidant tempol, a nitroxide antioxidant and a SOD mimetic, can delay the age of onset of epithelial tumors in Fancd2(-/-) knock-out mice model. More recently, this group (Zhang et al., 2010) also demonstrated that the antioxidant resveratrol maintains Fancd2(-/-) KSL cells in quiescence, improves the marrow microenvironment, partially corrects the abnormal cell cycle status, and significantly improves the spleen colony-forming capacity of Fancd2(-/-) bone marrow cells.

In spite of the promising results from previous studies, no therapy using antioxidants, megavitamins, or micronutrients has been shown to be effective in treatment of FA using evidence-based criteria. Thus, further studies with new protective agents are required for

the development of drugs with high effectiveness. Ideally, the protective agents/antioxidants should be already approved for human use or promptly bioavailable by dietary administration (Pagano et al., In press; Pagano and Korkina, 2000). For a rare disease like FA, insuperable obstacles to clinical chemoprevention trials may currently be faced due to the scanty numbers of patients susceptible to be recruited, compromising the best efforts in drawing adequate conclusions from too small scale clinical studies. As a possible means for overcoming the restraint imposed by the scarcity of human patients, and on ethical grounds, studies could be designed by utilizing knock-out mice from different complementation groups (FANCA, FANCC, FANCD2 and FANCG). However, animal models not always are a good approach, and *in vitro* studies with cells from FA patients could sometimes be a better way. Testing antioxidants to counteract the initial pro-oxidant state of FA cells or in cells of healthy donors (HD) subjected to the effects of alkylating agents, should rely on the choice of the best appropriate antioxidants to be used in animal models or even in clinical trials, with the ultimate goal of mitigating or delaying FA clinical progression.

PART II - OBJECTIVES OF THE THESIS



Objectives of the Thesis

The present work was divided in two parts, performed in two different laboratories, in which two complementary objectives were pursued.

The first objective, performed during the first two years, in the Laboratory of Toxicology of Faculty of Pharmacy of University of Porto (FFUP), was the following:

✓ Characterization of the acute toxicity of DEB in normal lymphocytes, by evaluation of cell viability as well as GSH and ATP levels in an *in vitro* cellular suspension model. Following the results obtained with the acute toxicity study, a mechanistic approach was further applied by using:

- Putative inhibitors of DEB bioactivation: cyclohexyl-3-dodecyl urea (CDU), elaidamide, Zinc (Zn^{2+})
- Antioxidant agents: NAC
- Mitochondrial protective agents: acetyl-L-carnitine (ALC) and α-lipoic acid (α-LA)
- Inhibitors of putative protective enzymes and inhibitors of protein synthesis: tannic acid, 3-amino-1,2,4-triazole (3-AT) and cycloheximide (CHX).

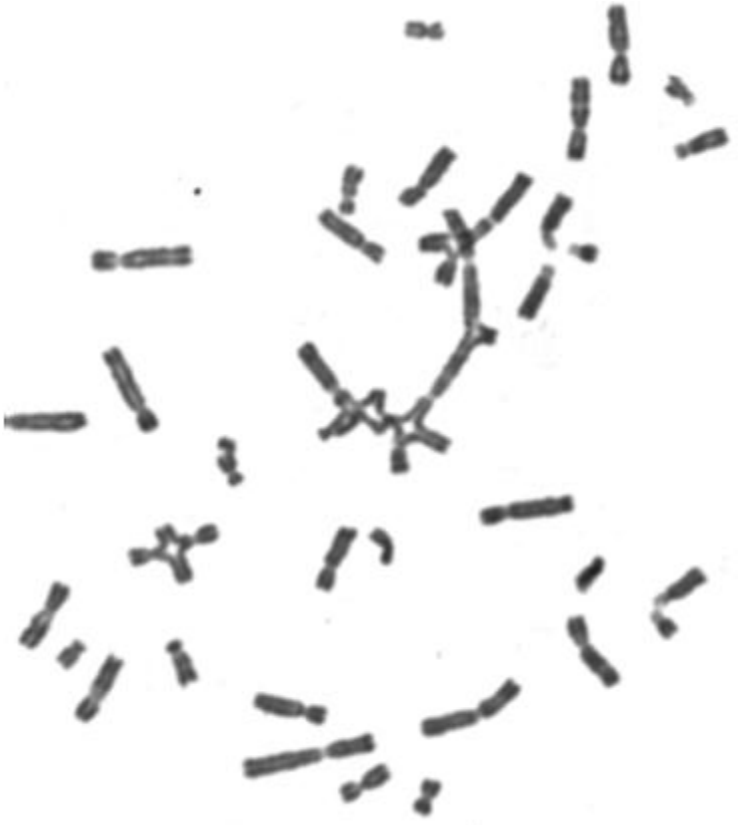
The global purpose of the first objective was to contribute for a better understanding of DEB-induced toxicity to human lymphocytes, by studying the putative contribution of biochemical pathways postulated to be involved in the reactivity of this compound. The specific goal was to search for protective drugs that could turn out to be useful to prevent spontaneous and DEB-induced CI in lymphocytes from FA patients.

The second objective, performed during the last two years, in the Laboratory of Cytogenetics of Institute of Biomedical Sciences Abel Salazar (ICBAS), was the following:

✓ Evaluation of the putative protective effect of previously selected drugs in spontaneous and DEB-induced CI in lymphocytes cultures from FA patients and control population.

✓ Optimization of a cocktail with the compounds that demonstrated better results in the decrease of CI in lymphocyte cultures from FA patients.

Since the available information points to an association of cellular phenotype and clinical features in the occurrence of both cancer-proneness and OS in FA disorder, the final goal was to find out an effective antioxidant prophylactic cocktail to be applied, *in vivo*, in FA patients, with a consequent block or delay in their characteristic BMF and predisposition to cancer development.



PART III – ORIGINAL RESEARCH

CHAPTER I

Protective effect of acetyl-L-carnitine and α-lipoic acid against toxicity of diepoxybutane to human lymphocytes

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Protective effect of acetyl-L-carnitine and α-lipoic acid against the acute toxicity of diepoxybutane to human lymphocytes

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ABSTRACT

The biotransformation and oxidative stress may contribute to 1,2:3,4-diepoxybutane (DEB)-induced toxicity to human lymphocytes of Fanconi Anemia (FA) patients. Thus, the identification of putative inhibitors of bioactivation, as well as the determination of the protective role of oxidant defenses, on DEB-induced toxicity, can help to understand what is failing in FA cells. In the present work we studied the contribution of several biochemical pathways for DEB-induced acute toxicity in human lymphocyte suspensions, by using inhibitors of epoxide hydrolases, inhibitors of protective enzymes as glutathione S-transferase and catalase, the depletion of glutathione (GSH), and the inhibition of protein synthesis; and a variety of putative protective compounds, including antioxidants, and mitochondrial protective agents. The present study reports two novel findings: (i) it was clearly evidenced, for the first time, that the acute exposure of freshly isolated human lymphocytes to DEB results in severe GSH depletion and loss of ATP, followed by cell death; (ii) acetyl-L-carnitine elicits a significant protective effect on DEB induced toxicity, which was potentiated by α-lipoic acid. Collectively, these findings contribute to increase our knowledge of DEB-induce toxicity and will be very useful when applied in studies with lymphocytes from FA patients, in order to find out a protective agent against spontaneous and DEB-induced chromosome instability.

1. Introduction

Fanconi Anemia (FA) is a rare recessive disorder, clinically characterized by progressive bone marrow failure, diverse congenital abnormalities and increased predisposition to cancer. Thirteen complementation groups have been identified and the genes for all of them (*FANCA*, *-B*, *-C*, *-D1*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J*, *-L*, *-M* and *-N*) appear to function in the maintenance of genomic stability (Moldovan and D'Andrea, 2009). The hypersensitivity of FA lymphocytes to the clastogenic (chromosome breaking) effect of DNA cross-linking agents, in particular to 1,2:3,4-diepoxybutane (DEB), provides a unique marker for the diagnosis (Auerbach et al., 1981, 1989).

It has been postulated that FA cell sensitivity to DEB should reflect some deficiency or deficiencies in coping with oxidative stress (Mukhopadhyay et al., 2006). In accordance, a direct association of oxidative stress with the primary genetic defect in FA was suggested through the interaction of the FANCC protein with NADPH cytochrome P450 reductase and glutathione S-transferase (GST), two enzymes involved in the detoxification of reactive intermediates (Cumming et al., 2001; Kruyt et al., 1998). On the other hand, the cellular and molecular mechanisms mediating DEB-induced cytotoxicity are not completely understood. Previous studies have shown that DEB acts as a bifunctional alkylating agent that exhibits both inter-strand and intra-strand DNA cross-linking ability (Millard et al., 2006). DEB is also redox-active, generating reactive oxygen species (ROS) that can damage DNA (Erexson and Tindall, 2000; Yadavilli et al., 2007).

DEB can form glutathione (GSH) conjugates in reactions driven by GST (Boogaard et al., 1996), this being an important mechanism of toxicity (Ahmad et al., 2002; Bogliolo et al., 2002b; Pagano et al., 2005). Since DEB has a particular structure, 2 epoxide rings, its metabolism may be also related with epoxide hydrolase (EH), mainly microsomal EH (mEH), which is

Abbreviations: α-LA, α-lipoic acid; 3-AT, 3-amino-1,2,4-triazole; 8OHdG, 8-hydroxydeoxyguanosine; ALC, acetyl-L-carnitine; BSA, bovine serum albumin; CDU, cyclohexyl-3-dodecyl urea; CHX, cycloheximide; DEB, 1,2:3,4-diepoxybutane; EH, epoxide hydrolase; FA, Fanconi Anemia; FBS, fetal bovine serum; mEH, microsomal EH; MTs, metallothioneins; NAC, N-acetyl-cysteine; PBS, phosphate buffered saline solution; ROS, reactive oxygen species; RT, room temperature; sEH, soluble EH; SH, sulfhydryl; SOD, superoxide dismutase.

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implicated in the majority of xenobiotic metabolism where epoxidation is involved (Boogaard and Bond, 1996; Morriseau and Hammock, 2005). Although mEH and soluble EH (sEH) are highly concentrated in the liver, they are found in nearly all tissues that were assayed for EH activity (Morriseau and Hammock, 2005), including lymphocytes. The relative contribution of these two enzymes to the toxic mechanism of DEB remains to be clarified.

DEB acts not only at cytoplasmic and nuclear level but also inside the mitochondria. This agent induces production of superoxide anion, causes mitochondrial DNA damage, and interferes with redox cellular balance and ATP production (Bogliolo et al., 2002a; Clarke et al., 1998). The consequences are reduction in cellular growth and cellular viability, as well as increase of apoptosis (Bogliolo et al., 2002a; Clarke et al., 1998; Yadavilli et al., 2007).

Knowing that biotransformation and oxidative stress contribute to DEB-induced toxicity (Dallapicola et al., 1985; Moldovan and D'Andrea, 2009; Porto et al., 2003; Takeuchi and Morimoto, 1993), the identification of putative inhibitors of bioactivation, as well as the determination of the protective role of oxidant defenses, on DEB-induced toxicity, can help to understand what is failing in FA cells. For that purpose, in the present work we studied the contribution of several biochemical pathways for DEB-induced acute toxicity in human lymphocyte suspensions, by using inhibitors of EHs, inhibitors of protective enzymes as GST and catalase, the depletion of GSH, and the inhibition of protein synthesis; and a variety of putative protective compounds, including antioxidants, and mitochondrial protective agents.

2. Materials and methods

2.1. Chemicals

Histopaque 1077, isotonic phosphate buffered saline solution (PBS), fetal calf serum (FBS), L-glutamine, luciferin, luciferase, reduced glutathione (GSH), glutathione reductase, reduced nicotinamide adeninedinucleotide phosphate (NADPH), bovine serum albumin (BSA), 1,2:3,4-diepoxybutane (DEB), adenosine triphosphate (ATP), ammonium chloride, cycloheximide (CHX), N-acetyl-cysteine (NAC), trypan blue, Giemsa, acetyl-L-carnitine (ALC), α-lipoic acid (α-LA), tannic acid, elaidamide, 3-amino-1,2,4-triazole (3-AT) and RPMI 1640 were obtained from Sigma (St. Louis, MO). Antibiotics (penicillin and streptomycin) were obtained from GIBCO. 1-Cyclohexyl-3-dodecyl urea (CDU) was obtained from Calbiochem. Sodium hydroxide (NaOH), perchloric acid ($HClO_4$), potassium chloride (KCl) and zinc sulphate ($ZnSO_4$) were obtained from Merck (Darmstadt, Germany). Tris-base was obtained from Promega Corporation (Madison, U.S.A.).

2.2. Blood samples

Blood samples were collected from normal subjects recruited among healthy male and female students and blood donors, aged 20–40 years, with the informed consent obtained from all the participants and according to the protocol approved by the Ethics Committee of Hospital Geral de Sto António. From each subject, 10 ml of venous blood was collected by antecubital venipuncture, into vacuum tubes with lithium heparin.

2.3. Isolation of lymphocytes

Lymphocytes were isolated from whole blood by a gradient density centrifugation method, using Histopaque solution 1077 in polypropylene centrifuge tubes, as previously described (Freitas et al., 2010). Briefly, 3 ml of collected blood was carefully layered on top of 3 ml of Histopaque 1077 in each 12 ml polypropylene tube. The tube was centrifuged at 890 × *g* for 30 min at room temperature (RT). The lymphocyte layer was carefully removed, using a glass Pasteur pipette, for another tube. The lymphocyte pellet was gently mixed with 10 ml of PBS and then centrifuged at 840 × *g* for 10 min at 4 °C, to remove platelets. The supernatant was decanted and 10 ml of a lysis solution (composed of Tris-base and ammonium chloride, in a pH of 7.2) was added to lymphocyte pellet, during 10 min, to lyse any remaining red blood cells. This suspension was centrifuged at 840 × *g* for 10 min at 4 °C, after which the supernatant was decanted and the lymphocyte pellet resuspended in 1 ml of RMPI 1640, supplemented with FBS, L-glutamine and antibiotics (penicillin and streptomycin). Isolated lymphocytes were kept on ice until use, within the following 30 min. Cell viability and cell yield were evaluated by the Trypan blue exclusion assay, using a neubauer chamber and an optic microscope (40×).

2.4. Experimental protocol for toxicological assays

Lymphocyte suspensions were diluted to a final concentration of 1×10^6 cells/ml, adding an adequate volume of supplemented culture medium. Subsequently, cell suspensions were equally distributed by 25 ml flat bottom polyethylene tubes (1×10^6 cells/ml) in a total volume of 2.5 ml/flask and incubated in a shaking water bath (90 oscillations/min) at 37 °C, for toxicological assays. Before any manipulation of lymphocyte suspensions, they were pre-incubated for 30 min, to adapt to new conditions.

2.4.1. Acute toxicity of DEB

Lymphocytes were exposed to DEB and 4 treatments were defined: a control group and three treatments with different DEB concentrations: 25 μg/ml, 100 μg/ml and 400 μg/ml. Lymphocytes were incubated for 5 h and samples were collected at the beginning of the experiment, $t = 0$ h, and 2, 3, 4 and 5 h after DEB exposure. The evaluated parameters were cell viability as well as glutathione and ATP levels. From the results obtained in these first experiments, 400 μg/ml DEB was established as the toxic concentration to study putative modulating drugs, since at this concentration the all measured toxicological parameters were severely affected.

2.4.2. Treatment with putative modulating drugs

Lymphocytes were exposed to putative modulating drugs, for possible prevention, or aggravation of DEB-induced toxicity. The use of both types of modulating drugs could give important clues on the mechanism of DEB-induced acute toxicity. To this end, we used concentrations that proved to be effective in *in vitro* studies, preferably with lymphocytes. In addition, prospective studies were always performed to find the most efficient concentrations.

The following putative protective treatments were used (drugs grouped according to their effects):

- Inhibitors of EHs: CDU (0.01 μM, 0.1 μM and 1 μM), as a sEH inhibitor (Davis et al., 2006), elaidamide (10 μM), as a mEH inhibitor (Morisseau et al., 2001). Zn^{2+} (10 mM), was also used as an inhibitor of both EHs (Draper and Hammock, 1999), though it is also known that this metal may behave as an antioxidant;
- Antioxidant: NAC (500 μM), as an antioxidant agent (Kligerman and Tennant, 2007);
- Mitochondrial protective agents: ALC (5 mM), a potent restorer of mitochondria energy production (Boerrigter et al., 1993) and α-LA (100 μM), a restorer of mitochondrial redox status (Han et al., 1997).

The following treatments were performed for putative aggravation of DEB-induced toxicity:

- Inhibitors of protective enzymes: tannic acid (5 μM) as a GST inhibitor (Wang et al., 2009), and 3-AT (20 mM) as a catalase inhibitor (Takeuchi and Morimoto, 1993);
- Inhibitor of protein synthesis: CHX (100 μg/ml) (Oda et al., 2007).

For each drug, 4 treatments were defined: (i) control, (ii) 400 μg/ml DEB, (iii) drug under study, (iv) drug under study plus 400 μg/ml DEB. The control groups were treated with the same vehicle used to dissolve the studied compounds, which was RPMI, except for α-LA that required 1% DMSO in RPMI. The putative modulating drugs were added to lymphocytes one hour before DEB exposure. Lymphocytes were then incubated 5 h and for each treatment, samples were collected at 2, 3, 4 and 5 h after DEB exposure.

2.5. Analytical techniques

2.5.1. Cell viability

Cell number and viability, as denoted by Trypan blue exclusion, were determined at all sampling points, on a neubauer chamber under an optic microscope (40×).

2.5.2. Sample processing for ATP and glutathione quantification

At the established sampling times, cell suspension aliquots were spinned (12,300 × *g*, 10 s, 4 °C) and the supernatants rejected. The pellet was then treated with $HClO_4$ 5%. After a brief vortexing, the homogenates were centrifuged (16,000 × *g*, 3 min, 4 °C). Aliquots of the resulting supernatants were immediately stored at −80 °C for the measurement of ATP and GSH, within one week. The pellet was dissolved in NaOH 0.3 M and stored at −20 °C for posterior protein quantification.

2.5.3. ATP quantification

ATP was measured using a bioluminescence assay, based in the fact that luciferase catalyzes the formation of light from ATP and luciferin (Capela et al., 2007). The emitted light intensity is linearly related to the ATP concentration and was measured using a 96-well Microplate Luminometer (BioTek Instruments).

2.5.4. Glutathione quantification

Glutathione quantification was performed by the DTNB-glutathione reductase recycling assay, based on the oxidation of GSH by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), as first described by Vandeputte et al. (1994) with some modifications (Carvalho et al., 2004). The rate of TNB formation is monitored at 415 nm and is proportional to the sum of GSH and GSSG present in the sample.

2.5.5. Protein quantification

Protein quantification was performed according to the method of Lowry et al. (1951) with some modifications (Dinis-Oliveira et al., 2007), using BSA as standard.

2.6. Statistical analysis

Results are expressed as mean ± SEM. The number of experiments for each assay is mentioned in the legend of each figure. Statistical comparison between groups was estimated using two-way ANOVA, followed the Bonferroni post hoc test with GraphPad Prism, version 5.0. *P* values lower than 0.05 were considered as statistically significant.

3. Results

3.1. Acute toxicity of DEB

Lymphocytes incubated for 5 h with DEB underwent glutathione depletion (Fig. 1A) and loss of ATP (Fig. 1B), followed by loss of cell viability (Fig. 1C). While glutathione depletion was already maximal at the second hour of incubation (down to about 35% of control levels), ATP depletion and cell death followed a time and concentration dependent pattern. Of note is also the severe depletion of ATP observed at the second hour of incubation (down to about 25% of control levels), and the fact that 400 μg/ml DEB was the only concentration achieving significant loss of viability in the studied human lymphocytes in the first 2 h of incubation. From these results, exposure of isolated human lymphocytes at the 400 μg/ml DEB concentration was chosen for the subsequent treatment with putative modulating drugs.

3.2. Epoxide hydrolase inhibition

Inhibition of sEH by CDU and inhibition of mEH by elaidamide had no effect on DEB (400 μg/ml)-induced toxicity, as measured by cellular levels of glutathione and ATP, as well as by cell viability (data not shown). Interestingly, pretreatment of lymphocytes with Zn^{2+}, an inhibitor of both EHs, prevented cell death in the first 3 h of incubation with DEB (Fig. 2), though it did not protect cells from DEB-induced depletion of glutathione and ATP (data not shown). After 3 h, Zn^{2+} showed to be toxic by itself and the protective effect was lost (Fig. 2).

3.3. Antioxidants

The antioxidant NAC protected lymphocytes from acute toxicity of DEB (400 μg/ml), as measured by cellular levels of glutathione and ATP, as well as by cell viability, though it only reached significance for glutathione at the third hour of exposure and viability at the fourth hour of exposure (Fig. 3).

3.4. Mitochondrial protective agents

ALC elicited a partial protective effect on DEB (400 μg/ml)-induced toxicity. Despite no significant differences were found in cellular levels of glutathione and ATP in DEB-induced lymphocytes co-exposed simultaneously to ALC (data not shown), ALC reduced cell death at the fourth and fifth hours of exposure to DEB (Fig. 4).

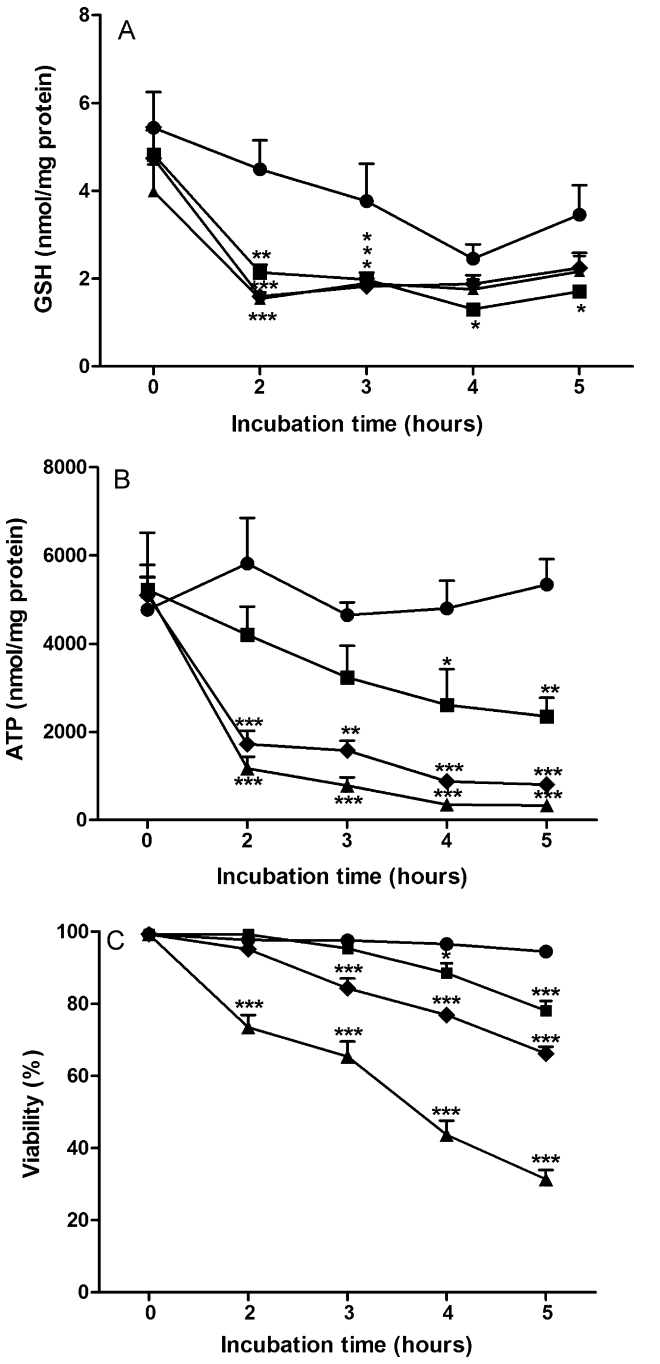

Fig. 1. DEB-induced acute toxicity in human lymphocytes, *in vitro*. (A) GSH levels, (B) ATP levels and (C) cellular viability in suspensions of human mononuclear lymphocytes (1×10^6 cells/ml) exposed during 5 h to DEB 25 μg/ml (■), DEB 100 μg/ml (♦), DEB 400 μg/ml (▲) and the control group (●). Results are expressed as the mean ± SEM of seven different experiments. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control.

α-LA alone did not protect lymphocytes from DEB-induced acute toxicity (data not shown). However, it potentiated ALC protective effect, since protection against DEB-induced cell death began earlier, at the third hour of incubation (Fig. 5).

3.5. Putative aggravation of DEB-induced toxicity

Inhibition of GST by tannic acid, inhibition of catalase by 3-AT and inhibition of protein synthesis by CHX had no effect on

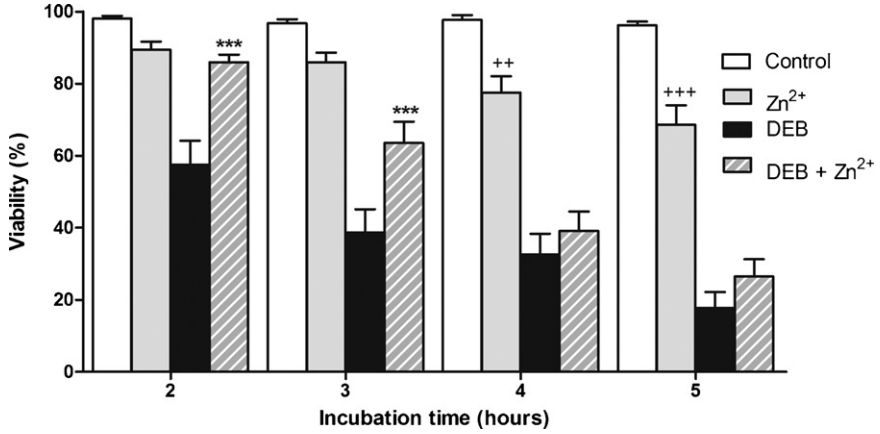

Fig. 2. Effect of Zn^{2+} on DEB-induced loss of viability in human lymphocytes, *in vitro*. Cellular viability in suspensions of human mononuclear lymphocytes (1×10^6 cells/ml), exposed to Zn^{2+} 10 mM, DEB 400 μg/ml, simultaneously to DEB 400 μg/ml and Zn^{2+} 10 mM, and the control group. Results are expressed as the mean ± SEM of six different experiments. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, compared with control. $^{***}P < 0.001$ compared with DEB 400 μg/ml.

DEB (400 μg/ml)-induced toxicity, as measured by cellular levels of glutathione and ATP, as well as by cell viability (data not shown).

4. Discussion

The purpose of the present study was to contribute for a better understanding of DEB-induced toxicity to human lymphocytes, by studying the putative contribution of biochemical pathways postulated to be involved in the reactivity of this compound. Another goal was to search for protective drugs that may turn out to be useful to prevent or delay the development of FA. The study reports two novel findings: (i) it was clearly evidenced, for the first time, that the acute exposure of freshly isolated human lymphocytes to DEB results in severe GSH depletion and loss of ATP, followed by cell death; (ii) ALC elicits a significant protective effect on DEB induced toxicity, which was potentiated by α-LA.

It is widely acknowledged that GSH-based pathways are involved in the metabolism and detoxification of DEB and its metabolites. For this assumption contributed pioneer studies performed by Boogaard et al. (1996), who demonstrated that human, rat and mouse liver cytosolic fractions were able to conjugate 10 mM [^{3}H] glutathione with DEB, in a concentration-dependent manner (DEB concentration ranging from 0.1 to 100 mM), and further confirmed this pathway in rat and mouse lung cytosolic fractions. Corroborating this assumption, GSH depletion was later reported to increase DEB-induced cytotoxicity on mouse germ cells (Spanò et al., 1998). DEB was also shown to deplete GSH in sea urchin embryos (Korkina et al., 2000). However, from the best of our knowledge, the depletion of GSH after acute exposure to DEB, in mammal cells, has never been demonstrated before. The present results, not only demonstrate this effect in the human target cells, the lymphocytes, but also show, for the first time, that following GSH depletion these cells suffer a severe loss of ATP levels, which lead to a significant cell death. It is noteworthy that the glutathione system has an important role in maintaining the integrity of mitochondria in these cells, well evidenced by the fact that GSH protects mtDNA from oxidative damage in human lymphocytes and that GSH depletion increases their susceptibility to mtDNA depletion (Hollins et al., 2006).

The depletion of glutathione will result in two major blows in the cell defense systems, one of them being the higher susceptibility to electrophilic agents, namely DEB itself and its metabolites, and the other being the higher susceptibility to prooxidant reactive species (which may also result from DEB reactivity), due to the loss of this important endogenous antioxidant. In fact, DEB-induced cytotoxicity, despite hitherto not completely understood, has been related to its ability to react with protein sulfhydryl (SH) groups (Loecken and Guengerich, 2008), to mediate the formation of DNA–DNA and DNA–protein cross-links (Michaelson-Richie et al., 2010), as well as to its capability to induce the production of ROS and induce oxidative stress (Erexson and Tindall, 2000; Yadavilli et al., 2007). The use of SH-donor antioxidants like NAC, may help to support this theory. In fact, NAC is a known precursor for glutathione synthesis that has also been shown to act by itself on redox balance and to significantly improve the antioxidant potential by increasing reduced glutathione (Mantovani et al., 2003). In accordance, in the present study we verified that NAC can protect human lymphocytes from acute toxicity of DEB at all measured parameters, corroborating a previous study showing that NAC inhibits ROS production and apoptosis induced by DEB in human lymphoblasts (Yadavilli et al., 2007).

From the results here obtained and those obtained by others using human TK6 lymphoblast as experimental model (Yadavilli et al., 2007), mitochondria seems to be substantially affected in the event of exposure to DEB. ALC and α-LA are potent antioxidants with their action reflected in mitochondrial stability, their beneficial effects being well established in several diseases related with oxidative stress, like diabetes mellitus, AIDS and various types of cancer (Calabrese et al., 2006; Devasagayam et al., 1993; Han et al., 1997; Mantovani et al., 2003). ALC, being a mitochondrial metabolite, improves the mitochondrial function and increases general metabolic activity (Calabrese et al., 2006). Here we demonstrated, for the first time, that ALC elicits a partial protective effect on DEB induced toxicity. α-LA, besides its potential to induce a substantial increase in cellular reduced glutathione, is a scavenger of hydroxyl radicals, singlet oxygen and hypochlorous acid (Han et al., 1997) and functions as an essential cofactor in metabolic reactions involving energy utilization in mitochondria (Prahalathan et al., 2006). Our results showed that α-LA did not protect lymphocytes from DEB-induced acute toxicity. However, the combined use of α-LA and ALC resulted in a potentiation of ALC protective effect, which agrees with the assumption that α-LA shows beneficial effects in oxidative stress conditions, own to its synergistic action with other antioxidants (Hagen et al., 2002; Mantovani et al., 2003; Prahalathan et al., 2006).

Boogaard and Bond (1996) suggested that DEB metabolism and detoxification in humans are in its majority effectuated by EH enzymes in liver and lungs, mainly mEH, and that metabolites formed in this process are less toxic than metabolites formed by DEB conjugation with GSH. Our results indicate that inhibition of sEH and mEH, in human lymphocytes, by CDU and elaidamide, respectively, does not affect DEB toxicity. Tentative inhibition of

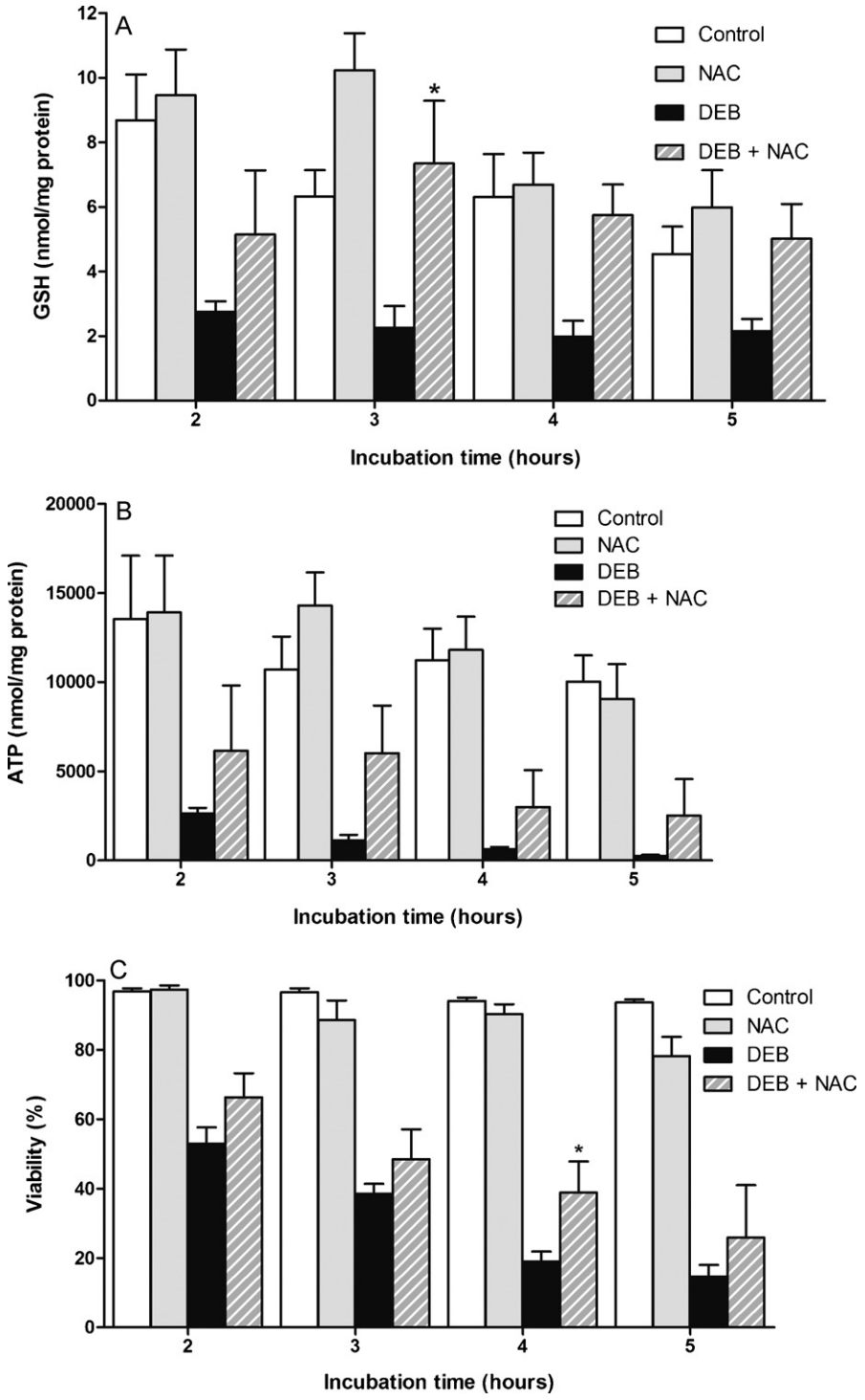

Fig. 3. Effect of NAC on DEB-induced acute toxicity in human lymphocytes, *in vitro*. (A) GSH levels, (B) ATP levels and (C) cellular viability in suspensions of human mononuclear lymphocytes (1×10^6 cells/ml) exposed to NAC 500 μM, DEB 400 μg/ml, simultaneously to DEB 400 μg/ml and NAC 500 μM, and the control group. Results are expressed as the mean ± SEM of six different experiments. *$P < 0.05$ compared with DEB 400 μg/ml.

both EHs by Zn^{2+} elicited a protective effect in the first 3 h of incubation, avoiding cell death. However, Zn^{2+} does not protect lymphocytes from depletion of glutathione and ATP, which suggests that this protective effect may be related with a mechanism other than inhibition of EHs. In fact, it has recently been recognized that Zn^{2+} has a protective role by counteracting oxidative stress (Bray and Bettger, 1990; Prasad, 2008; Prasad et al., 2004; Stehbens, 2003). Zn^{2+} decreases ROS generation through inhibition of NADPH oxidase, is an excellent scavenger of HO•, and is a co-factor of cytosolic superoxide dismutase (SOD) (Prasad, 2008; Prasad et al., 2004). Zn^{2+} may further protect lymphocytes by inducing the synthesis of metallothioneins (MTs) (Yamada and Koizumi, 1991). Since MTs contain a high amount of cysteine (to the extent of one third of the total amino acids), this effect may contribute for the antioxidant effect of Zn^{2+}. In line with this, Zn^{2+} was previously shown to protect human peripheral blood lymphocytes from Cr(III)(phenanthroline)3-induced oxidative stress and apoptosis (Sankaramanivel et al., 2010). Together, these findings can

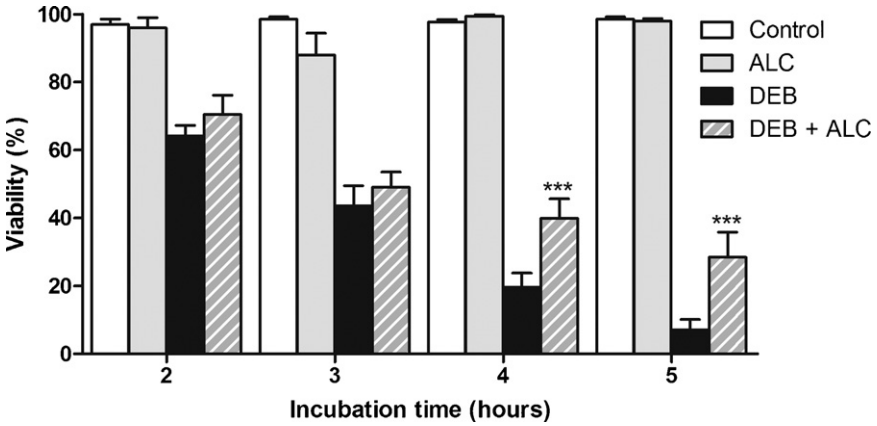

Fig. 4. Effect of ALC on DEB-induced loss of viability in human lymphocytes, *in vitro*. Cellular viability in suspensions of human mononuclear lymphocytes (1×10^6 cells/ml) exposed to ALC 5 mM, DEB 400 μg/ml, simultaneously to DEB 400 μg/ml and ALC 5 mM, and the control group. Results are expressed as the mean ± SEM of five different experiments. $^{***}P < 0.001$ compared with DEB 400 μg/ml.

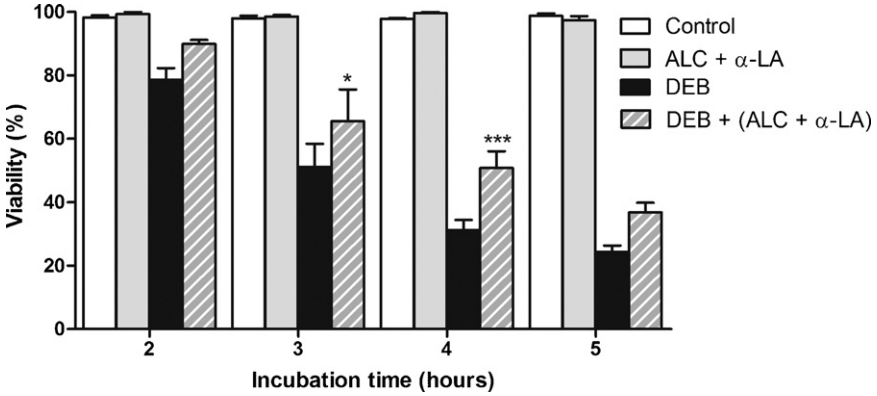

Fig. 5. Effect of ALC plus α-LA on DEB-induced loss of viability in human lymphocytes, *in vitro*. Cellular viability in suspensions of human mononuclear lymphocytes (1×10^6 cells/ml) exposed to ALC 5 mM + α-LA 100 μM, DEB 400 μg/ml, simultaneously to DEB 400 μg/ml and ALC 5 mM + α-LA 100 μM, and the control group. Results are expressed as the mean ± SEM of five different experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with DEB 400 μg/ml.

help to explain the partial protective effects of Zn^{2+} observed in the present study. Besides the antioxidant effects of Zn^{2+}, it is important to keep in mind that the other route of DEB detoxification, GSH conjugation, is not affected by EHs inhibition. Furthermore, our results suggest that hydrolysis of DEB by EH in lymphocytes, in opposition to what occurs in lungs and liver, is not the most important route of detoxification.

Under the present experimental conditions, the inhibition of GST by tannic acid, inhibition of catalase by 3-AT and inhibition of protein synthesis by CHX had no effect on DEB-induced toxicity in human lymphocytes. The lack of tannic acid modulatory effect on DEB toxicity may reflect insufficient inhibition or redundancy in the metabolism of this diepoxide by GST isoforms in human lymphocytes. These cells contain moderate levels of GSTμ and GSTπ (Van Lieshout and Peters, 1998). Tannic acid as variable inhibitory potencies for the different GST isoforms but only partial effects have been achieved *in vitro* (Zhang et al., 1997) and *in vivo* (Krajka-Kuźniak et al., 2008), which indicates that the inhibition of GST activity is probably not a good approach to prevent clastogenesis in FA patients. Concerning the inhibition of catalase by 3-AT, it was previously demonstrated that it increases the levels of 8-hydroxydeoxyguanosine (8OHdG), typical of oxidative DNA damage, in Epstein-Barr virus transformed FA lymphoblasts and normal controls exposed to 20 mM H_2O_2 (Takeuchi and Morimoto, 1993). The absence of aggravating effects in the present study indicates that H_2O_2 production is not a major contributor for the observed deleterious effects. The fact that CHX, a prototypical protein synthesis inhibitor commonly used to study cell signal transduction had no effect on DEB toxicity indicates absence of regulatory synthesis of protective proteins following the acute effects of DEB.

In conclusion, the results obtained in the present study clearly demonstrate that acute exposure to DEB causes glutathione depletion and loss of ATP, followed by cell death in human lymphocytes. We also demonstrated, for the first time, that ALC elicits a partial protective effect on DEB induced toxicity. In addition, while α-LA alone did not protect lymphocytes from DEB-induced acute toxicity, the combined use of α-LA and ALC resulted in a potentiation of ALC protective effect. Collectively, these results contribute to increase our knowledge of DEB-induce toxicity, which may prove to be useful to find new ways to prevent spontaneous and DEB-induced chromosome instability, and ultimately to prevent clastogenesis in lymphocytes from patients with FA.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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CHAPTER II

Improvement of genetic stability in lymphocytes from Fanconi Anemia patients through the combined effect of α-lipoic acid and N-acetylcysteine

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Improvement of genetic stability in lymphocytes from Fanconi anemia patients through the combined effect of α-lipoic acid and N-acetylcysteine

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Abstract

Fanconi Anemia (FA) is a rare genetic disorder, characterized by progressive bone marrow failure and increased predisposition to cancer. Despite being highly heterogeneous, all FA patients are hypersensitive to alkylating agents, in particular to 1,2:3,4-diepoxybutane (DEB), and to oxidative damage. Recent studies point to defective mitochondria in FA cells, which is closely related with increased production of reactive oxygen species (ROS) and concomitant depletion of antioxidant defenses, of which glutathione is a well-known biomarker.

The objective of the present work is to evaluate the putative protective effect of α-lipoic acid (α-LA), a mitochondrial protective agent, and N-acetylcysteine (NAC), a direct antioxidant and a known precursor for glutathione synthesis, in spontaneous and DEB-induced chromosome instability (CI) in lymphocyte cultures from FA patients.

For that purpose, lymphocyte cultures from 15 FA patients and 24 healthy controls were pre-treated with 20 μM α-LA, 500 μM NAC and α-LA plus NAC at the same concentrations, and some of them exposed to DEB (0.05 μg/ml). A hundred metaphases per treatment were scored to estimate the relative frequency of spontaneous and DEB-induced chromosome breakage.

The obtained results reveal that a cocktail of α-LA and NAC can drastically improve the genetic stability in FA lymphocytes *in vitro,* decreasing CI by 60% and 80% in cultures from FA patients and FA mosaic/chimera patients, respectively. These results suggest that the studied cocktail can be used as a prophylactic approach to delay progressive clinical symptoms in FA patients caused by CI, which can culminate in the delay of the progressive bone marrow failure and early cancer development.

Keywords

Fanconi Anemia, oxidative stress, antioxidants, α-lipoic acid, N-acetylcysteine, chromosome instability, bone marrow failure, cancer susceptibility.

Introduction

Fanconi Anemia (FA) is a rare genetic disorder characterized by chromosome instability (CI), several congenital malformations, progressive bone marrow failure, and increased predisposition to cancer, particularly acute myelogenous leukemia [1]. Genetically, FA is a highly heterogeneous disease with 15 genes so far characterized and cloned (FA-A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P) [2] and that appears to influence genomic stability. The clinical manifestations and the onset of disease are also extremely variable. However, all FA patients have, in common, unique markers that characterize the disease: hypersensitivity to the clastogenic effect of DNA cross-linking agents, in particular to diepoxybutane (DEB), [3] and hypersensitivity to oxidative damage [4].

DEB-induced cytotoxicity, despite hitherto not completely understood, has been related to oxidative damage and to glutathione (GSH) depletion [5-7]. The activation of the mitochondrial apoptotic pathway in the event of DEB-induced oxidative stress (OS) was more recently demonstrated [8]. Importantly, DEB-induced DNA-DNA and DNA-protein cross-links [9] are a characteristic feature of ROS-mediated damage. In accordance to the involvement of mitochondria and OS in the mechanisms of DEB toxicity, we have recently demonstrated that acute exposure to DEB both causes glutathione (GSH) depletion and loss of ATP, followed by cell death in lymphocytes collected from healthy humans [10].

The role of oxygen and oxidative damage in FA cells was described for the first time by Nordenson [11] followed by Joenje et al [12]. Two years later, Joenje and Oostra [13] established a direct correlation between high tensions of oxygen and increased damage in FA lymphocytes. A direct association of OS with the primary genetic defect in FA was suggested through the interaction of the FANCC protein with NADPH cytochrome P450 reductase [14] and glutathione S-transferase [15], two enzymes involved in the detoxification of reactive intermediates. At least FANCC, FANCG and FANCA have been shown to be associated with redox-related imbalance and to a unique functional and structural sensitivity to OS [16] and, altogether, these proteins are also related with mitochondrial dysfunction [14-18]. Recently, Mukhopadhyay et al [18] reported that FANCG protein physically interacts with the mitochondrial peroxidase peroxiredoxin 3 (PRDX3), FA-G cells display distorted structures, and FA-A, -C and -G have PRDX3 cleavage and decreased peroxidase activity.

Mitochondrial dysfunction ultimately leads to oxidative modifications of DNA, proteins and lipids. It is recognized that FA cells show abnormal accumulation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a sub-product of oxidative DNA damage, and also a mutagenic substance by itself [19]. However, Castillo et al [20] concluded that the FA pathway is redundant for the repair of oxidative DNA damage (7,8-dihydro-8-hydroxyguanine, 8-oxoG). Therefore, the accumulation of 8-oxoG in FA cells reflects sustained ROS overproduction rather than defective processing of oxidized bases. These evidences, altogether, point towards a pro-oxidant state associated with dysfunctional mitochondria, in FA cells (reviewed by Pagano et al) [4].

Some studies have been performed testing the use of antioxidants for decreasing chromosome damage in FA cells. Dallapiccola et al [21] obtained a partial correction of CI in FA lymphocytes treated with the antioxidants sodium ascorbate, L-cysteine, 2-mercaptoethanol, α-mercaptopropionylglycine, and GSH. Later, the same group [22] extended this study to desferoxamine, also with partial correction of CI. Pincheira et al [23] demonstrated that Vitamin E decreases the frequency of chromosomal damage and the duration of G2 in FA lymphocytes. Some *in vivo* studies were also performed in animal models. Zhang et al [24] showed that dietary supplements with antioxidants can delay the age of onset of epithelial tumors in FA mouse models. More recently, this group [25] also demonstrated that the antioxidant resveratrol maintains Fancd2(−/−) KSL cells in quiescence, improves the marrow

microenvironment, partially corrects the abnormal cell cycle status, and significantly improves the spleen colony-forming capacity of Fancd2(−/−) bone marrow cells. In spite of the promising results from previous studies, no therapy using antioxidants, megavitamins, or micronutrients has been shown to be effective in treatment of FA using evidence-based criteria. Thus, further studies with new protective agents are required for the application of drugs with high effectiveness.
Among a number of potential candidate molecules with antioxidant properties, some can be identified as "mitochondrial nutrients" and substances that normalize GSH balance. α-Lipoic acid (α-LA) is a natural and endogenous compound that occurs in mitochondria and is an essential cofactor for many mitochondrially localized enzyme complexes. Furthermore, α-LA features a cyclic disulfide moiety which exists in redox equilibrium with its reduced dithiol form, dihydrolipoic acid. Dihydrolipoic acid acts in GSH replenishment and is a potent antioxidant in a redox-cycling environment, by reducing vitamin C, vitamin E and coenzyme Q_{10} [26,27]. Previous studies also proved that α-LA can be a protective agent against clastogenic [28] and genotoxic agents [29]. N-acetylcysteine (NAC), the acetylated variant of the amino acid L-cysteine, is an excellent source of sulfhydryl groups, and is converted in the body into metabolites capable of stimulating GSH synthesis, promoting detoxification, and acting directly as free radical scavengers [30]. Recently, a study from Yadavilli et al [8] showed that NAC has the ability to inhibit ROS production and apoptosis induced by DEB in human lymphoblasts. Importantly, there are some clinical studies referring the benefit of the combined use of α-LA and NAC by ameliorating the hematological response in advanced cancer patients [31] and in the event of muscle-damaging exercise [32].
In a previous study [10], we showed that α-LA and NAC have a partial protective effect on acute DEB-induced toxicity to normal human lymphocytes, though the effect of these compounds in CI of lymphocytes has never been tested before. Thus, the aim of the present work was to evaluate the putative protective effect of α-LA and NAC, in spontaneous and DEB induced CI lymphocytes cultures from FA patients.

Methods

Subjects

This study includes 15 FA patients, referred by several Hospitals, and already diagnosed with certainty on the basis of DEB-induced cytogenetic tests, and 24 healthy donors (HD), recruited among healthy male and female donors, aged 20–40 years. The study was approved by the Ethical Committe of Hospital Geral de Santo António, CHP, Porto. All procedures were done with the written informed consent of the participants.

Cells and cell cultures

From each HD participant, 10 ml of venous blood was collected by antecubital venipuncture, into vacuum tubes with lithium heparin. From FA patients, 3 ml to 6 ml of venous blood were collected, depending on the age and healthy conditions of the patients, into vacuum tubes with lithium heparin.
Whole blood (0.5 ml) was cultured in RPMI 1640 (Sigma) complete medium supplemented with 15% fetal calf serum (GIBCO), antibiotics (10,000 units/ml of penicillin and 10,000 μg/ml of streptomycin) (GIBCO) and 29 mg/ml of L-glutamine (Sigma). Cultures were stimulated with 5 μg/ml of phytohemagglutinin (GIBCO) and placed in an incubator at 37°C with 5% CO_2 atmosphere, for 72 h.

Exposure to DEB
DEB ((±)-1,2:3,4-diepoxybutane, [298-18-0], D-7019 Lot 34 H3683, Sigma), prepared in RPMI 1640, was added to lymphocyte cultures 24 h after their initiation, thus exposing cells to the chemical for 48 h. In lymphocyte cultures from FA patients, DEB was added at the concentration of 0.05 μg/ml (the same

concentration used in the diagnostic test). As the lymphocytes from HD are not so sensitive to DEB at that concentration, and have variable responses, 3 higher concentrations were used to obtain a significant toxic response (see Figure 1 for a description of DEB-induced CI pattern in HD and FA patients). Therefore, a concentration of 0.1 μg/ml was selected in cultures from 5 individuals (HD1-5), a concentration of 0.2 μg/ml was selected in cultures from 11 individuals (HD6-11 and HD14-18) and a concentration of 0.4 μg/ml was selected in cultures from 8 individuals (HD12-13 and HD19-24).

Since DEB is a suspected carcinogen with unknown risk, appropriate precautions were taken. All culture procedures were handled using appropriate gloves and in a vertical laminar flow hood. As DEB is rapidly inactivated by concentrated HCl, all disposable culture bottles and pipettes were rinsed with HCl before being discarded.

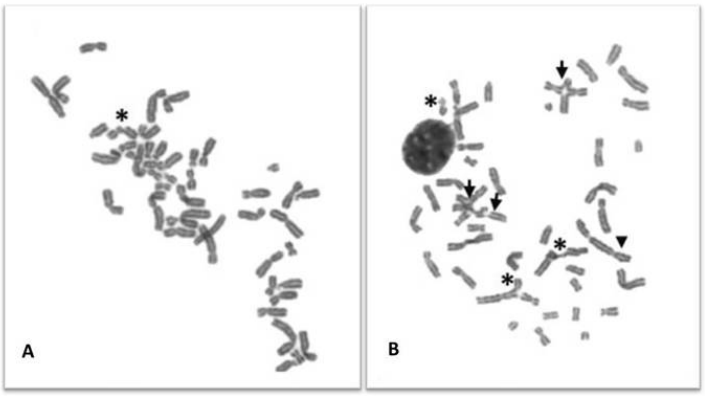

Figure 1. CI pattern in metaphases from DEB-induced lymphocyte cultures from a healthy donor and FA patient. Chromosomes were stained in a 4% Giemsa solution. The images were observed with an optical microscope (Olympus CX31) and captured with a digital camera (Nikon Sightds-smc), with the software DP20-5E microscope digital camera. (A) Selected metaphase from a HD lymphocyte culture exposed to 0.2 μg/ml of DEB for 48 h. One chromatid break can be seen (asterisk). (B) Selected metaphase from a FA patient lymphocyte culture exposed to 0.05 μg/ml of DEB for 48 h. High level of chromosome instability can be visualized, especially being important the tri and tetraradial figures (arrow) that are the hallmark for the diagnosis of FA. It can also be seen a dicentric chromosome (head arrow) and 3 chromatid breaks (asterisks).

Antioxidant treatments

We used two antioxidants, α-LA 20 μM [33] (Sigma) and NAC 500 μM [10] (Sigma) at concentrations that proved to be effective in *in vitro* studies, preferably with lymphocytes. In addition, prospective studies were always performed to find the most efficient concentrations (data not shown). Additionally, α-LA and NAC concentrations used in the present study are within the plasma concentrations found in previous human pharmacokinetic studies using these drugs [34,35]. α-LA was dissolved in 1% dimetilsulfoxide (DMSO) in RPMI 1640 and NAC was dissolved in RPMI 1640. α-LA was stored in the dark at room temperature. NAC was stored at 4°C. The two antioxidants were used from the same batch in all experiments.

Antioxidant treatments in DEB-exposed lymphocyte cultures from healthy donors

Two sets of experiments were done. In the first set, lymphocyte cultures were pre-treated with α-LA 20 μM 1.5 h before DEB exposure. In the second set of experiments lymphocyte cultures were pre-treated with NAC 500 μM, 1.5 h before DEB exposure. DMSO toxicity was found to be absent within our experimental conditions.

Antioxidant treatments in spontaneous and DEB-exposed lymphocyte cultures from FA patients

Three sets of experiments were done. In the first set, lymphocyte cultures were treated with α-LA 20 μM for 48 h. In DEB-induced lymphocyte cultures α-LA was added 1.5 h before DEB exposure. In the second, lymphocyte cultures were treated with NAC 500 μM for 48 h. In DEB-exposed lymphocyte cultures, NAC was added 1.5 h before DEB

exposure. In the last set, both antioxidants were added simultaneously to lymphocyte cultures, at the same concentrations, for 48 h. In DEB-exposed lymphocyte cultures antioxidants were added 1.5 h before DEB exposure. DMSO toxicity was found to be absent within our experimental conditions.

Cytogenetic analysis

After 3 days of culture, cells were harvested after 1 h of incubation with colcemid (GIBCO) (4 μg/ml), followed by hypotonic treatment with 75 mM KCl (Sigma) and fixed 3 times in a 1:3 iced solution of acetic acid (Merck): methanol (Merck). The resulting suspensions were dropped onto microscope slides and stained for 5 min in a 4% Giemsa solution (Merck) diluted in phosphate buffer saline solution (Sigma).
Analysis was performed on 50–100 metaphases from each experiment, by an independent scorer and in a blinded fashion. Only when the mitotic index (MI) was very low and the number of breaks was high, a minimum of 22 metaphases was counted. Each cell was scored for chromosome number and the number and type of structural abnormalities. Gaps (acromatic areas less than a chromatid in width) were excluded in the calculation of chromosome breakage frequencies, and rearrangements (triradials and quadriradials, dicentrics and ring chromosomes) were scored as two breaks.

Mitotic index

From the same microscope stained slides, used for chromosome breakage analysis, a thousand cells were randomly scored, distinguishing between interphase nuclei and metaphase nuclei. MI was calculated as follows: number of metaphase nuclei /number of total nuclei.

Statistical analysis

Graph results are expressed as mean ± SEM and table results are expressed as mean ± SD. Statistical comparison among groups was estimated using one-way ANOVA, followed by the Bonferroni post hoc test, and comparison between two groups was estimated using paired t-test, both with GraphPad Prism, version 5.0 software. P values lower than 0.05 were considered as statistically significant.

Results

Effect of α-lipoic acid and N-acetylcysteine on DEB-induced CI in lymphocyte cultures from healthy donors

As shown in Table 1, lymphocyte cultures from HD exposed to DEB and pre-treated with α-LA or NAC showed a reduction in the number of breaks per cell compared to those only exposed to DEB ($P > 0.05$ and $P < 0.05$, respectively). Noteworthy, two distinct groups can be considered concerning the protective effect: a group of responders and another one of non-responders. Among DEB-induced cultured lymphocytes pre-treated with α-LA 54% are responders whereas among those pre-treated with NAC, 82% are responders. However, the response with α-LA treatment is greater, while with NAC the potency is not only lower but also more variable. Globally, the percentage of reduction in lymphocyte cultures pre-treated with α-LA was 25% and with NAC was 20%.

Effect of α-lipoic acid, N-acetylcysteine and α-lipoic acid plus N-acetylcysteine on spontaneous CI in lymphocyte cultures from FA patients

As shown in Table 2, lymphocyte cultures from FA patients treated with α-LA, NAC and α-LA plus NAC showed a significant reduction in the number of breaks per cell, compared to cultures without antioxidant treatment ($P < 0.01$, $P < 0.01$ and $P < 0.01$, respectively). Comparing α-LA *vs* NAC treatments, no difference ($P > 0.05$) was found in the reduction of the number of breaks per cell. However, comparing both isolated α-LA

and NAC treatments with the combined α-LA plus NAC treatment, the reduction in the number of breaks per cell was highly significant ($P < 0.05$ and $P < 0.01$, respectively). Globally, the percentage of reduction in lymphocyte cultures treated with α-LA was 34% ($P < 0.01$), with NAC was 40% ($P < 0.01$) and with α-LA plus NAC was 58% ($P < 0.001$), as shown in Figure 2.

Concerning the α-LA antioxidant effect two distinct groups were, once more, observed: responders and non-responders; in a total of 13 lymphocyte cultures, 85% were responders, and in two of them CI decreased to normal values (below 0.1). In NAC and α-LA plus NAC treated cultures only responders were observed. Besides, in two cultures treated with NAC and four treated with α-LA plus NAC a decrease in the number of breaks per cell to normal values was also observed (Table 2).

Table 1. Number of DEB-induced breaks per cell in cultured lymphocytes from healthy donors.

Number of breaks per cell							
Healthy donors	Control group (n)	α-LA 20 µM (n)	% reduction	Healthy donors	Control group (n)	NAC 500 µM (n)	% reduction
HD1	0,09 (100)	0,09 (100)	0,00	HD14	0,20 (41)	0,14 (50)	30,00
HD2	0,09 (100)	0,06 (100)	33,33	HD15	0,14 (50)	0,12 (50)	14,29
HD3	0,07 (100)	0,07 (100)	0,00	HD16	0,12 (50)	0,04 (50)	66,67
HD4	0,08 (100)	0,03 (100)	62,50	HD17	0,10 (50)	0,12 (50)	0,00
HD5	0,04 (100)	0,01 (100)	75,00	HD18	0,12 (50)	0,08 (50)	33,33
HD6	0,44 (100)	0,12 (100)	72,73	HD19	0,22 (50)	0,16 (50)	27,27
HD7	0,12 (100)	0,11 (100)	8,33	HD20	0,32 (50)	0,14 (50)	56,25
HD8	0,08 (100)	0,09 (100)	0,00	HD21	0,14 (50)	0,10 (50)	28,57
HD9	0,09 (100)	0,10 (100)	0,00	HD22	1,14 (50)	1,00 (50)	12,28
HD10	0,10 (100)	0,11 (100)	0,00	HD23	0,18 (51)	0,12 (50)	33,33
HD11	0,18 (50)	0,08 (100)	55,56	HD24	0,13 (100)	0,20 (50)	0,00
HD12	0,19 (53)	0,22 (100)	0,00	–	–	–	–
HD13	0,15 (100)	0,06 (100)	60,00	–	–	–	–
Mean±SD	0.132±0.102	0.088±0.051		Mean±SD	0.255±0.300	0.202±0.268	

In control group no antioxidant treatment was done. The effect of the antioxidant treatments was calculated by the percentage of reduction of the number of breaks per cell in α-LA and NAC treatments relatively to control group. — Unavailable data; α-LA indicates α-lipoic acid; NAC indicates N-acetylcysteine; n indicates the number of cells analyzed. Paired t-test: Control group vs NAC 500 µM $P<0.05$

Table 2. Number of breaks per cell in cultured lymphocytes from FA patients.

	Mean number of breaks per cell						
Patients	Control group (n)	α-LA 20 μM (n)	% reduction	NAC 500 μM (n)	% reduction	α-LA 20 μM + NAC 500 μM (n)	% reduction
FA1	0.26 (100)	0.09 (100)	65.38	–	–	–	–
FA2	0.27 (100)	0.14 (100)	48.15	–	–	–	–
FA3	0.28 (100)	0.15 (100)	46.43	–	–	–	–
FA4	0.62 (91)	0.29 (92)	53.23	–	–	–	–
FA5	1.50 (100)	0.94 (100)	37.33	–	–	–	–
FA6	0.75 (100)	0.50 (76)	33.33	–	–	–	–
FA7	1.50 (50)	1.50 (50)	0.00	–	–	–	–
FA8	0.19 (100)	0.16 (50)	15.79	0.16 (50)	15.79	0.16 (50)	15.79
FA9	0.26 (50)	0.12 (50)	53.85	0.06 (50)	76.92	0.04 (50)	84.62
FA10	0.30 (50)	0.14 (50)	53.33	0.08 (50)	73.33	0.04 (50)	86.67
FA11	0.22 (50)	0.18 (50)	18.18	0.10 (50)	54.55	0.06 (50)	72.73
FA12	0.24 (50)	0.08 (50)	66.67	0.18 (50)	25.00	0.06 (50)	75.00
FA13	0.30 (50)	0.44 (50)	0.00	0.26 (50)	13.33	0.20 (50)	33.33
FA14	0.31 (100)	–	–	0.24 (50)	22.58	0.20 (50)	35.48
Mean±SD	0.500±0.452	0.364±0.417		0.154±0.078		0.109±0.075	

In control group no antioxidant treatment was done. The effect of the antioxidants was calculated by the percentage of reduction of the number of breaks per cell in the α-LA, NAC and α-LA plus NAC treatments relatively to control group. — Unavailable data. Abbreviations are explained in Table 1. Paired t-test: Control group vs α-LA 20 μM $P < 0.01$; Control group vs NAC 500 μM $P < 0.01$; Control group vs α-LA 20 μM + NAC 500 μM $P<0.01$; α-LA 20 μM vs α-LA 20 μM + NAC 500 μM $P<0.05$; NAC 500 μM vs α-LA 20 μM + NAC 500 μM $P<0.01$.

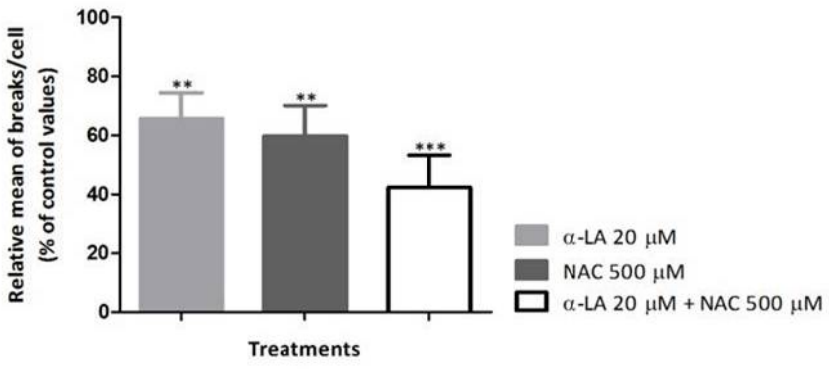

Figure 2. Effect of α-LA, NAC and α-LA+NAC on chromosome breaks in cultured lymphocytes from FA patients. Lymphocyte cultures were treated with 20 μM α-LA, 500 μM NAC, and both antioxidants simultaneously at the same concentrations. The results are presented as the relative mean of breaks per cell (percen- tage of control values). Comparison of the relative mean of breaks/cell was made between control group and each antioxidant treatment (*** $P<0.001$ and ** $P < 0.01$) and between treatments ($P>0.05$). Results are representative of the mean of 14 experiments depicted from Table 2.

Effect of α-lipoic acid, N-acetylcysteine and α-lipoic acid plus N-acetylcysteine on spontaneous CI in lymphocyte cultures from a particular group of FA patients - mosaics and chimera

Considering the cytogenetic diagnosis of the patients referred in Table 2, patients FA9 and FA10 were classified as mosaics and FA11 as a post-transplant chimera. Thus, they have a higher frequency of normal cells. What we can see is that, for these patients, the number of breaks per cell decreased to normal values with α-LA plus NAC treatment, which translates in an even greater reduction in the number of breaks per cell, of about 80%, $P<0.001$ (Figure 3).

Effect of α-lipoic acid plus N-acetylcysteine on cell cycle progression in cultured lymphocytes from FA patients

MI was measured in lymphocyte cultures submitted to the three types of antioxidant treatments. The results displayed in Table 3 show that, in average, the α-LA treatment increased the MI from 0.17 (in control group) to 0.18, while NAC treatment increased to 0.19, and, most importantly, the combined use of both antioxidants resulted in a significant increase to 0.27, which clearly demonstrates that α-LA plus NAC treatment significantly increased the MI of cultured lymphocytes, $P < 0.05$ (Figure 4).

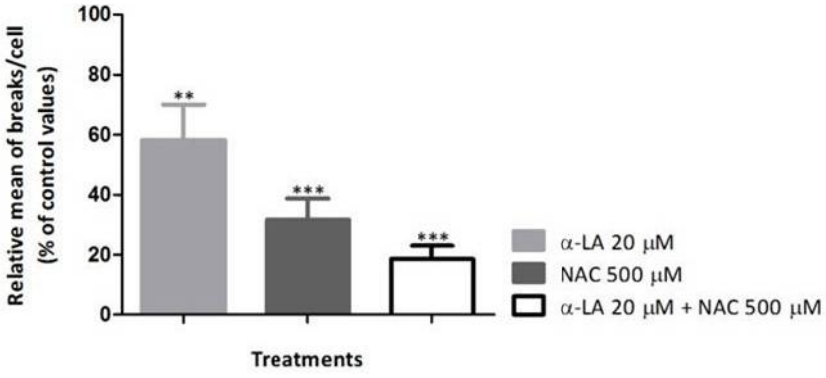

Figure 3 Effect of α-LA, NAC and α-LA + NAC on chromosome breaks in cultured lymphocytes from FA mosaics/chimeras. Lymphocyte cultures were treated with 20 μM α-LA, 500 μM NAC, and both antioxidants simultaneous- ly at the same concentrations. The results are presented as the relative mean of breaks per cell (percentage of control values). Comparison of the relative mean of breaks/cell was made between control group and each antioxidant treatment (*** $P<0.001$ and ** $P<0.01$) and between treatments ($P>0.05$). Results are representative of the mean of 3 experiments depicted from Table 2.

Table 3. Determination of mitotic index in cultured lymphocytes from FA patients.

	Mitotic index			
Patients	Control group	α-LA 20 μM	NAC 500 μM	α-LA 20 μM + NAC 500 μM
FA8	0,013	0,019	0,023	0,025
FA9	0,029	0,028	0,033	0,037
FA10	0,019	0,019	0,009	0,027
FA11	0,009	0,006	0,013	0,014
FA13	0,011	0,019	0,026	0,036
FA14	0,019	0,018	0,012	0,020
Mean±SD	0.017±0.007	0.018±0.007	0.019±0.009	0.026±0.009

In control group no antioxidant treatment was done. Abbreviations are explained in Table 1.
Paired t-test: Control group vs α-LA 20 μM + NAC 500 μM $P<0.05$

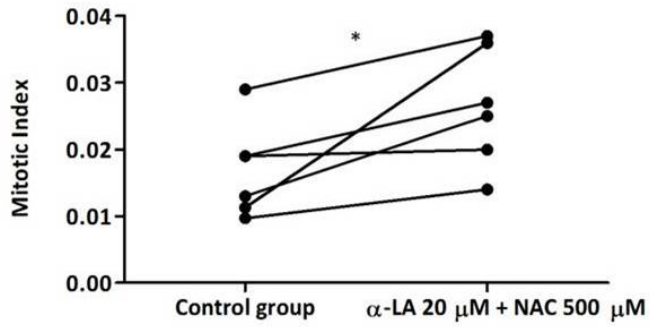

Figure 4. **Effect of α-LA + NAC on mitotic index of cultured lymphocytes from FA patients.** Lymphocyte cultures were treated with 20 μM α-LA plus 500 μM NAC. Mitotic index was evaluated as described in "methods" and the results are presented as absolute frequencies depicted from Table 3 (*$P < 0.05$).

Effect of α-lipoic acid, N-acetylcysteine and α-lipoic acid plus N-acetylcysteine on DEB-induced CI in lymphocyte cultures from FA patients

As shown in Table 4, lymphocyte cultures from FA patients pre-treated with α-LA, NAC, and α-LA plus NAC, and after exposure to DEB, showed a significant reduction in the number of breaks per cell compared to the control group ($P < 0.05$, $P < 0.05$ and $P < 0.05$, respectively). Comparing α-LA *vs* NAC treatments ($P > 0.05$), α-LA *vs* α-LA plus NAC treatments ($P < 0.05$) and NAC *vs* α-LA plus NAC treatments ($P > 0.05$) no differences were found in the reduction in the number of breaks per cell. Globally, the percentage of reduction in lymphocyte cultures pre-treated with α-LA was 28% ($P > 0.05$), with NAC was 54% ($P < 0.001$) and with α-LA plus NAC was 60% ($P < 0.001$), as shown in Figure 5. Once more, two distinct groups can be considered, a group of responders and another one of non-responders. In a total of 12 α-LA pre-treated lymphocyte cultures, 67% were responders, while in a total of 7 NAC pre-treated lymphocyte cultures, 86% were responders. In α-LA plus NAC pre-treated cultures 100% were responders. In a culture from one patient belonging to this last group, the number of breaks per cell decreased to values considered normal (below 0.1).

Discussion

In the present work we evaluated the protective effect of α-LA and NAC and, more importantly, the protective effect of a cocktail with these two compounds, against spontaneous and DEB-induced CI in lymphocyte cultures from FA patients. The obtained results provide an important and novel finding: it was clearly evident, for the first time, that the concomitant exposure to α-LA and NAC can drastically improve the genetic stability in lymphocytes from FA patients, *in vitro.*

Various reports have previously shown mitochondrial dysfunctions in FA cells, including distorted mitochondrial structures and peroxyredoxin 3 deregulation in FA-A, FA-C and FA-G cells[18] and mitochondrial matrix densification in FA-A fibroblasts after 8-methoxypsolaren photoreaction or UVA irradiation [17]. It is known that mitochondrial dysfunctions ultimately lead to a defect in energy transduction, with increased formation of ROS, which, by itself, can lead to lower cellular energy charge, oxidative modification of DNA, protein and lipids. In addition, there is also a possibility of a positive feedback cycle between ROS formation and mitochondrial damage, exacerbating the processes of cellular dysfunction (reviewed in Tarnopolsky) [26]. It was already demonstrated that GSH protects mitochondrial DNA from oxidative damage in human lymphocytes and that GSH depletion increases their susceptibility to mitochondrial DNA damage [36]. It is noteworthy that GSH levels are downregulated in FA patients [37]. Therefore, it may be postulated that such imbalance in the redox state of FA cells can be counteracted by antioxidants, in particular mitochondrial nutrients, like α-LA, and other antioxidants that can replace or replenish GSH levels, like NAC, as it was demonstrated in the present study.

Hypersensitivity of lymphocytes to the clastogenic effect of DEB is a common biomarker of FA. Previous studies have shown

Table 4. Number of DEB-induced breaks per cell in cultured lymphocytes from FA patients.

	Mean number of breaks per cell						
Patients	Control group (n)	α-LA 20 μM (n)	% reduction	NAC 500 μM (n)	% reduction	α-LA 20 μM + NAC 500 μM (n)	% reduction
FA1	4.36 (100)	1.16 (100)	73.39	–	–	–	–
FA2	1.85 (100)	1.99 (100)	0.00	–	–	–	–
FA3	1.80 (100)	1.50 (100)	16.67	–	–	–	–
FA4	0.78 (100)	0.57 (100)	26.92	–	–	–	–
FA6	1.00 (80)	1.16 (56)	0.00	–	–	–	–
FA7	7.20 (25)	5.50 (22)	23.61	–	–	–	–
FA8	1.74 (50)	2.10 (50)	0.00	1.16 (50)	33.33	0.70 (50)	59.77
FA9	0.60 (50)	0.30 (50)	50.00	0.22 (50)	63.33	0.30 (50)	50.00
FA10	1.32 (50)	1.60 (50)	0.00	1.60 (50)	0.00	–	–
FA11	0.40 (100)	0.10 (50)	75.00	0.14 (44)	65.00	0.24 (45)	40.00
FA12	3.76 (50)	–	–	–	–	1.48 (50)	60.64
FA13	5.06 (50)	–	–	0.20 (50)	96.05	2.26 (50)	55.34
FA14	2.26 (50)	1.46 (37)	35.40	1.28 (50)	43.36	1.10 (50)	51.33
FA15	4.31 (100)	0.14 (50)	96.75	0.14 (50)	96.75	0.02 (50)	99.54
Mean±SD	2.603 ± 2.019	1.465 ± 1.444		0.677 ± 0.641		0.871 ± 0.799	

In control group no antioxidant treatment was done and lymphocytes were exposed to DEB 0.05 μg/ml. The effect of the antioxidants was calculated by the percentage of reduction of the number of breaks per cell in the α-LA, NAC and α-LA plus NAC treatments relatively to control group. — Unavailable data. Abbreviations are explained in Table 1. Paired t-test: Control group vs α-LA 20 μM $P<0.05$; Control group vs NAC 500 μM $P<0.05$; Control group vs α-LA 20 μM + NAC 500 μM $P<0.05$.

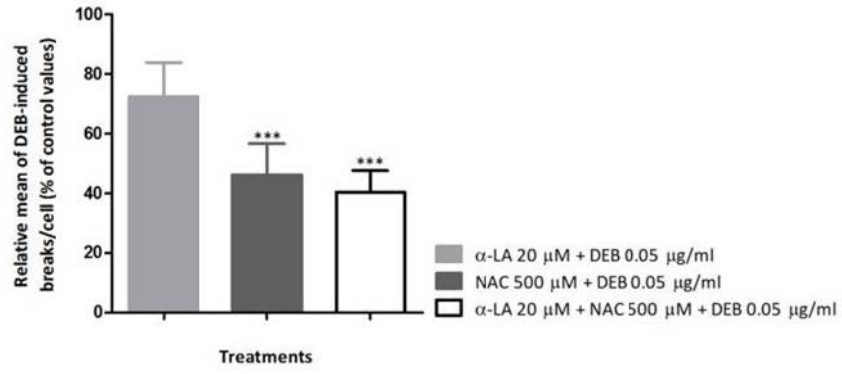

Figure 5. Effect of α-LA, NAC and α-LA+NAC on chromosome breaks in DEB-induced cultured lymphocytes from FA patients. Lymphocyte cultures were pre-treated with 20 μM α-LA, 500 μM NAC, and both antioxidants simul- taneously at the same concentrations, 1.5 h before exposure to DEB 0.05 μg/ml for 48 h. The results are presented as the relative mean of breaks per cell (percentage of control values). Compari- son of the relative mean of breaks/cell was made between control group and each antioxidant treatment (*** $P<0.001$) and between treatments ($P>0.05$). Results are representative of the mean of 14 experiments depicted from Table 4.

that DEB acts as a bifunctional alkylating agent, exhibiting both inter-strand and intra-strand DNA cross-linking ability [38] and that is redox-active, generating ROS that can damage DNA [8]. Furthermore, acute exposure of normal human lymphocytes to DEB is associated with GSH and ATP depletion [10], which indicates that DEB can also cause mitochondrial dysfunction. The exacerbation of OS and DNA cross-linking in FA lymphocytes, through DEB-mediated effects, can reproduce damaging conditions present in severe clinical FA situations, which theoretically extends the applicability of the present results from bench to bedside.

Under the present experimental conditions, the α-LA effect, measured by the reduction in the number of breaks per cell, was similar (around 30%) in the three studied groups: DEB-induced lymphocyte cultures from HD and spontaneous and DEB-induced cultures from FA patients (Figure 6A). This indicates that α-LA effect was independent of DEB exposure or health condition of the studied subject. On the other hand, we verified that NAC effect in these same three groups seems to be dependent of the treatment and health condition (Figure 6B), since the reduction in the number of breaks per cell was greater in DEB-induced lymphocytes from FA patients. These results are in agreement with previous studies, showing that NAC inhibits ROS production and apoptosis induced by DEB in human lymphoblasts [8] and that NAC protects human lymphocytes from acute *in vitro* exposure to DEB [10]. Last but not least, we also verified that the effect of the combined use of α-LA plus NAC is independent of DEB exposure, as shown in

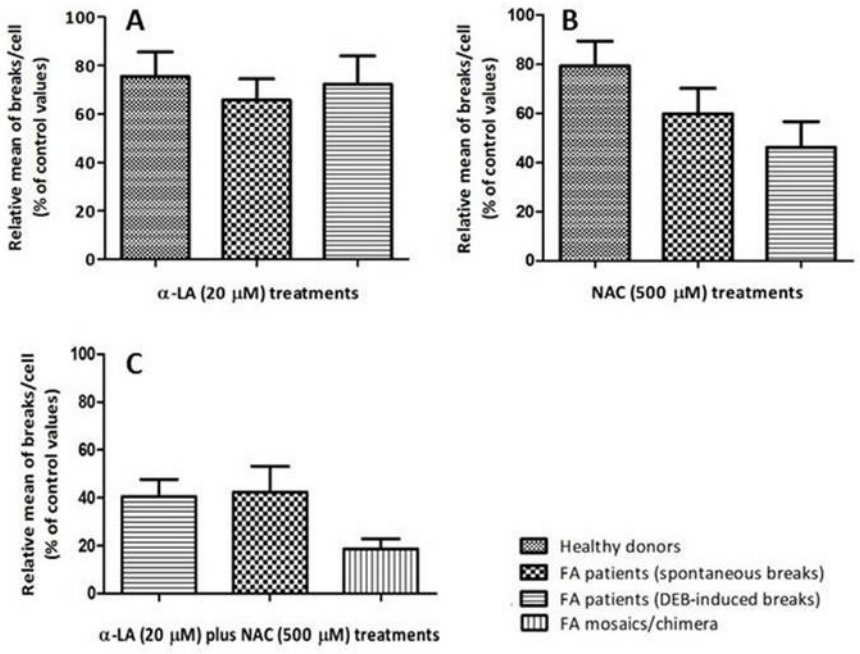

Figure 6. Comparison between the different studied groups according to the antioxidant treatments. Comparative analysis of the effect of α-LA (A), NAC (B) and α-LA plus NAC treatments was performed between lymphocyte cultures from the four studied groups: healthy donors, FA patients (spontaneous breaks), FA patients (DEB-induced breaks) and FA mosaic/chimera patients. All values were depicted from Figures 2, 3 and 5.

Figure 6C, with a 60% of reduction in the number of breaks per cell in both groups of FA patients, and even more effective in cultures from FA patients with higher frequency of normal cells, such as FA mosaics or FA chimera, in which the reduction was of about 80%. Furthermore, the measurement of spontaneous chromosomal breaks was of particular importance, as it rules out the possibility that the main anti-chromosomal break effect of α-LA and NAC is due to a direct interaction of these agents with DEB. Additionally, MI was measured in lymphocyte cultures from FA patients submitted to all antioxidant treatments. The reason for this assay was to evaluate if the decrease in CI was due to an increase in mitotic proliferation, with a consequent shortening of the cell cycle arrest

in G2, as it was demonstrated for α-tocopherol by Pincheira et al [23]. What we observed is that MI increased in all antioxidant treatments, especially with α-LA plus NAC treatment, suggesting a relation between a decrease in CI and a shortening of cell arrest in G2. We cannot exclude, however, that, in lymphocyte cultures from mosaic and chimera patients, the increase in MI can be due to a positive selection of normal cells, with a consequent increase in apoptosis.

The results now obtained suggest that α-LA plus NAC cocktail may be useful to keep chromosome stability in FA patients, which can be very important to block or delay the progression of the disease. This cocktail may be even more effective when applied to FA mosaics and FA chimeras, after bone marrow transplant, as we observed *in vitro*. It must be stressed that bone marrow transplant only resolves bone marrow failure, curing the hematological manifestations. However, CI is not mitigated, and predisposition to cancer, other than leukemia, persists, especially solid tumors [3,39,40]. Thus, controlling CI in FA patients after bone marrow transplant could be very important.

Additionally to increasing chromosomal stability, α-LA has also important cellular roles in the regulation of gene transcription, inhibition of the activation of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and activating protein 1, [41,42] while NAC apparently modulates cytokine concentrations, as interleukin(IL)-1, TNF-α (tumor necrosis factor-alpha) and IFN-γ (interferon-gamma) [30]. These pharmacological effects may further contribute for the therapeutic usefulness of α-LA plus NAC cocktail by avoiding the inflammatory process, mostly frequent in FA children patients [43,44]. Of note, some studies concerning the immunological response of FA patients demonstrated lymphocyte dysfunction and high levels of TNF-α [45]. Low production of IL including IL1, IL2, and IL6, as well as IFN-γ and granulocyte-macrophage colony stimulating factor have also been reported [46].

The present work might open a novel strategy to deal with FA disease. The choice of these antioxidants, apart from their pharmacodynamics, takes into account that both α-LA and NAC have been already approved for safe human use with minimal or no adverse effect, showing an excellent safety profile. NAC has been in clinical use for more than 30 years, in conditions characterized by depletion of GSH or OS, as cancer, AIDS, pulmonary diseases, heart diseases, as well as in acetaminophen overdose [30,47,48]. Thus, in conditions characterized by a chronic excessive OS and in clinical situations where GSH levels are decreased, NAC appears to be a highly effective drug, and may be also recommendable in the prevention/treatment of FA. α-LA has been used, especially in Germany, for over 30 years, in the treatment of diabetic polyneuropathies. Several evidences support a role for α-LA as a mitigator of OS in type 2 diabetes [49], this drug being also used in the treatment of atherosclerosis, cardiovascular diseases, cataract, neurodegenerative diseases, liver diseases and AIDS [50]. Besides the well-known characteristics as antioxidant and as mitochondrial nutrient, α-LA can be administrated orally, since it is easily absorbed in the stomach, it crosses blood–brain barrier, due to its amphipathic properties, and is not toxic at doses used for prophylactic and therapeutic purposes [50]. Furthermore, α-LA and NAC are also inexpensive drugs, which is a great advantage compared with other proposed therapies, namely gene therapy [51], and others that are now available, such as androgen therapy [52], allowing an easy access by all FA patients. Therefore, this cocktail may be immediately tested in phase III clinical trials for treating chromosome instability. Moreover, a FA prevention trial could also be designed to see whether the administration of these drugs can prevent the development of leukemia, in an initial phase, and solid tumors in an advanced form of the disease. The proposed clinical trial, using the combined administration of α-LA and NAC also poses a few challenges. FA is a rare disease, which implies a small population of

patients, most of them children or teenagers. This requires a strong adhesion to the new approach by parents and family. In addition, the efficacy may vary among FA patients, as we observed by the presence of responders and non responders. However, this challenge may be easily overcome by studying the response of each individual in an *in vitro* assay before starting the therapy.

A large number of epidemiological studies have shown health improvements in association with the consumption of dietary antioxidants as part of food. Several studies have shown that the provision of individual antioxidants as supplements are either ineffective [53], or can be deleterious to health [54]. Part of the reason that large doses of exogenous single antioxidants can be deleterious is that every antioxidant can also function as a pro-oxidant and antioxidants function optimally when several are present and can function as redox couples [26]. This fact may explain the success of our cocktail, α-LA plus NAC. They act as an effective redox couple, and in two distinct but closely related processes: α-LA acts as a mitochondrial nutrient and NAC as a GSH precursor and direct ROS scavenger.

Conclusions

The present study provides an important and novel finding that may have immediate clinical applicability: it was clearly evident, for the first time, that the concomitant exposure to α-LA and NAC can drastically improve the genetic stability in lymphocytes from FA patients. Since the available information points to an association of cellular phenotype and clinical features with the occurrence of both cancer-proneness and OS in FA disorder, these results suggest that α-LA plus NAC can be an effective antioxidant prophylactic cocktail to be applied, *in vivo*, in FA patients, with a consequent block or delay in their characteristic bone marrow failure and early cancer development. On the pre-clinical perspective, mechanistic studies are still required to understand why these antioxidants together are so effective in the reduction of CI in lymphocytes from FA patients, providing, this way, further clues to know what is falling in FA cells.

Abbreviations

8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-oxoG, 7,8-dihydro-8-hydroxyguanine; AIDS, Acquired immune deficiency syndrome; CI, Chromosome instability; DEB, Diepoxybutane; DMSO, Dimetilsulfoxide; FA, Fanconi anemia; GSH, Reduced glutathione; HD, Healthy donor; IL, Interleukin; INF-γ, Interferon-gamma; MI, Mitotic index; NAC, N-acetylcysteine; NF-kB, Nuclear factor kappa-light-chain-enhancer of activated B cells; OS, Oxidative stress; PRDX3, Peroxiredoxin 3; ROS, Reactive oxygen species; TNF-α, Tumor necrosis factor alpha; α-LA, α-lipoic acid.

Competing interests

The authors declare no competing financial interest.

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Authors' contributions

FP designed experiments, performed research, analyzed data and drafted the manuscript. RS

performed research and analyzed data. APF, CG and JB provided blood samples and clinical data from FA patients, analyzed the experimental data. BP and FC coordinated the study, designed experiments, analyzed data, critically revised the manuscript and gave final approval of the version to be published. All authors read and approved the final manuscript.

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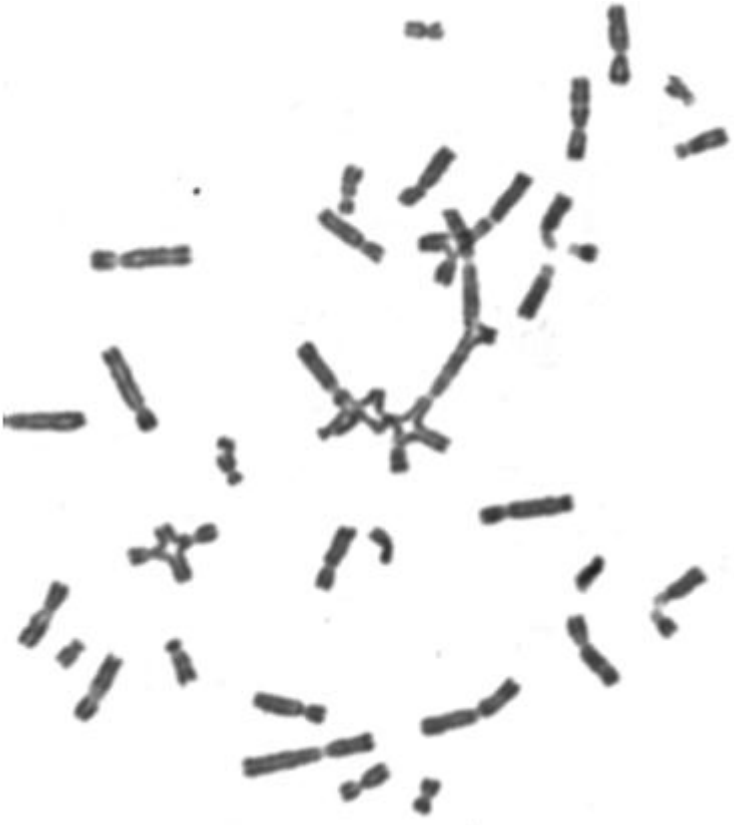


PART IV – GLOBAL DISCUSSION

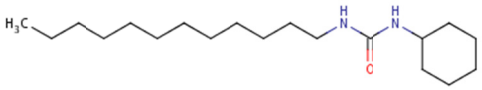
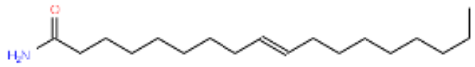
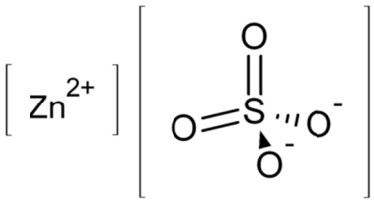
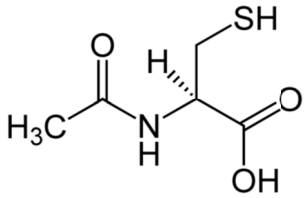
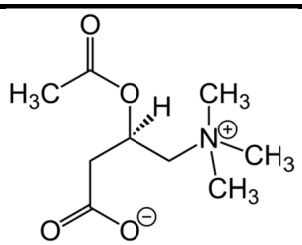
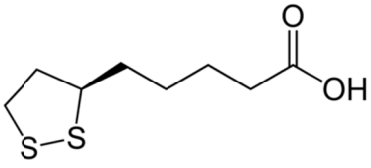
Global Discussion

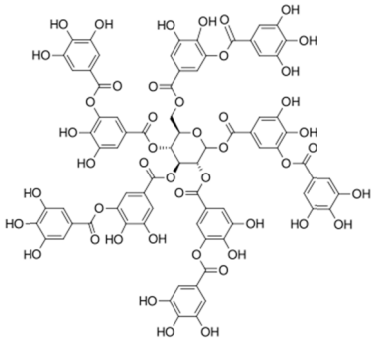
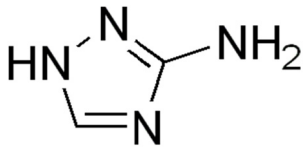
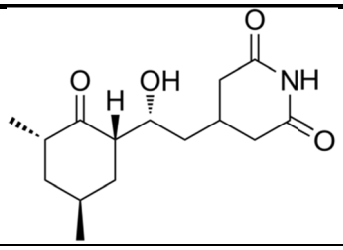
In order to achieve the ultimate goal of the present thesis, that is, to develop protective and/or curative therapeutic measures for the characteristic CI in cells from FA patients, two complementary objectives were considered. In the first objective the global purpose was to contribute for a better understanding of DEB-induced toxicity to normal human lymphocytes, by studying the putative contribution of biochemical pathways postulated to be involved in the reactivity of this compound, and to select the better compounds to be applied in lymphocyte cultures from FA patients. In the second objective the global purpose was to find which of the compounds selected in the first objective could turn out to be useful to prevent spontaneous and DEB-induced CI in lymphocytes from FA patients.

In order to reach the first objective, cell viability as well as GSH and ATP levels were evaluated in an *in vitro* cellular suspension model. The results reported in article I allowed us to establish that the acute exposure of human lymphocytes, freshly isolated from human volunteers, to DEB, results in severe GSH depletion and loss of ATP, followed by cell death. Besides the novelty of these findings, they also provided a better clarification of DEB's mechanism of toxicity to lymphocytes. The pro-oxidant nature of DEB is recognized and characterized in other cell types (Bartók and Láng, 1980; Erexson and Tindall, 2000b; Korkina et al., 2000; Pagano et al., 2001; Spanò et al., 1998). In addition, it is also known that DEB may interact with mitochondria (Yadavilli et al., 2007), which could explain ATP depletion observed in our study. Moreover, it has been referred that the levels of GSH in FA cells may be lower than in normal cells (Dallapiccola et al., 1985) and some studies have shown that there are already mitochondrial dysfunctions in FA cells (Afanas'ev, 2006; Bogliolo et al., 2002a; Mukhopadhyay et al., 2006; Paulin-Levasseur et al., 1998; Rousset et al., 2002), especially at the membrane level, which compromises its normal function.

Following the results obtained with the acute toxicity study, a mechanistic approach was further applied, by studying the putative contribution of biochemical pathways postulated to be involved in the reactivity of this compound. Furthermore, the final goal of the first objective was to search for protective drugs that could turn out to be useful to prevent spontaneous and DEB-induced CI in lymphocytes from FA patients. Therefore, we chose putative inhibitors of DEB bioactivation, antioxidant agents and mitochondrial protective agents. Besides, inhibitors of protective enzymes and an inhibitor of protein synthesis were also tested. The compounds and their principal functions are summarized in Table 5.

Table 5. Compounds and their principal functions and chemical structure used in the mechanistic approach of DEB-induced toxicity. CDU, Cyclohexyl-3-dodecyl urea; Zn^{2+}, Zinc; NAC, N-acetylcysteine; ALC, Acetyl-L-carnitine; 3-AT, 3-amino-1,2,4-triazole; CHX, Cycloheximide.

CATEGORIES	COMPOUNDS NAME	PRINCIPAL FUNCTIONS	CHEMICAL STRUCTURE
Putative inhibitors of DEB bioactivation	CDU	Inhibitor of sEH	
	Elaidamide	Inhibitor of mEH	
	Zn^{2+}	Inhibitor of both EH; Antioxidant	
Antioxidant agent	NAC	Precursor for GSH synthesis; ROS scavenger	
Mitochondrial protective agents	ALC	Mitochondrial metabolite	
	α-LA	Cofactor of many mitochondrial enzymes; scavenger of ROS	

Inhibitors of protective enzymes	Tannic acid	Inhibitor of GST	
	3-AT	Inhibitor of catalase	
Inhibitor of protein synthesis	CHX	Interfere with the translocation step in protein synthesis, blocking translational elongation	

Among all the compounds tested, only four showed positive results. We considered a positive result when DEB-induced toxicity, measured by its effects on GSH, ATP levels and cell viability, was attenuated by putative inhibitors of DEB bioactivation, antioxidant agents and mitochondrial protective agents, and when DEB-induced toxicity was increased by inhibitors of protective enzymes and an inhibitor of protein synthesis. Interestingly, the compounds that showed positive results can be integrated in the same categories: Zn^{2+} and NAC as antioxidant agents and α-LA and ALC as mitochondrial protective agents. The rationale behind the effectiveness of these compounds in modulating DEB-induced toxicity will be focused in the remaining discussion.

Zn^{2+} is a known inhibitor of both EHs, the soluble and the microsomal (Draper and Hammock, 1999). As mentioned before, Boogaard and Bond (Boogaard and Bond, 1996) suggested that DEB metabolism and detoxification in human liver and lungs are, in its majority, effectuated by EH enzymes, mainly mEH, and that metabolites formed in this process are less toxic than metabolites formed by DEB conjugation with GSH. It was therefore expected that inhibition of EHs would increase DEB-induced toxicity to lymphocytes. However, we showed that inhibition of sEH and mEH by CDU and elaidamide, respectively, did not affect DEB toxicity. On the other hand, tentative inhibition with Zn^{2+} elicited a partial protective effect concerning cell viability, though it did not protect lymphocytes from depletion of GSH and ATP. From these results, we assumed

that this protective effect with Zn^{2+} is related to a mechanism other than inhibition of EHs. We suggested that the mechanism behind this result could be due to the antioxidant activity of this metal, already demonstrated by several authors (Bray and Bettger, 1990; Prasad, 2008; Prasad et al., 2004; Sankaramanivel et al., 2010; Stehbens, 2003; Yamada and Koizumi, 1991). In fact, all these authors recognized that Zn^{2+} has a protective role by counteracting OS by four principal ways: it can decrease ROS generation, it is a scavenger of •OH, it can induce the synthesis of metallothioneins (that contain a high amount of cysteine) and finally, it acts as a co-factor of SOD. Additionally, the tentative inhibition of DEB detoxification by EHs inhibitors also showed that hydrolysis of DEB by EHs in lymphocytes, in opposition to what occurs in lungs and liver, is not the most important route of DEB detoxification and that the GSH system can be more relevant than it was firstly supposed.

The use of SH-donor antioxidants, like NAC, in an attempt to reduce DEB acute toxicity in human lymphocytes can be justified by two principal ways. First, NAC is a known precursor for GSH synthesis, increasing its intracellular levels and, therefore, significantly improving the cellular antioxidant potential of the cells (Atkuri et al., 2007). Secondly, NAC can act directly as free radical scavenger (Kelly, 1998). GSH depletion by DEB compromises endogenous antioxidant system of the cells, making lymphocytes more susceptible to pro-oxidant reactive species as well as to electrophilic agents, like DEB and its metabolites. In fact, DEB-induced cytotoxicity, despite hitherto not completely understood, has been related to its ability to react with protein SH groups (Loecken and Guengerich, 2007), to mediate the formation of DNA-DNA and DNA-protein cross-links (Michaelson-Richie et al., 2010) as well as to its capability to induce the production of ROS and induce OS (Erexson and Tindall, 2000a; Yadavilli et al., 2007). In accordance, in the present study we verified that NAC can protect human lymphocytes from acute toxicity of DEB at all measured parameters, corroborating a previous study showing that NAC inhibits ROS production and apoptosis induced by DEB in human lymphoblasts (Yadavilli et al., 2007). Furthermore, the protective effect of NAC also suggests how relevant is DEB conjugation with GSH in the process of detoxification of this compound, at least in lymphocytes.

Mitochondria seems to be substantially affected in the event of exposure to DEB. This postulate can be assumed from the results obtained with the characterization of the acute toxicity of DEB in lymphocytes, showing a depletion on ATP levels, and from those obtained by others, being especially relevant the work of Yadavilli and co-workers (Yadavilli et al., 2007), that using human TK6 lymphoblast as experimental model, reported that DEB-induced apoptosis is related with dissipation of mitochondrial membrane potential and depletion of mitochondrial cytochrome c levels. Considering this

rationale, the use of mitochondrial protective agents to counteract DEB-induced toxicity seems to be a logic and pertinent way. ALC, an ester of L-carnitine, serves as a source of acetylcholine and L-glutamate, and also contributes to energy-producing reactions (Calabrese et al., 2006). In article I we demonstrated, for the first time, that ALC elicits a partial protective effect on DEB induced toxicity, specifically avoiding cell death, while not presenting ATP depletion. However, it had been recently reported that ALC may lead to SOD stabilization (Haorah et al., 2011) and it can significantly prevent the decrease of SOD activity (Zhang et al., 2012). This means that ALC can also indirectly control ROS levels and this may reduce mitochondrial injury, although it cannot increase ATP production. Therefore, these facts can explain why ALC only prevents cell death, similarly of the effect of NAC in the study of Yadavilli, and once more related with OS. α-LA, the other mitochondrial protective agent in study, functions as an essential cofactor in metabolic reactions involving energy utilization in mitochondria, being an essential cofactor for many mitochondrially localized enzyme complexes (Prahalathan et al., 2006). Besides, it has the potential to induce a substantial increase in intracellular GSH and is a scavenger of •OH, singlet oxygen ($^1O_2$) and hypochlorous acid (HClO) (Han et al., 1997). Our results showed that α-LA did not protect lymphocytes from DEB-induced acute toxicity, in spite of its wide range of action. However, the combined use of α-LA and ALC resulted in a potentiation of ALC protective effect, which agrees with the assumption that α-LA shows beneficial effects in OS conditions, own to its synergistic action with other antioxidants (Hagen et al., 2002; Mantovani et al., 2003; Prahalathan et al., 2006). These results suggest that α-LA and ALC may eventually increase the total antioxidant status of the lymphocytes, instead of increasing directly GSH and ATP levels, interacting with some antioxidant systems, both directly and by regulation of gene transcription: ALC modulates SOD activity, as previously explained, and α-LA, either in its oxidized or reduced form, upregulates the transcription of the GSTP gene, (Lii et al., 2010) and also increases the GPx, glutathione reductase (GR) and SOD activities (Odabasoglu et al., 2011), all enzymes involved directly or indirectly in defense against OS.

The other three compounds tested were supposed to aggravate the deleterious effect of DEB-induced toxicity: tannic acid by inhibition of GST, 3-AT by inhibition of catalase and CHX by inhibition of protein synthesis. In fact, no aggravation was observed in ATP and GSH levels and cell death. Firstly, these results show that the lack of tannic acid modulatory effect on DEB toxicity may reflect redundancy on biotransformation of DEB by GST isoforms in human lymphocytes. Besides, these cells contain moderate levels of GSTμ and GSTπ (van Lieshout and Peters, 1998) and tannic acid has variable inhibitory potencies for the different GST isoforms. Secondly, the results show that H_2O_2 production is not a major contributor for the observed DEB deleterious effects. Finally, cell

signal transduction had no effect on DEB toxicity, indicating absence of regulatory synthesis of protective proteins following the acute effects of DEB.

Thereafter, the first objective of the present thesis was achieved: to increase the knowledge of DEB-induced toxicity as a potential way to find out protective drugs that could turn out to be useful to prevent spontaneous and DEB-induced CI in cultured lymphocytes from FA patients. The selected drugs were Zn^{2+}, NAC, ALC and α-LA.

In order to reach the second objective the putative protective effect of the previously selected drugs, against spontaneous and DEB-induced CI in lymphocytes cultures from FA patients, was evaluated. Considering the previous results we started to evaluate the effect of ALC and α-LA, alone and in combination, in DEB-induced lymphocyte cultures from HD. While α-LA (20 μM) was mostly effective in reducing the DEB-induced CI, the response to ALC (5 mM) was not so linear, and the conjugation of the two mitochondrial nutrients wasn't better than α-LA alone. Some studies *in vivo* and *in vitro* demonstrated amelioration in OS and mitochondrial activity after treatment with ALC and α-LA. An *in vitro* study from Lal and co-workers (Lal et al., 2008) in primary human fibroblast demonstrated that a combination of ALC and α-LA is highly effective in reversing OS arising from iron overload. An *in vivo* study of Hagen and co-worker (Hagen et al., 1998) in old rats with the aim to improve mitochondrial-supported bioenergetics decline and OS increase during aging, demonstrated that feeding old rats with ALC in combination with α-LA increased metabolism and lowered OS more than either compound alone. In human lymphocytes exposed to DEB the effect of both compounds didn't corroborate these studies, at least they didn't show a relevant capability to reduce CI.

In the present work it was firstly observed that α-LA alone, at the concentration of 20 μM, showed anti-clastogenic activity in DEB-induced lymphocyte cultures from HD. These results are in accordance with a study of Prahalathan and co-workers (Prahalathan et al., 2006) who demonstrated that α-LA can be a protective compound against adriamycin, a clastogenic agent, in the erythropoietic system of rats. Another study from Dadhania and co-workers (Dadhania et al., 2010) reported that α-LA pretreatment ameliorates methotrexate-induced intestinal toxicity in rats. Afterwards the study was enlarged, in the control population, testing one more concentration of α-LA, 60 μM, to analyze if its effect was concentration dependent. It was verified that α-LA effect in DEB-induced lymphocyte cultures was not concentration dependent (data not shown), once the results obtained for both concentrations were similar. Therefore, the concentration of 20 μM was chosen to study the effect of α-LA in cells from FA patients. The effect in spontaneous and DEB-induced lymphocyte cultures from FA patients, as stated in article II, was effective in the reduction of CI in both types of cultures, being mostly pronounced

in spontaneous cultures. However, under the present experimental conditions, the α-LA effect, measured by the reduction in the number of breaks per cell, was similar (around 30 %) in the three studied groups: DEB-induced lymphocyte cultures from HD and spontaneous and DEB-induced cultures from FA patients.

α-LA, an endogenous and natural compound, is a mitochondrial nutrient, with a great range of action. Liu (Liu, 2008) described a mitochondrial nutrient as an agent that can directly or indirectly protect mitochondria from oxidative damage and improve mitochondrial function. α-LA and its reduced form, dihydrolipoic acid (DHLA), form a redox couple, DHLA being the predominant form (Gorąca et al., 2011). Both are small molecules that contain two oxidized or reduced SH groups. Direct protection includes its capability to act as a potent antioxidant in redox-cycling environment, reducing vitamin C, vitamin E and coenzyme Q_{10} (Tarnopolsky, 2008), as a ROS scavenger (Han et al., 1997), as a possible chelator of redox active metals (Gorąca et al., 2011) and as an essential cofactor for many mitochondrial localized enzymes (Prahalathan et al., 2006). Indirect protection includes regulation of enzymes that act in biotransformation of xenobiotics or have an antioxidant activity, as the case of GST, GPx, SOD and GR (Lii et al., 2010; Odabasoglu et al., 2011). Furthermore, the α-LA/DHLA couple has the ability to increase or maintain GSH levels, by acting as a transcriptional inducer of genes governing GSH synthesis and, potentially, by increasing substrate availability, mainly cysteine (Han et al., 1997; Shay et al., 2008). Consequently, α-LA has the potential to maintain the antioxidant status of the cells, as demonstrated by Zembron-Lacny and co-workers (Zembron-Lacny et al., 2009), protecting mtDNA from oxidation, since its activity occurs specially in mitochondria. In turn, it is commonly known that FA cells, as mentioned in the introduction of this thesis, show overproduction of ROS, resulting in decrease in the cellular antioxidant defense systems, increase in cellular free iron levels and a deficiency in erythrocyte SOD (Korkina et al., 1992). Catalase activity is also decreased (Takeuchi and Morimoto, 1993) and an association of FANCC gene with enzymes involved in the process of biotransformation is also recognized (Kruyt et al., 1998), being related with the transcription of GSTP1 gene (Cumming et al., 2001). It is also noteworthy that GSH levels are downregulated in FA patients (Pagano et al., 2004). Moreover, many reports had provided evidence for mitochondrial dysfunction in FA cells, either in its metabolism as well as in its structure (Afanas'ev, 2006; Bogliolo et al., 2002a; Mukhopadhyay et al., 2006; Paulin-Levasseur et al., 1998; Rousset et al., 2002). Additionally, α-LA/DHLA may reduce the proinflammatory conditions by its interaction with NF-kB, inhibiting its activity via TNF-α (Shay et al., 2008). In fact, FA cells show enhanced intracellular NF-kB activation and transcriptional activity compared to normal cells (Macé et al., 2007), presenting an impairment in immunological system. NF-kB, besides being a potent indicator of stress factors such as ROS, is also a

transcription factor that induces the expression of many genes involved in inflammation pathway, endothelial cell migration and apoptosis.

The next step was to evaluate the effect of NAC in DEB-induced lymphocyte cultures from HD, at the same concentration tested in lymphocyte suspensions. The results stated in article II showed that NAC has the capability to decrease the CI in HD, although the effect demonstrated by NAC was not only lower but also more variable comparing with α-LA. However, concerning the effect of NAC in spontaneous and DEB-induced lymphocyte cultures from FA patients, the results clearly demonstrated that NAC has a better capability in decreasing the CI, being the effect most pronounced in DEB-induced lymphocyte cultures. In opposite to the effect of α-LA, NAC effect seems to be dependent of the treatment and health condition of the donor, since the reduction in the number of breaks per cell was greater in DEB-induced lymphocytes from FA patients.

NAC, the acetylated variant of the amino acid L-cysteine, has been shown to increase GSH levels, the body's major antioxidant, as mentioned before. Of glutathione's three component amino acids (glutamate, glycine, and cysteine), cysteine has the lowest intracellular concentration. Because *de novo* synthesis is the primary mechanism by which GSH is replenished, cysteine availability can limit the rate of GSH synthesis in the event of OS (Millea, 2009). Moreover, NAC has the ability to reduce extracellular cystine to cysteine (Kelly, 1998). For this activity, also contributes the capability of NAC to be a source of SH metabolites. As a source of SH groups, NAC can also enhance GST activity, promote detoxification, and act directly on ROS. Thus, it is not surprising that NAC acts as powerful scavenger of HClO and is capable of reducing •OH and H_2O_2 (Kelly, 1998). Once more, NAC abilities partially correct FA cells deficiencies, mainly overproduction of ROS, low levels of GSH and an obviously imbalance in OS defense. Additionally, NAC apparently modulates proinflammatory cytokine concentrations, as IL-1, TNF-α and IFN-γ (interferon gamma) (Kelly, 1998), which can be very useful to prevent infections in FA patients, similarly to the effect of α-LA. A recent report also demonstrated that NAC can modulate the hematological response in healthy and active males (Zembron-Lacny et al., 2009), by measuring some hematological parameters, in particular erythropoietin (EPO), which is a cytokine that controls erythropoiesis. It has been shown that EPO production and erythroid differentiation are regulated by ROS, especially H_2O_2, which are involved in redox-sensitive signaling pathways through downregulation of transcription factors (Fandrey et al., 1994; Huang et al., 1996; Nagata et al., 2007). This means that ROS generation can suppress EPO synthesis, whereas antioxidants can stimulate its synthesis. This particularity can be very useful to regulate the erythropoiesis in FA cells, avoiding the characteristic BMF.

Finally the last goal was to find out a cocktail with the compounds that demonstrated better results in the decrease of CI. Therefore, the combined effect of α-LA and NAC in spontaneous and DEB-induced cultured lymphocytes from FA patients, was evaluated. The results were surprising, especially in spontaneous breaks, in which the reduction was about 60% globally, and in patients with mosaicism or chimerism of about 80%, as reported in article II. The measurement of spontaneous chromosomal breaks was of particular importance, as it goes beyond the possibility of the main anti-chromosomal break effect of α-LA and NAC being due to a direct interaction of these agents with DEB. Moreover, the mitotic index increased with all antioxidant treatments, especially with α-LA plus NAC treatment, suggesting a relation between a decrease in CI and a shortening of cell arrest in G2. It was also tried to optimize the cocktail adding Zn^{2+}, however CI was not improved relatively of the effect of α-LA and NAC (data not shown).

In conclusion, the main objective, to find an effective antioxidant prophylactic cocktail to be applied, *in vivo*, in FA patients, was achieved. The results obtained suggest that α-LA plus NAC cocktail may be useful to keep chromosome stability in FA patients, which can be very important to block or delay the progression of the disease. This cocktail may be even more effective when applied to FA mosaics and FA chimeras, after BMT, as observed *in vitro*. It must be stressed that BMT only resolves BMF, curing the hematological manifestations. However, CI is not mitigated, and predisposition to cancer, other than leukemia, persists, especially solid tumors (Alter and Kupfer, 2002 (update 2011); Auerbach, 2009; Spanier et al., In press). Thus, controlling CI in FA patients after BMT could be very important. Thereby, with the present thesis it is expected to open a novel strategy to deal with FA disease.

As previously described, α-LA and NAC have excellent properties, and their use has already shown great advantages. Both have been already approved for safe human use with minimal or no adverse effect, showing an excellent safety profile, and they are also inexpensive drugs. Because of that, NAC and α-LA have been in clinical use for more than 30 years. The use of NAC is recommendable in conditions characterized by depletion of GSH or OS, as cancer, AIDS, pulmonary diseases, influenza, heart diseases, as well as in acetaminophen overdose (Atkuri et al., 2007; Kelly, 1998; Millea, 2009), demonstrating to be a highly effective drug. α-LA has been used in the treatment of diabetic polyneuropathies. Several evidences support a role for α-LA as a mitigator of OS in type 2 diabetes (Golbidi and Laher, 2010), this drug being also used in the treatment of atherosclerosis, cardiovascular diseases, cataract, neurodegenerative diseases, liver diseases, AIDS and cancer (Bilska and Wlodek, 2005; Gorąca et al., 2011). Besides,

considering its mitochondrial activity, α-LA is used for aging prevention. Mitochondria provide energy for basic metabolic processes, and their decay with age impairs cellular metabolism and leads to cellular decline, with an increase production of damaging free radicals. α-LA reverses the age-associated decline in mitochondrial enzymes and, therefore, may lower the increased risk of oxidative damage that occurs during the aging process (Arivazhagan et al., 2003). Both drugs show excellent pharmacokinetics. NAC is rapidly absorbed following an oral dose. Although its deacetylation in the intestinal mucosa and lumen probably limits the absorption of intact molecules of NAC, this apparent low bioavailability is misleading, since in the degradation process a variety of physiologically-beneficial, SH-containing metabolites are formed (Kelly, 1998). Humans can synthesize α-LA *de novo* from fatty acids and cysteine, but only in very small amounts. Therefore, α-LA needs to be absorbed from exogenous sources, food or nutritional complements, being easily absorbed in the stomach and crossing blood-brain barrier, due to its amphipathic properties, which allows prevention of lipid peroxidation (Bilska and Wlodek, 2005; Gorąca et al., 2011).

Although the question posed in the present thesis is not “new”, in that a long line of studies, dating back to Nordenson in 1977, have been trying to correct CI with a variety of antioxidants (see Introduction), in this work it was presented for the first time a cocktail focused on the effects of two well-selected antioxidants in compensating CI in primary lymphocyte cultures from FA patients.

The success of this cocktail is that α-LA and NAC act as an effective redox couple and in two distinct but closely related processes: α-LA acts principally as a mitochondrial nutrient and NAC as a pro-glutathione dietary supplement, being both direct ROS scavengers. It is also very important the fact that both compounds may modulate proinflammatory cytokine activities and concentrations, which opens a possibility that they could prevent infections in FA patients, particularly FA children, which are even more susceptible than normal children. Additionally, NAC also modulates EPO, a cytokine responsible for hematopoiesis response, which could be of great importance for FA patients, with an impaired hematopoietic system, that leads to BMF. Therefore it is suggested that this cocktail can be tested in phase III clinical trials for decreasing CI in FA patients, a preventive approach that has not been proposed before (Figure 10).

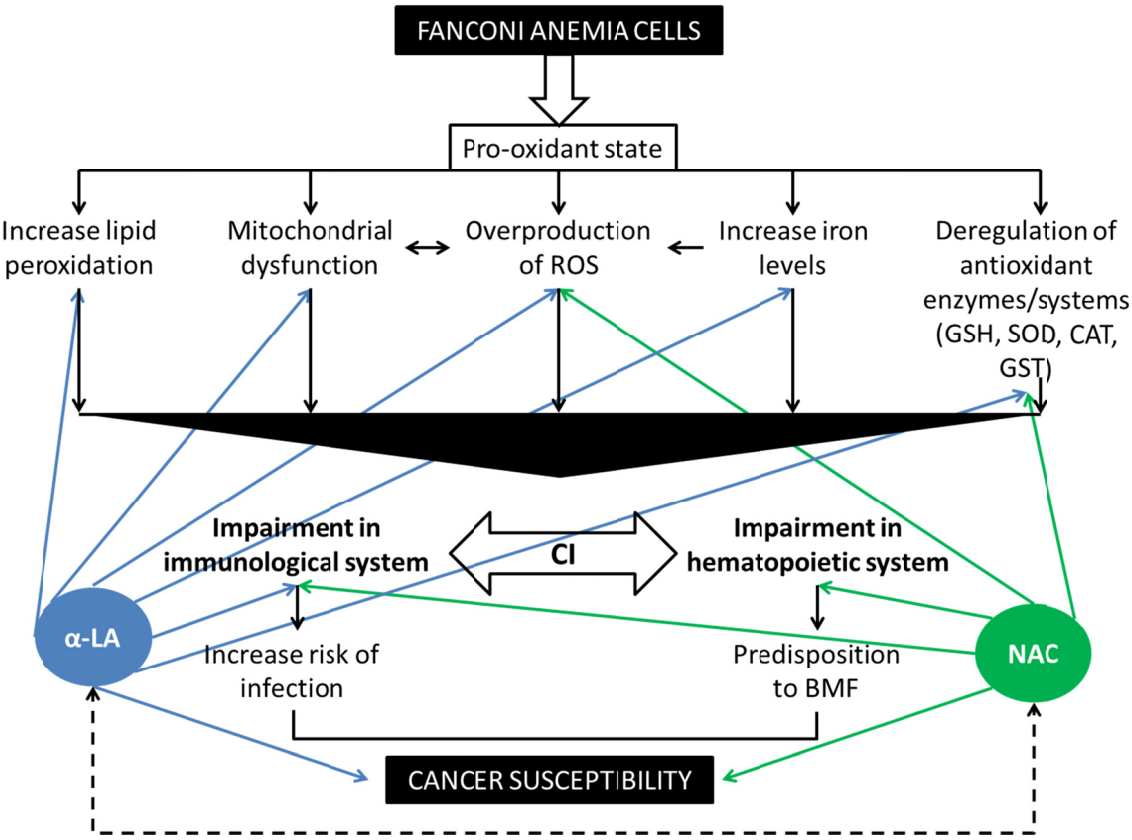


Figure 10. Proposed scheme for α-LA and NAC action in Fanconi Anemia cells (original figure). Fanconi anemia cells are in permanent pro-oxidant state. These cells show increased levels of lipid peroxidation, demonstrate mitochondrial dysfunction, show increased iron levels, being the last closely related with overproduction of ROS. Besides, FA cells demonstrate deregulation in antioxidant enzymes and systems. These characteristics result in chromosome instability, followed by impairment in immunological system and hematopoietic system, increasing the risk of infection and predisposition to BMF, respectively. This condition culminates in higher cancer susceptibility. α-LA and NAC can potentially prevent all this FA features. Moreover, α-LA and NAC can regenerate itself together. CI, chromosome instability; ROS, reactive oxygen species; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GST, glutathione S-transferase; α-LA, α-lipoic acid; NAC, N-acetylcysteine; BMF, bone marrow failure.

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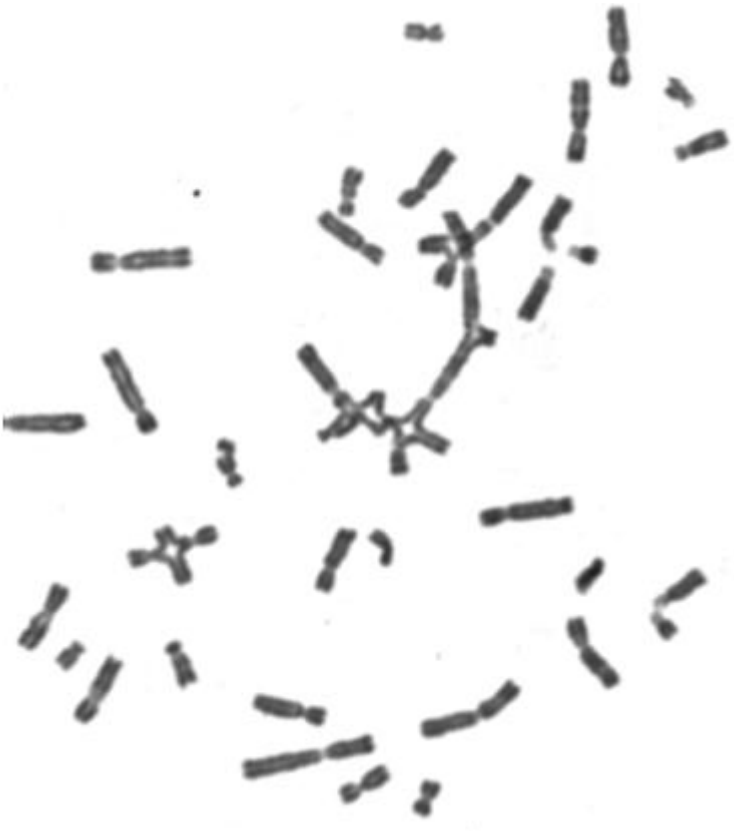


PART V – FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

Final Conclusions and Future Perspectives

The present thesis provides an important and novel finding that may have immediate clinical applicability: it was clearly evident, for the first time, that the concomitant exposure to α-LA and NAC can drastically improve the genetic stability in lymphocytes from FA patients. Since the available information points to an association of cellular phenotype and clinical features with the occurrence of both cancer-proneness and OS in FA disorder, the results presented here suggest that α-LA plus NAC can be an effective antioxidant prophylactic cocktail to be applied, *in vivo*, in FA patients, with a consequent block or delay in their characteristic BMF and early cancer development.

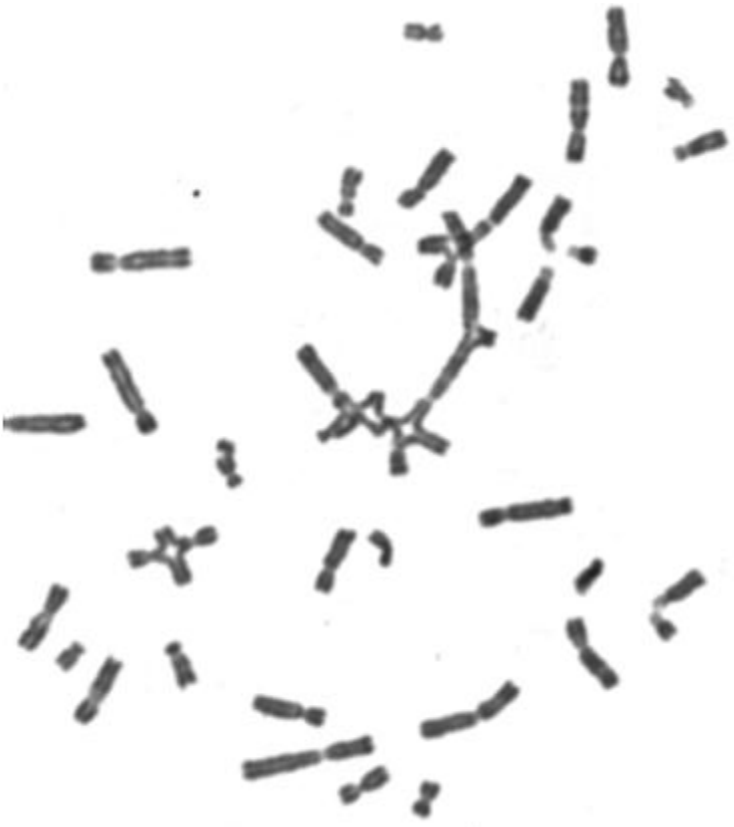
Because α-LA and NAC are already approved for human use and they are also inexpensive drugs, allowing an easy access by all FA patients, the preventive application of the cocktail proposed in this study can be of a great advantage, before the use of other proposed therapies, namely gene therapy (Tolar et al., 2012), and others that are now available, such as androgen therapy (Scheckenbach et al., 2012). Being this study of great clinical potential, it is expected that it will hopefully inspire clinicians to perform the required clinical trials. Moreover, with the application of the proposed α-LA and NAC cocktail, future prospective studies can be designed in order to see if this preventive approach can decrease the incidence of leukemia, in early stages of the disease, and the incidence of solid tumors in an advanced stage of the disease.

The proposed clinical trial, using the combined administration of α-LA and NAC, poses a few challenges. FA is a rare disease, which implies a small population of patients, most of them children or teenagers. This requires a strong adhesion to the new approach by parents and families. In addition, the efficacy may vary among FA patients, as it was observed by the presence of responders and non-responders in the present studies (see article II). However, this challenge may be easily overcome by studying the response of each individual in an *in vitro* assay before starting the therapy.

On the pre-clinical perspective, mechanistic studies are still required to evaluate if the improvement of CI due to α-LA and NAC is accompanied by an improvement of the hematopoietic status of the patients. To achieve this goal various parameters should be measured in primary lymphocytes from FA patients before and after the exposure to α-LA and NAC cocktail. An initial approach would be the characterization of the general antioxidant status of those cells, measuring haemoglobin oxidation, RBC protein oxidation (carbonyl groups) and lipid peroxidation. Characterization of non-enzymatic antioxidant defence (GST/GSSG ratio and endogenous levels of α-LA) and enzymatic antioxidant

defense (catalase, SOD, GSTP and GSH-Px GR) should also be performed. Another approach would be the study of cell signal transduction, which can be helpful to see if the exposure to the α-LA and NAC cocktail can lead to synthesis of regulatory and protective proteins. In a recent study, persistence of HbF was related with increased expression of the FOXO3a pathway involved in OS response. Knowing that FA patients are characterized by increased levels of HbF, the evaluation of FOXO3a pathway expression can answer the question if it is overexpressed in FA cells and if the cocktail modulates its regulation.

With the present thesis we expect to open a novel strategy to deal with FA disease, focused essentially on prophylactic therapeutics. It is our hope to increase the life expectancy of FA patients and improve their life quality, avoiding the early onset of BMF, the recurrent infections and the development of early cancer. We tightly think that our aim can be achieved and our mission fulfilled. With simple thoughts, simple methods and simple ways, shown with the present thesis, we believe to have contributed to an important advance in Fanconi anemia research.



PART VI - REFERENCES

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