HUMAN SPERM MITOCHONDRIAL FUNCTION:

IMPLICATIONS FOR MALE (IN)FERTILITY

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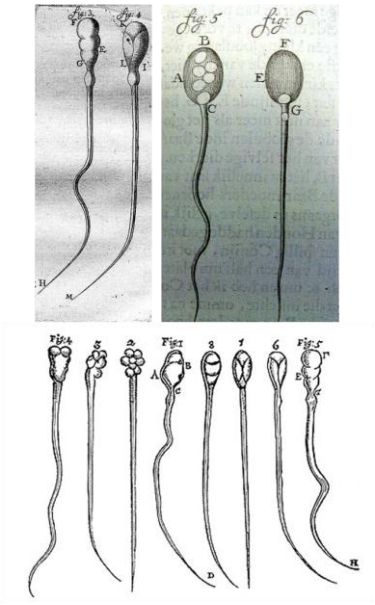
"Science must set limits to knowledge, but should not set limits to imagination."

Bertrand Russel (1872-1970)

"Whenever a theory appears to you as the only possible one, take this as a sign that you have neither understood the theory, nor the problem which it was intended to solve."

Karl Popper (1902-1994)



First drawings of mammalian sperm (Antonius Leeuwenhoek, 1677).

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RESUMO

As mitocôndrias são organelos dinâmicos que geram ATP por fosforilação oxidativa. Este processo depende da existência de um gradiente electroquímico ao longo da membrana mitocondrial interna, gerado pelo bom funcionamento da cadeia respiratória mitocondrial, e cujos complexos proteicos são codificados por dois genomas: o genoma nuclear e o genoma mitocondrial. Os espermatozóides humanos contêm um número variável de mitocôndrias na peça intermédia, que são responsáveis pelo fornecimento de energia para as mais diversas funções celulares, incluindo a mobilidade dos gâmetas masculinos. Com o objectivo de clarificar o papel da mitocôndria e do seu genoma (ADNmt) no funcionamento dos espermatozóides e na (in)fertilidade masculina, procedeu-se à análise de uma série de aspectos celulares e moleculares relacionados com a mitocôndria. Para isso, usaram-se amostras de esperma de qualidade distinta, fornecidas por pacientes que recorreram a um laboratório de reprodução assistida.

Mostrou-se, através da técnica de PCR em tempo real, que os espermatozóides humanos possuem um número variável de moléculas de ADNmt, e que o conteúdo em ADNmt é baixo, quando comparado com células somáticas. Além disso, este conteúdo parece ser inversamente proporcional à qualidade espermática. Numa tentativa de entender a razão pela qual amostras de diferente qualidade apresentam conteúdos dissimilares de ADNmt, procedeu-se ao estudo de duas proteínas relacionadas com a sua manutenção: a ADN polimerase gama (POLG, a única ADN polimerase existente na mitocôndria) e o factor de transcrição mitocondrial A (TFAM; que está envolvido nos processos de transcrição e replicação do ADNmt). Antes de mais, usando as técnicas de imunocitoquímica e "western-blotting", mostrou-se que espermatozóides humanos expressam estas duas proteínas na peça intermédia. Para além disso, a expressão destes dois factores parece estar correlacionada com

os principais parâmetros de avaliação da qualidade seminal (concentração, mobilidade e morfologia dos espermatozóides). Resultados semelhantes foram obtidos para a expressão de duas proteínas da cadeia respiratória: subunidades I e VIc da citocromo c oxidase (COXI e COXVIc, respectivamente). Paradoxalmente, a presença de POLG parece predizer, de forma inversa, o conteúdo em ADNmt. Este factor poderá, então, ter um papel na regulação do número de cópias de ADNmt em espermatozóides, embora os mecanismos desta possível regulação não tenham sido determinados.

A relevância da POLG na função espermática foi também estudada a nível genético, tendo este estudo incidido particularmente numa região do gene que contém repetições de nucleótidos CAG de tamanho variável. Para isso, usaram-se as técnicas de PCR de fluorescência e sequenciação de ADN. Os resultados mostraram que, embora as amostras de pior qualidade apresentem uma maior incidência de heterozigotia, este polimorfismo não parece afectar os principais parâmetros seminíferos, nem o conteúdo em ADNmt, ou a expressão de POLG. Assim, e apesar do significado biológico destes variantes genéticos não ter sido clarificado, a sua análise não parece ter relevância clínica.

Por outro lado, a análise de subpopulações de espermatozóides com capacidade de movimentação distinta (isoladas usando o método clássico de "swim-up") confirmou a correlação entre a expressão das proteínas POLG e COXI e a mobilidade espermática. Além disso, o estudo de fracções de espermatozóides com diferente potencial de membrana mitocondrial (usando a sonda "MitoTracker Green" e um aparelho de "sorting" celular) sugeriu que a expressão destas proteínas contribui para a manutenção da função mitocondrial.

A capacidade da sonda "MitoTracker Green" (bem como das sondas "MitoTracker Red" e "JC-1") monitorizar o potencial de membrana mitocondrial em espermatozóides foi determinada usando microscopia de fluorescência, através da incubação de espermatozóides com disruptores do potencial, tanto antes como após a incubação com as sondas. Qualquer

uma das três sondas parece monitorizar o potencial de membrana de espermatozóides no momento da incubação. No entanto, nenhuma delas é suficientemente dinâmica para revelar alterações no potencial que ocorram após a incubação dos espermatozóides com as sondas.

Por último, procedeu-se a um estudo preliminar usando diferentes marcadores celulares, com o intuito de determinar o possível valor clínico da análise dos seguintes parâmetros funcionais: potencial de membrana mitocondrial, integridade membranar, estado do acrossoma e estado da cromatina. Neste estudo foram usadas, não apenas amostras de pacientes que recorreram a um laboratório de reprodução assistida, mas também amostras de dadores saudáveis. Os resultados sugerem que todos os parâmetros avaliados são significantes, em especial a análise do potencial de membrana mitocondrial e da integridade membranar.

ABSTRACT

Mitochondria are dynamic organelles that generate ATP through oxidative phosphorylation, which takes place within the electron transfer chain (ETC) and depends on the existence of an electrochemical potential across the inner mitochondrial membrane. The ETC is the only cellular apparatus that is encoded by both the nuclear and the mitochondrial (mtDNA) genomes. Human sperm contain a variable number of mitochondria in the midpiece, where these organelles might supply sperm with energy for several proposes, including sperm motility. In order to better understand the role of mitochondria and its genome in human sperm function and male (in)fertility, a number of mitochondrial-related features was analysed in the present study, using distinct quality sperm samples from men attending an infertility clinic.

Using real-time PCR, it was shown that human sperm possess a variable number of mtDNA molecules, which is invariable low when compared to somatic cells, and seems to be inversely proportional to sperm quality. In an attempt to understand why distinct quality sperm samples present dissimilar mtDNA content, two proteins related with its maintenance were studied: DNA polymerase gamma (POLG; the sole DNA polymerase in mitochondria) and mitochondrial transcription factor A (TFAM; which is involved in both the transcription and replication of mtDNA). Using both immunocytochemistry and western blotting, it was shown that human sperm express these proteins in the midpiece. Moreover, the expression of these factors seems to be correlated with sperm concentration, motility and morphology (the three main sperm quality parameters). Similar outcomes were obtained for two proteins of the ETC: cytochrome c oxidase subunits I and VIc (COXI and COXVIc, respectively). Paradoxically, the presence of POLG seems to negatively predict mtDNA copy number.

POLG may, therefore, play a role in regulating sperm mtDNA copy number, although the exact mechanism of this possible regulation remains undetermined.

The relevance of POLG in sperm function was additional studied at the gene level, particularly on a variable-length CAG-repeat region, using both fluorescent PCR and DNA sequencing. Although poorer quality sperm samples presented a higher incidence of heterozygosity for the *POLG* CAG-repeat genotype, these polymorphisms did not seem to affect the three sperm principal parameters, nor mtDNA copy number or POLG expression. Thus, and while the biological meaning of these variants remains unclear, their clinical significant seems irrelevant.

The analysis of subpopulations of sperm presenting distinct ability to swim (isolated using the classical swim-up procedure) confirmed the correlation between the expression of both POLG and COXI and sperm motility. Moreover, the study of sperm fractions with distinct mitochondrial membrane potential (MMP; using MitoTracker Green and a cell-sorter) suggested that the expression of these proteins contributes for the maintenance of mitochondrial function.

The ability of MitoTracker Green (as well as MitoTracker Red and JC-1) to monitor sperm MMP was determined, using fluorescence microscopy, by incubating sperm with distinct MMP disrupters, either before or after the incubation with the probes. The three mitochondrial probes provided similar results, and seemed to monitor sperm MMP at the time of incubation. However, none of them was dynamic enough to reveal MMP alterations occurring after the incubation with the probes.

Finally, a preliminary approach was conducted using distinct cellular markers, to determine the clinical value of the assessment of different sperm functional parameters: MMP, membrane integrity, acrosomal status and chromatin status. Importantly, these parameters were analysed not only in sperm samples from patients, but also from healthy

donors. All the traits analysed seem to be significant, with emphasis on both MMP and membrane integrity.

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ABBREVIATIONS USED

A – Adenine

aa – Amino acid

AD - Alzheimer's disease

ADP - Adenosine diphosphate

Ala – Alanine

ANT - Adenine nucleotide translocator

AOT - Acridine orange test

AR- Androgen receptor

Arg- Arginine

ART - Assisted reproductive techniques

ATP -Adenosine triphosphate

bp – Base pairs

BER - Base excision repair

BSA – Bovine serum albumin

C - Cytosine

CAC – Citric acid cycle

CEQ – Capillary electrophoretic genetic analysis system

CFTR - Cystic fibrosis transmembrane conductance regulator gene

CLAP – Chymostatin, leupeptin, antipain and pepstatin mixture

CM-H_2XRos – Chloromethyl-dihydro-X-rosamine

CMT - Charcot-Marie-Tooth disease

CMX-Ros – Chloromethyl-X-rosamine

COMET – Single cell gel electrophoresis assay

COX – Cytochrome c oxidase

CSB – Conserved sequence box

CV – Coefficient of variance

Cyt – Cytochrome

DAPI – 4’,6-diamidino-2-phenylindole

dGK - Deoxyguanosine kinase

$DiOC_6(3)$ - 3,3’-dihexyloxacarbocyanine iodide

D-loop – Displacement loop

DMSO- Dimethylsulfoxide

DNA – Deoxirribonucleic acid

mtDNA – Mitochondrial DNA

nDNA – Nuclear DNA

dNTP – Deoxyribonucleotide

DTT – Dithiothreitol

ECF – Enhanced chemifluorescence

ETC – Electron transfer chain

FCCP – p-trifluoromethoxy carbonyl cyanide phenylhydrazone

FITC – Fluorescein isothiocyanate

G – Guanine

HMG – High mobility group

HOS – Hypo-osmotic swelling

H-strand – mtDNA heavy strand

HSP – H-strand promoter

ICC - Immunocytochemistry

ICSI – Intracytoplasmic sperm injection

Ig – Immunoglobulin

IRB – Internal review board

IVF – *In vitro* fertilisation

JC-1 - 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide

Kb – Kilo base pairs

KCN – Potassium cyanide

KDa - Kilodaltons

KSS - Kearns-Sayre Syndrome

LDH – Lactate dehydrogenase

LHON- Leber Hereditary Optic Neuropathy

L-strand – mtDNA light strand

LSP – L-strand promoter

MELAS – Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes

MERRF – Myoclonic epilepsy and ragged red fibres

MMP- Mitochondrial membrane potential

MTERF - Mitochondrial transcription termination factor

MT-G – MitoTracker Green FM

MT-R – MitoTracker Red CM-H_2XRos

mtSSB – Mitochondrial single-strand binding protein

NADH - Nicotinamide dinucleotide

NARP – Neuropathy, ataxia and retinis pigmentosa

ND – NADH dehydrogenase

NRF – Nuclear respiratory factor

OA - Oligoasthenozoospermic

OAT – Oligoasthenoteratozoospermic

O_H – Origin of replication of the H-strand

O_L - Origin of replication of the L-strand

OXPHOS – Oxidative phosphorylation

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PD - Parkinson's disease

PEO - Progressive external ophthalmoplegia

adPEO – Autosomal dominant PEO

arPEO – Autosomal recessive PEO

PHGP - Phospholipid hydroperoxidase glutathione peroxidase

Pi – Inorganic phosphate

PI- Propidium iodide

PMSF – Phenylmethanesulphonyl fluoride

POLG – DNA polymerase gamma

POLG – Gene encoding the catalytic subunit of POLG

POLG2 – Gene encoding the accessory subunit of POLG

POLRMT - Mitochondrial RNA polymerase

PS- Pearson's syndrome

PSA – *Pisum sativum* agglutinin

PVDF – Polyvinylidene diflouride

Rh123 - Rodamine 123

RNA – Ribonucleic acid

mRNA – Messenger RNA

rRNA- Ribossomal RNA

siRNA – Small interfering RNA

tRNA- Transfer RNA

RNase MRP – RNA-processing endoribonuclease

ROS – Reactive oxygen species

SCSA - Sperm chromatin structure assay

SDS - Sodium dodecyl sulphate

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SNHL - Sensorineural hearing loss

T – Timidine

TAS – Termination-associate sequence

TBST – Tris buffer saline with Tween 20

TEMED – N, N, N',N'-Tetramethylethylenediamine

TES - Trishydroxymethylmethyl-2-aminosulfonate

TEST – TES and Tris

TFAM – Mitochondrial transcription factor A

TFB1M - Mitochondrial transcription factor 1

TFB2M - Mitochondrial transcription factor 2

TK - Thymidine kinase translocator

TMRE – Tetramethylrhodamine ethyl ester

TRIS – Trishydroxymethylaminomethane

TUNEL - Terminal deoxynucleotidyl transferase-mediated transferase dUTP nick end labelling

Tyr - Tyrosine

U – Uracil

Ubi - Ubiquinone

WHO - World Health Organization

CHAPTER I

INTRODUCTION

Some of the contents of this Chapter have been originally published in:

St John, J.C., Bowles, E.J. and Amaral, A. (2007) Sperm mitochondria and fertilisation. *Soc Reprod Fertil Suppl*. **65**: 399-416.

1. The mammalian sperm and male (in)fertility

The mammalian sperm is the result of a series of cells divisions and maturational modifications that coordinately occur in the seminiferous tubules, within the testis, and which are subjected to hormonal control from the hypothalamic-pituitary axis. During spermatogenesis, diploid spermatogonial stem cells differentiate and undergo meiosis, producing haploid spermatids. These, in turn, and after complex morphological modifications, finally give rise to sperm (reviewed in de Kretser and Kerr, 1994). In this post-meiotic final process (termed spermiogenesis), the majority of the cellular components are remodelled: 1) the nucleus becomes tightly compacted, as nuclear histones are partially and sequentially replaced by transition proteins and protamines, enhancing chromatin condensation (reviewed in Dadoune, 2003); 2) the majority of the cytoplasm and typical somatic cell-type organelles are lost in the so called residual bodies, which are phagocyted by Sertoli cells; 3) the Golgi apparatus suffers conformational changes, contributing to the formation of the acrosome, a large secretory vesicle on the sperm head (Sinowatz and Wrobel, 1981; Moreno *et al.*, 2000); 4) a flagellum is formed; 5) some mitochondria are lost in the residual bodies, while the remaining rearrange in elongated tubular structures and are packed helically around the anterior portion of the flagellum (Otani *et al.*, 1988); 6) the mitochondrial DNA copy number is reduced (Hecht *et al.*, 1984). In the end, a functional sperm is a simple, differentiated and hydrodynamic-shaped cell perfectly adapted to its purpose: to fertilise an oocyte.

This remarkable cell is composed of three main regions: the head, the midpiece and the tail (Fig. 1). The head comprises the nucleus (with a haploid set of chromosomes) and the acrosome (which covers the anterior part of the nucleus and contains hydrolytic enzymes that aid sperm penetration through oocyte vestments). The tail (or flagellum), essential for sperm motility, is composed by a central axoneme (consisting of a central pair of single microtubules surrounded by nine outer doublets) encircled by outer dense fibers and a fibrous sheath. In the

midpiece, apart from the axoneme, there are also a variable number of mitochondria wrapped around the outer dense fibers.

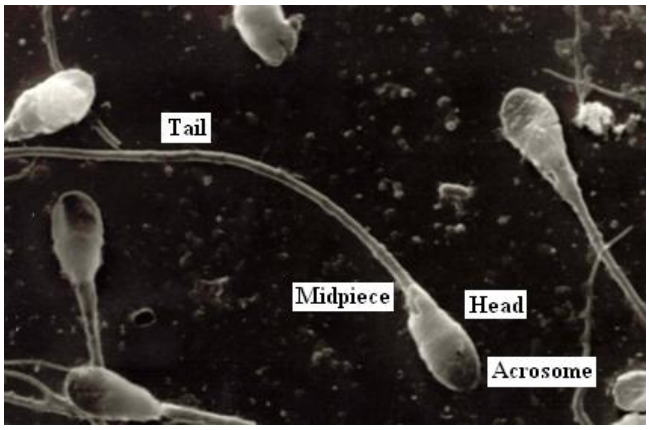


Figure 1. Electron microscopy picture of human sperm (*image captured by Dr Aurora Gabriel and provided by Dr Pedro Caballero-Campo, from Clinica Tambre Andrology Laboratory, Madrid, Spain*). Human sperm is composed of three main regions: the head (which comprises the nucleus and the acrosome), the midpiece (where mitochondria are localized) and the tail (or flagellum).

All the events occurring in spermatogenesis and spermiogenesis are supported by an accurate gene expression regulation, which includes the testis-specific expression of several genes, that either encode isoforms of somatic cell proteins such as, for example, lactate dehydrogenase subunit C – LDH-C_4 (Machado de Domenech *et al.*, 1972; Millan *et al.*, 1987) and cytochrome C_T (Goldberg *et al.*, 1977), or proteins with no homologues in somatic cells, such as protamines 1 and 2 (Wykes *et al.*, 1995) and acrosin (Kashiwabara *et al.*, 1990). On the other hand, the chromatin condensation caused by the replacement of histones by protamines ultimately results in cessation of transcription in mid-spermiogenesis (Kierszenbaum and Tres, 1978). To counteract this, the beginning of spermiogenesis is characterized by an elevated transcription activity, which is controlled by testis-specific transcription factors and coactivators (for reviews see Sassone-Corsi, 2002; Tanaka and Baba, 2005). Also, there are a number of posttranscriptional events, including the processing and storage of mRNAs, that permit protein synthesis in transcriptionally silent spermatids

(reviewed in Braun, 1998; Eddy, 1998; Hecht, 1998; Grootegoed *et al.*, 2000; Steger, 2001). Accordingly, in the last stages of spemiogenesis, gene expression is effectively controlled via translation.

At the end of spermiogenesis, sperm are released from the seminiferous epithelium, an event called spermiation (Beardsley and O'Donnell, 2003), and undergo posttesticular maturation in the epididymis, where important physiological attributes are acquired, including the ability to swim and to fertilise an oocyte (reviewed in Yanagimachi, 1994; Cooper, 2007). The epididymis is also where sperm are stored until ejaculation. At ejaculation, sperm leave the epididymis and are mixed with the secretions of the accessory glands (seminal vesicles, prostate and bulbourethral glands), constituting the semen. In order to eventually fertilise an oocyte, ejaculated sperm must even endure a poorly defined process of final maturation in the female reproductive tract. This fertilising competence acquisition, which can also be consummated *in vitro* with appropriate media, is known as capacitation, and involves the activation of intracellular signalling cascades and the phosphorylation of certain protein residues, particularly tyrosines (reviewed in Visconti and Kopf, 1998). Capacitation is also associated with striking changes in sperm motility, designated as sperm hyperactivation (reviewed in Suarez, 1996; Suarez and Pacey, 2006). Only capacitated sperm are able to undergo the acrosome reaction (reviewed in Yanagimachi, 1994; Breitbart *et al.*, 1997; Breitbart and Spungin, 1997; Nolan and Hammerstedt, 1997), an exocytotic episode by which the acrosomal membrane fuses with the sperm plasma membrane, resulting in the release of acrosomal contents. This event assists sperm-oocyte interaction, by aiding the penetration of sperm through the oocytes's protective layers.

The sperm cell was long-established as a highly specialised cell merely committed to deliver the paternal genome to the oocyte, which contributes to the developing embryo, not only the maternal genome, but also the cytoplasm, organelles, and all the necessary proteins

and RNAs (at least until the embryo be able to produce its own). However, mounting evidence is changing this traditional view and sperm is no longer considered a simple "DNA-carrier-cell". Definitely, it is now known that sperm is responsible for introducing the sperm-specific phospholipase C-zeta, an essential activating factor, without which the oocyte does not complete meiosis (Saunders *et al.*, 2002; Swann *et al.*, 2004; Knott *et al.*, 2005; Saunders *et al.*, 2007). Furthermore, in most mammals (excluding rodents) sperm also contributes with the centrosome (Schatten, 1994; Simerly *et al.*, 1995; Palermo *et al.*, 1997) and it is the sperm aster that brings together the male and female pronulei (Navara *et al.*, 1994; Sutovsky *et al.*, 1996a).

In addition, recent findings suggest a possible role for sperm RNA in early embryo development. Indeed, and although sperm are considered transcriptionally and translationally silent, the presence of a complex population of RNAs in ejaculated sperm is well documented, which is composed not only by mRNAs (Pessot *et al.*, 1989; Kumar *et al.*, 1993; Miller *et al.*, 1994; Wykes *et al.*, 1997; Miller *et al.*, 1999), but also by microRNAs (Ostermeier *et al.*, 2005b). While attempts to fully characterize the sperm transcriptome (Dadoune *et al.*, 2005; Zhao *et al.*, 2006) and to evaluate its possible prognostic value in male factor infertility (Ostermeier *et al.*, 2002; Lambard *et al.*, 2004; Ostermeier *et al.*, 2005a; Carreau *et al.*, 2007; Platts *et al.*, 2007) have been carried out, the actual role of sperm RNA is a topic under debate (for recent reviews see Miller and Ostermeier, 2006; Boerke *et al.*, 2007). Contradicting the established idea that the RNAs present in ejaculated sperm are merely the remains of spermiogenesis, with no functional meaning, it has been shown that, at least *in vitro*, human paternal mRNAs are delivered to the egg at fertilisation (Ostermeier *et al.*, 2004). Moreover, significant amounts of a sperm RNA was shown to be responsible for a non-mendelian inheritance of an epigenetic change in mice (Rassoulzadegan *et al.*, 2006), suggesting that paternal RNA can influence embryo development. Apart from this, sperm RNAs may

possibly be required by the sperm itself, to replace degraded proteins (Miller and Ostermeier, 2006), which would imply that mature sperm could be able to synthesise proteins.

Nevertheless, it is generally accepted that gene expression in mature sperm is restricted to the mitochondria. In fact, mammalian sperm seem to be able to synthesise both mitochondrial-encoded RNAs (MacLaughlin and Terner, 1973; Hecht and Williams, 1978; Alcivar *et al.*, 1989) and proteins (Ahmed *et al.*, 1984; Twaina-Bechor and Bartoov, 1994), although sperm mitochondrial translation failed to be proven at least in human (Diez-Sanchez *et al.*, 2003b). More recently, however, incorporation of amino acids into polypeptides during sperm capacitation has been shown in different mammals, including humans, suggesting, for the first time, that mammalian sperm are able to synthesise nuclear-encoded proteins (Gur and Breitbart, 2006; 2008). These outcomes are particularly odd, not only because they seem to contradict the dogma that sperm are translationally silent cells (at least for nuclear-encoded proteins), but also because the results also suggest that translation of nuclear-encoded proteins occurs in mitochondrial-type ribosomes, an event with no equivalent in any other cell-type. Consequently, these data need confirmation and this issue is certainly controversial.

To sum up, all the distinctive traits stated so far make sperm a very unusual cell, distinct, at different levels, from both somatic cells and oocytes. Although the uniqueness of sperm is well established, and despite the increasing knowledge on sperm biology, the fully characterisation of the features that distinguishes a good-quality sperm (*i.e.*, with high fertilisation potential), are just beginning to be elucidated. This complete characterisation is imperative for applications such as the development of an effective male contraceptive, or a better diagnosis of male infertility (as well as the advance of new strategies to try to overcome this problem).

Human infertility, generally defined as the failure to conceive after one year of regular intercourse in the absence of any kind of contraception, is a growing problem in developing

and industrialised countries (Skakkebaek *et al.*, 2006), affecting approximately 15 % of couples. Concomitant with this, the use of assisted reproductive techniques (ART) experienced a widespread increase in the last years (Andersen and Erb, 2006). Of these, *in vitro* fertilisation (IVF), in which sperm is placed in contact with unfertilised oocytes *in vitro*, in order to facilitate fertilisation (Lopata *et al.*, 1978); and intracytoplasmic sperm injection (ICSI), a micromanipulation procedure where a single sperm is inject into an oocyte (Palermo *et al.*, 1992), are the most used techniques (Andersen *et al.*, 2008).

Mounting data from both clinical and epidemiological studies suggest that male reproductive health is diminishing, with a remarkable decline in semen quality (Andersson *et al.*, 2008). The diagnosis of male infertility is usually based on the traditional analysis of semen quality, according to the World Health Organization guidelines (WHO, 1999) which defined the procedures for semen handling and processing and the parameters to evaluate (that together constitute a relatively standardised laboratory analysis called spermiogram) and also threshold values for these parameters (below which the sample is considered abnormal; Table I). This classical analysis focuses mainly in three principal parameters: sperm concentration, sperm motility and sperm morphology, rooted in which different aetiologies are defined. To this extent, a sample is considered normal (normozoospermic) if it contains at least 20 millions of sperm per ml of semen, at least 50 % sperm with progressive motility, and at least 15 % morphologically normal sperm. Low values of concentration, motility and morphology are designated oligozoospermia, asthenozoospermia and teratozoospermia, respectively. As theses aetiologies often occur in combination, combined names are also used; for example, a semen sample abnormal for the three parameters is called oligoasthenoteratozoospermic (OAT).

Table I – Reference values for the evaluation of human sperm quality (World Health Organization, 1999). Samples below these values are considered abnormal. The three principal parameters are sperm concentration, motility and morphology.

Parameter	Reference value (WHO, 1999)
Ejaculate volume	≥ 2 ml
Ejaculate pH	≥ 7.2
Total sperm number	≥ 40 million sperm/ejaculate
Sperm concentration	≥ 20 million sperm/ml ejaculate
Sperm motility	≥ 50% motile sperm
Sperm morphology	≥ 15% normal forms*
Sperm vitality	≥ 50% live sperm

* According to the strict criteria (Kruger *et al.*, 1986).

The possible causes behind low sperm quality are numerous and, indeed, male infertility is a complex disease, with a multitude of origins, that can be congenital or acquired, and genetic or non-genetic (Iammarrone *et al.*, 2003). Genetic causes are usually studied at the chromosomal level, to expose possible abnormal karyotypes and/or meiotic disorders. In fact, the incidence of chromosomal anomalies in men attending infertility clinics is more frequent than in the general population (reviewed in Egozcue *et al.*, 2000). Moreover, there are other well documented genetic anomalies that are correlated with male infertility, such as microdeletions in specific regions of the Y chromosome, and *CFTR* mutations (reviewed in Huynh *et al.*, 2002), all of which manly related with low sperm counts.

Noteworthy, the evaluation of semen quality, while relevant, is not sufficient to a complete diagnosis of male infertility, nor does it have an absolute prognostication value (*i.e.*: certain individuals presenting high semen quality cannot conceive, and others whose semen is considered abnormal can). Therefore, in recent years, a number of tests have been developed to monitor different aspects of sperm function, including motion parameters, capacitation,

acrosome reaction and sperm-oocyte interaction, among others (reviewed in Aitken, 2006; Lewis, 2007). The introduction of these already existing "functional" tests and the development of news ones will certainly contribute to a better understanding of male infertility.

2. The mitochondrion

2.1. The generation of cellular ATP

Mitochondria are dynamic organelles present in almost all eukaryotic cells. It is generally accepted that these organelles have originated from primitive bacteria that formed endosymbiotic interactions with ancestors of eukaryotic cells, and ultimately became essential to the normal function of the host cells (Margulis, 1975; reviewed in Gray *et al.*, 1999). The number, size, shape and distribution of mitochondria present in the cytoplasm differ not only from cell to cell, but also in a same cell, and seem to be a function of the cell's energy demands (Moyes *et al.*, 1998; Okamoto and Shaw, 2005). This dynamics is achieved by a series of fission and fusion events (reviewed in Polyakov *et al.*, 2003; Rube and van der Bliek, 2004), whose regulation is essential for maintaining the functionality of a mitochondrial population (reviewed in Detmer and Chan, 2007). While mitochondria are implicated in different cellular functions, such as homeostasis and cell death regulation (reviewed in McBride *et al.*, 2006), its main purpose is believed to be the production of energy in the form of adenosine triphosphate (ATP), through oxidative phosphorylation (OXPHOS).

The structure of a mitochondrion is intimately related with its function. It contains two membranes, an outer and an inner membrane, that together regulate the transport of molecules and create two different regions in the organelle: the matrix and the intermembrane space (Fig. 2). Due to the presence of porin channels, the outer membrane is permeable to molecules

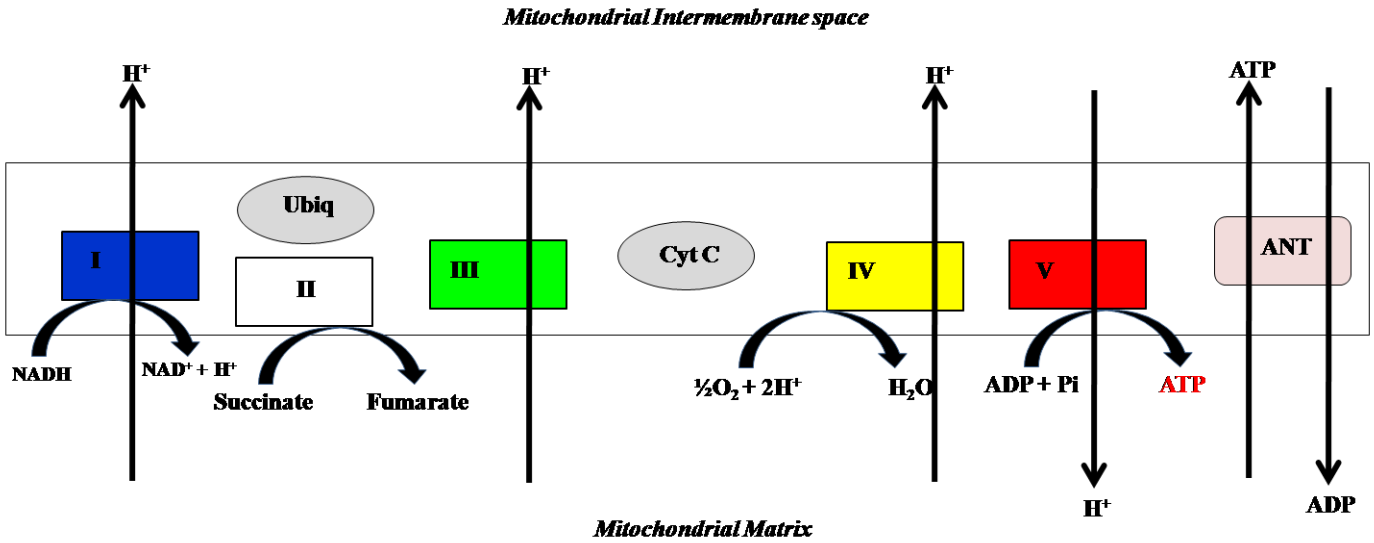
and ions. On the contrary, the inner membrane, which is utterly infolded, forming cristae, is mostly impermeable, even to small molecules. As a result, the transport of molecules to the matrix depends on the activity of specific transporters within the inner membrane. The enzymes of the electron transfer chain (ETC), involved in the OXPHOS, are also localized in this membrane. Moreover, and except for glycolysis that occurs in the cytosol, all the other metabolic pathways of fuel oxidation, such as the Citric Acid Cycle (CAC), and the oxidation of both fatty acids and amino acids, occur in the mitochondrial matrix (Nelson and Cox, 2000).

Figure 2. Schematic representation of the structure of a mitochondrion. This organelle is composed of an outer and an inner membrane. The inner membrane encloses the matrix and is extensive infolded, forming cristae. The region between the two membranes is called intermembrane space. *Adapted from http://www.sciencehelpdesk.com/unit/bg3/1*.

Mitochondrial OXPHOS is the major contributor to cellular ATP, being far more productive than the anaerobic ATP-generator processes (Pfeiffer *et al.*, 2001). It depends on the activity of the five complexes of the ETC (Fig. 3, complexes I-V), four of which are involved in a series of oxidation and reduction steps that culminate in the reduction of molecular oxygen (O_2) to produce water (H_2O). Complex I – NADH-dehydrogenase (ND) - oxidizes nicotinamide dinucleotide (NADH; produced during different catabolic pathways,

such as glycolysis, the CAC and fatty acids oxidation), and complex II – Succinate dehydrogenase – and enzyme also operating in the CAC, accepts electrons from succinate. Electrons are then transferred from both Complexes I and II to Ubiquinone, which is subsequently oxidised by Complex III – Cytochrome c dehydrogenase. After this, there is a transfer of electrons to Cytochrome c and from this to Complex IV – Cytochrome c oxidase (COX) - and ultimately to O_2, with the formation of H_2O. The electron flow through Complexes I, III and IV is accompanied by the pumping of protons from the matrix to the intermembrane space, creating a transmembrane electrochemical gradient. The energy stored in this gradient is then used by Complex V – ATP synthase - to catalyse the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). The transport of ADP in and ATP out of the mitochondrial matrix is accomplished by adenine nucleotide translocator (ANT).

Importantly, the ETC is also the main source of reactive oxygen species (ROS) generation, which, in normal conditions, is balanced by various cellular antioxidant defences (reviewed in Turrens, 2003). At moderate concentrations, ROS play important roles in signalling pathways (reviewed in Thannickal and Fanburg, 2000). However, imbalanced ROS production results in cellular oxidative stress, which may cause damage to cellular macromolecules, such as lipids, DNA and proteins, and ultimately result in cell death (reviewed in Droge, 2002).



Complex	I	II	III	IV	V
nDNA subunits	>18	4	8	10	10
mtDNA subunits	7	0	1	3	2

Figure 3. The electron transfer chain. Electrons flow along the chain, resulting in the production of H_2O. Protons (H^+) are pumped into the mitochondrial intermembrane space, creating an electrochemical gradient across the inner mitochondrial membrane, which provides the energy for ATP synthesis. Most of the subunits for the complexes are encoded by nDNA. However, all of the complexes except for Complex II also have subunits encoded by mtDNA. Abbreviations: I, II, III, IV, V – Complexes I, II, III, IV and V, respectively. Ubiq – Ubiquinone. Cyt C – Cytochrome c. ANT – Adenine nucleotide translocator. nDNA- nuclear DNA. mtDNA – Mitochondrial DNA. *Adapted from St. John et al. (2007).*

2.2. The function of sperm mitochondria

Mature mammalian sperm possess between 22 and 75 crescent-shaped mitochondria arranged end to end in the midpiece, where these organelles are packed helically around the anterior portion of the flagellum (Otani *et al.*, 1988). The anchorage of the mitochondrial sheath to the axoneme is supported by the sub-mitochondrial reticulum, a complex of filaments that seems to sustain the mitochondrial organization in the midpiece (Olson and Winfrey, 1990). Furthermore, the outer membranes of sperm mitochondria are enclosed in a keratinous structure, the mitochondrial capsule, formed by disulfide bonds between cysteine- and proline-rich selenoproteins, including the sperm-specific phospholipid hydroperoxidase glutathione peroxidase (PHGPx; Ursini *et al.*, 1999). Apparently, this structure confers mechanical stability, and is responsible for some distinctive features of sperm mitochondria, namely the resistance to hypo-osmotic stress, and the unfeasibility of completely isolating these organelles.

As mentioned before, the organization of mitochondria in a sperm-like manner takes place during spermiogenesis, concomitantly with the loss of the majority of the cytoplasm. The fact that some mitochondria are retained in a very specialized region of sperm, while most of the cytoplasm is lost, suggests that these organelles might have a crucial role on sperm function. As mitochondria generate ATP through OXPHOS, these organelles might supply sperm with energy for several proposes, including flagellar propulsion and thus sperm motility (Summers and Gibbons, 1971). In accordance with this, electron microscopy observations have revealed that sperm from asthenozoospermic samples presented disordered mitochondrial appearance along with significantly shorter midpieces and fewer mitochondrial gyres than normozoospermic counterparts, while midpiece widths and tail lengths were similar (Mundy *et al.*, 1995). However, the absolute reliance of sperm motility in OXPHOS-derived ATP has been questioned, especially in those species whose sperm possess a very

long tail, such as mice, as it is doubtful that sufficient ATP can diffuse from the mitochondria to the distal end of the flagellum. In accordance, mammalian sperm possess various glycolitic enzymes in the principal piece (Krisfalusi *et al.*, 2006; Kim *et al.*, 2007). Moreover, the oxygen levels in the female reproductive tract, which sperm need to go through, are relatively low (Fischer and Bavister, 1993).

In fact, the nature of the ATP generated for mammalian sperm motility, *i.e.*, the relative significance of OXPHOS *versus* glycolysis, is a long-term debate that has been elegantly reviewed by others (Ruiz-Pesini *et al.*, 2007; Storey, 2008). Early biochemical studies on the energy metabolism of mammalian sperm were done using hypotonically treated cells, *i.e.*, with disrupted membranes, a simple method developed by Keyhani and Storey (1973a) to resolve the difficulty in isolating sperm mitochondria. These authors analysed respiration rates and oxygen consumption to assess mitochondrial activities with various substrates, in sperm from different species (Keyhani and Storey, 1973b; Storey and Keyhani, 1974; Storey and Kayne, 1977; Storey, 1978; Storey and Kayne, 1978; Storey, 1980; Carey *et al.*, 1981). Mitochondrial activity was observed in all cases, suggesting that OXPHOS is operating in mammalian sperm, and is responsible for the production of at least some of the ATP needed for sperm motility. The results were, though, species-specific, *i.e.*, sperm from distinct species have dissimilar capabilities to metabolize different substrates. This led to the hypothesis that the mitochondrial activity of sperm of a certain species is adjusted to the substrate content of the female reproductive tract (Storey, 1980). Whatever the case may be, it has been shown that oxygen consumption strongly correlates with sperm motility (Bohnensack and Halangk, 1986; Halangk *et al.*, 1990). The relevance of OXPHOS to mammalian sperm motility has also been analysed using mitochondrial inhibitors, namely rotenone, antimycine A, potassium cyanide, olygomicin, and others. Incubation of sperm with these drugs resulted in ATP depletion and/or decreased sperm motility, both in humans (Ford

and Harrison, 1981; de Lamirande and Gagnon, 1992; Ruiz-Pesini *et al.*, 2000a; St John *et al.*, 2005), and other mammals (Halangk and Bohnensack, 1986; Pascual *et al.*, 1996; Krzyzosiak *et al.*, 1999), evidently showing that OXPHOS function is crucial to sperm motility. Clear evidence came also from a patient harbouring a mitochondrial point mutation causing a reduced activity of the Complex I of the ETC, and whose sperm presented low motility. The supplementation of the patient's sperm with succinate, a substrate for Complex II, circumventing the effect of the mutation, resulted in an increase in sperm motility (Folgero *et al.*, 1993). The analysis of the enzymatic activities of the mitochondrial complexes have shown a positive correlation between those activities and sperm quality, particularly with sperm motility, and have also evidenced that control subjects present higher activities than asthenozoospermic individuals (Ruiz-Pesini *et al.*, 1998; 2000b).

On the other hand, and more recently, studies using mice models have questioned the importance of OXPHOS-derived ATP. In fact, it has been suggested that most of the energy required for sperm motility is definitely generated by the glycolytic pathway (Miki *et al.*, 2004; Mukai and Okuno, 2004). However, this has been challenged by others (Ford, 2006; Ruiz-Pesini *et al.*, 2007). To this extent, Ford has pointed out some ideas that strongly reinforce the significance of OXPHOS to sperm motility, namely the fact that sperm can remain motile in sugar-free media, and that motility is not affected when glycolysis is inhibited. More than that, this author has proposed that the ATP produced in the midpiece may reach the distant parts of the sperm tail by using an adenylate kinase shuttle, or other shuttles (Ford, 2006).

To conclude, the controversy on the importance of OXPHOS *versus* glycolysis in the sperm biology field has been confounded by possible changes during the lifetime of the male gamete, as well as by species-specific discrepancies. It is, however, undoubtedly well established that human sperm motility relies on OXPHOS-derived ATP. Then again,

mammalian sperm, like other cell types, seem to be versatile and able to use distinct metabolic pathways according to the substrates available. Similarly to somatic cells, the distinct metabolic pathways in sperm might be connected. For instance, the inactivation of transdolase (an enzyme of the pentose phosphate pathway) in a mice model resulted in loss of mitochondrial function, diminished levels of ATP, reduced sperm motility and, thus, male sterility (Perl *et al.*, 2006).

The link between mitochondrial-generated ATP and sperm motility and fertilisation competence has certainly been illustrated by the assessment of the sperm mitochondrial activity through the evaluation of the electrical potential across the inner mitochondrial membrane, usually referred as mitochondrial membrane potential (MMP). To this extent, diverse mitochondrial fluorescent probes have been used, such as Rodamine 123 (Rh123), 3,3'-dihexyloxacarbocyanide iodide [$DiOC_6(3)$], the well-known range of MitoTracker probes and 5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1). All these probes accumulate in mitochondria of living cells depending on the MMP and thus can be used to monitor functional mitochondria. In sperm, multiple studies, using either flow cytometry or fluorescence microscopy, have revealed a strong correlation between loss of mitochondrial function, monitored by low/inexistent MMP, and diminished motility in human (Evenson *et al.*, 1982; Auger *et al.*, 1989; Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Marchetti *et al.*, 2004) and other mammals (Garner *et al.*, 1997; Gravance *et al.*, 2001; Love *et al.*, 2003; Spinaci *et al.*, 2005). More than that, both sperm quality and fertilisation ability (measured as fertilisation rates after IVF) are strongly related with MMP, and thus with mitochondrial functionality (Kasai *et al.*, 2002; Marchetti *et al.*, 2002; Wang *et al.*, 2003; Liu *et al.*, 2004; Marchetti *et al.*, 2004). Additionally, the results of one of these studies proposed that MMP is the most sensitive test to determine sperm quality (Marchetti *et al.*, 2002). Indeed, and mirroring the importance of mitochondria to sperm function, subpopulations of

sperm with high MMP, isolated using a cell-sorter, seem to be enriched in cells with elevated fertilisation capabilities (Gallon *et al.*, 2006).

3. The mitochondrial genome

3.1. Mitochondrial DNA structure and function

Mitochondria possess their own genome, the mitochondrial DNA (mtDNA), which is present in multiple copies, usually in the range of 10^3-10^4 copies per human somatic cell (Bogenhagen and Clayton, 1974; Shmookler Reis and Goldstein, 1983; Miller *et al.*, 2003). Although not packaged with histones, this genome is organized in a DNA-protein structure called the nucleoid, each containing 1-10 mtDNA molecules (Satoh and Kuroiwa, 1991; Legros *et al.*, 2004). Human mtDNA (Fig. 4) is a circular, double-stranded molecule containing 16,569 base pairs (bp), and encoding 13 polypeptides of the ETC (seven subunits of complex I, one component of complex III, the 3 catalytic subunits of complex IV, and two subunits of complex V; Fig. 3), along with the RNAs necessary for mtDNA translation, namely, 2 rRNAs (12S and 16S) and 22 tRNAs (Anderson *et al.*, 1981). All other components of the ETC are nuclear-encoded and need to be targeted to the mitochondria. This makes the ETC unique in the cell, implying a correct intercommunication between the two genomes (nuclear and mitochondrial) for its proper assembly and function. The two mtDNA strands are referred as heavy (H-strand) and light (L-strand) because they possess different G+T content, and can, therefore, be separated on denaturing caesium chloride gradients. The H-strand encodes most of the information: the two rRNAs, 14 tRNAs and 12 of the 13 polypeptides; while the L-strand encodes the remaining tRNAs and a single polypeptide (Attardi and Schatz, 1988). The mitochondrial genome is extremely compact, presenting no introns, and possessing only a major non-coding region – the displacement-loop (D-loop; Figs. 4, 5), which contains most of the cis-acting control elements of the replication and transcription

processes. The extreme economy of organization of this genome is also illustrated by the fact that some genes even overlap (ND4 and ND4L, ATPase 6 and 8; Anderson *et al.*, 1981) and that others do not contain termination codons, as these are generated by post-transcriptional polyadenylation (Ojala *et al.*, 1981).

3.2. MtDNA expression

As noted above, mitochondria are not self-sufficient components in the cell, depending on nuclear-encoded proteins for their maintenance. These include mtDNA transcription and replication factors that are translocated to the mitochondria (Clayton, 1998). The replication of the mitochondrial genome is, however, independent from the cell cycle, and both transcription and replication of mtDNA occur at a greater rate than what is described for nuclear genes (Bogenhagen and Clayton, 1977). Moreover, and also particularly to this genome, mtDNA replication is intimately related to its transcription, as the initiation of replication depends on the transcription having occurred previously.

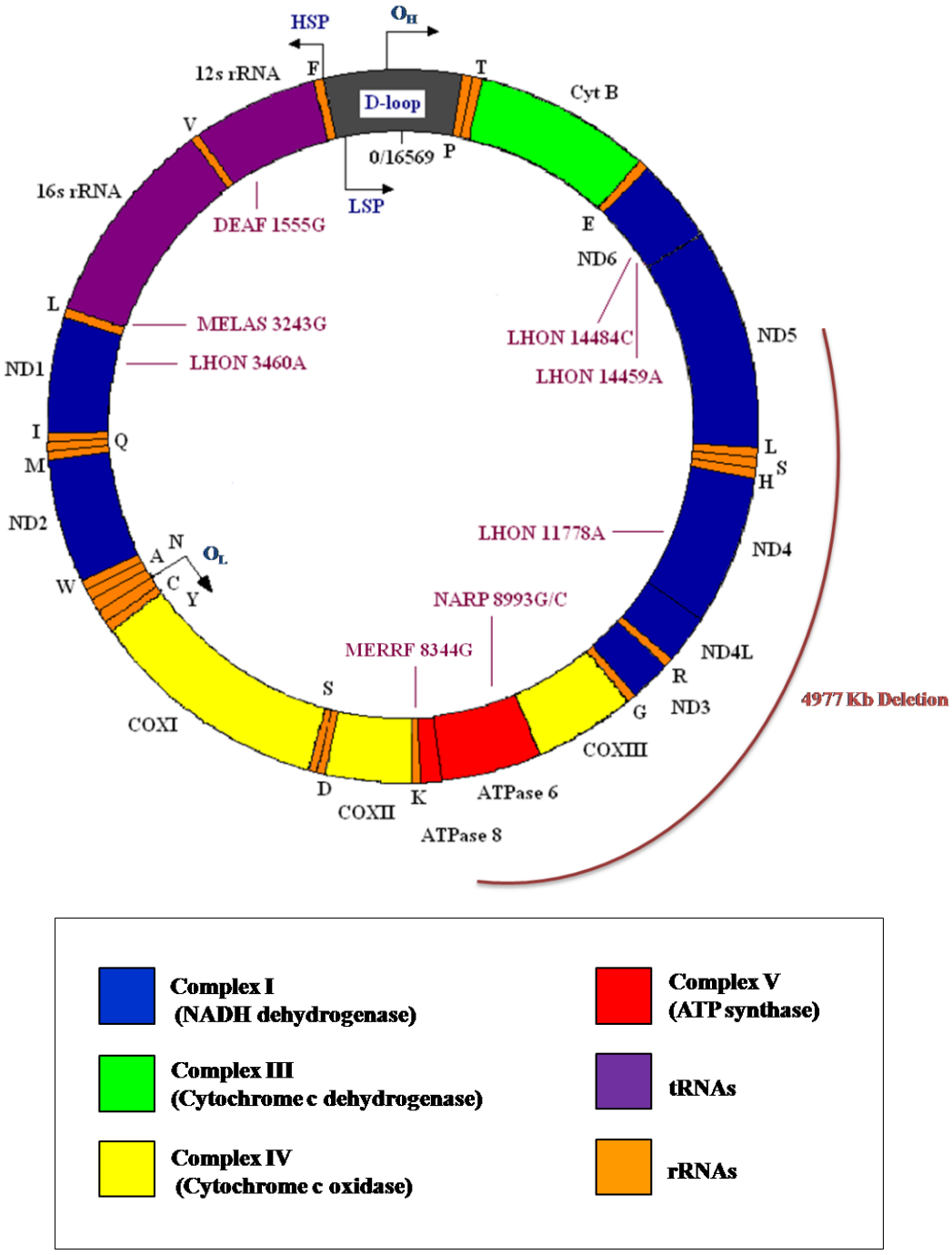


Figure 4. The human mitochondrial genome. This double-stranded molecule encodes 13 polypeptides of the electron transfer chain. The heavy (H) strand encodes 12 of these polypeptides, along with the 14 tRNAs and the 2 rRNAs (outside circle), while the light (L) strand encodes a single polypeptide (ND6) and 8 tRNAs (inside circle). The D-loop is the major non-coding region and contains the origin of replication of the H-strand (O_H), as well as the promoter regions of both strands (HSP, LSP). The origin of replication of the L-strand (O_L) is located at about 2/3 of the molecule length, in between tRNAs genes. tRNAs are identified by their respective one-letter codes. The localisation of the common 4977 Kb deletion and of some point mutations and their associated diseases are also depicted. *Adapted from Mitomap (2008).*

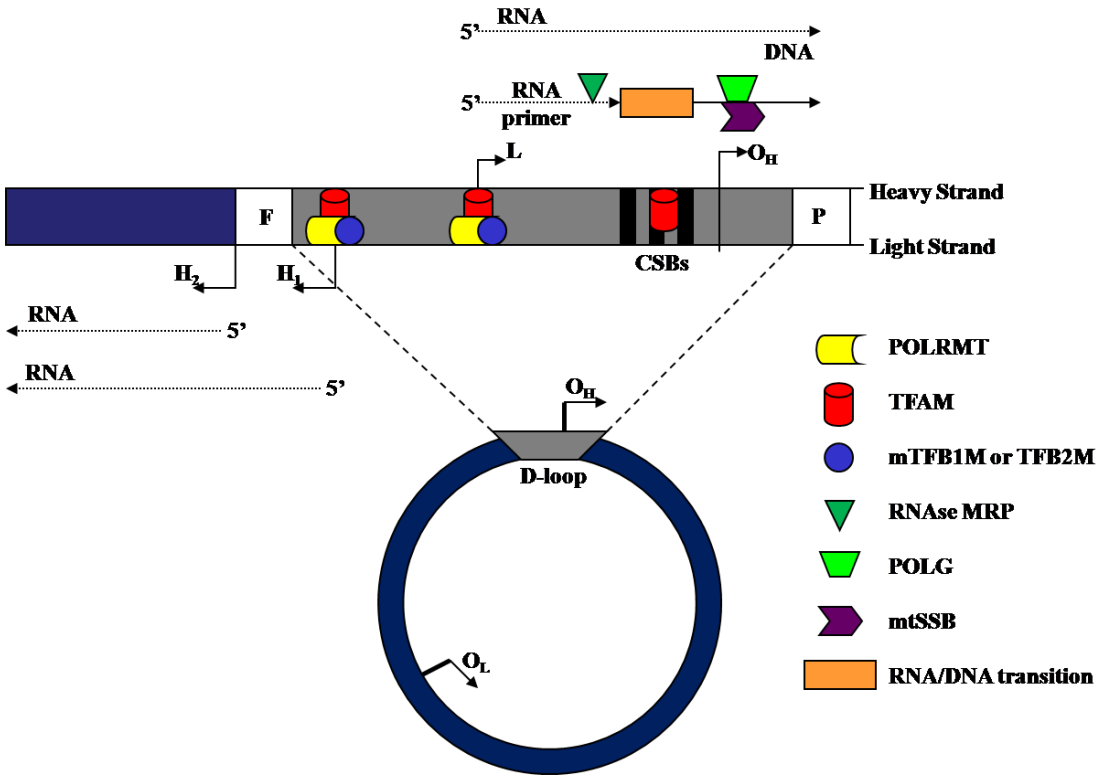


Figure 5. Transcription and replication of the mitochondrial genome. These processes are dependent on a number of nuclear-encoded factors that are translocated to the mitochondria, including: RNA polymerase (POLRMT); mitochondrial transcription factor A (TFAM); mitochondrial transcription factors 1 or 2 (TFB1M, TFB2M); RNA-processing endoribonuclease (RNase MRP); DNA polymerase gamma (POLG); mitochondrial single-strand binding protein (mtSSB). Other abbreviations: L, H_1, H_2 - transcription initiation sites; O_H, O_L – origin of replication sites; CSBs – conserved sequence boxes; F – phenylalanine tRNA gene; P – proline tRNA gene. See text for details. *Adapted from St. John et al. (2007).*

Transcription of the mitochondrial genome (Fig. 5), which is mediated by the mitochondrial-specific RNA polymerase (POLRMT; Tiranti *et al.*, 1997), requires at least two transcription factors: mitochondrial transcription factor A (TFAM; Fisher and Clayton, 1985; 1988) and either mitochondrial transcription factor 1 or 2 (TFB1M and TFB2M, respectively; Falkenberg *et al.*, 2002; McCulloch *et al.*, 2002). Transcription can start at three different initiation sites localized in the D-loop, two for the H-strand (H_1 and H_2) and one for the L-strand (L) (Montoya *et al.*, 1982; 1983; Bogenhagen *et al.*, 1984). H_1 and L are located within independent promoter regions: the H-strand promoter (HSP) and the L-strand promoter (LSP),

respectively (Fig. 4), both of which contain enhancer elements that include binding sites for TFAM (Parisi and Clayton, 1991; Dairaghi *et al.*, 1995). The interaction of TFAM with the enhancers permits POLRMT binding to mtDNA, in the presence of either TFB1M or TFB2M, and activation of transcription. Additionally, transcription termination seems to require mitochondrial transcription termination factors (MTERFs; Daga *et al.*, 1993; Fernandez-Silva *et al.*, 1997; Linder *et al.*, 2005). The transcripts originated from H_2 and L are long polycistronic molecules covering almost the whole strands that are then processed to originate mature RNAs (Ojala *et al.*, 1981). In contrast, transcription commencing at H_1, which occurs much more frequently than at H_2, is linked to a transcription termination event occurring downstream from 16S rRNA, giving rise to a smaller molecule coding for the 2 rRNAs, along with 2 tRNAs (Montoya *et al.*, 1983; Kruse *et al.*, 1989). These distinct features of H_1 and H_2 might have an important functional meaning, and seem to explain the differential regulation of rRNA *versus* mRNA transcription (Montoya *et al.*, 1983).

Mature transcripts are translated in mitocondrial ribosomes, or mitoribosomes, that are localized in the matrix. Mitoribosomes are distinct from cytosolic counterparts, but share some characteristics with bacterial ribosomes, and are sensitive to antibiotics that inhibit prokaryotic translation. However, they have a particularly small RNA content and, therefore, a low sedimentation coefficient (55S; Attardi and Ojala, 1971; Brega and Vesco, 1971). Mitochondrial translation arises by the use of the 22 mtDNA-encoded tRNAs only, which is possible because of a simplified codon-anticodon pairing scheme (Attardi and Schatz, 1988), and using a genetic code that differs from the "universal" one (Barrell *et al.*, 1979).

The regulation of mtDNA expression in individual tissues, and in response to distinct cellular energy demands, is far from being understood, and might be very complex (Bonawitz *et al.*, 2006; Asin-Cayuela and Gustafsson, 2007). Regulation at the mitochondrial transcription level may involve distinct factors, and while TFAM has been proposed to play a

key role (Garstka *et al.*, 1994; Montoya *et al.*, 1997), recent reports are showing that other elements also contribute for this regulation, including mtDNA transcription activators (as TFB2M; Cotney *et al.*, 2007) and repressors (as MTERF3; Park *et al.*, 2007). Also, for the proper function of the mitochondrial ETC, the expression of mitochondrial and nuclear-encoded subunits must be highly coordinated. Such a control seems to be accomplished by transcription factors, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2), that regulate the expression of both nuclear-encoded ETC genes and of mitochondrial transcription and replication factors (Scarpulla, 1997; 2002). These activators, are, in turn, regulated by coactivators that respond to distinct physiological signals (Wu *et al.*, 1999; Andersson and Scarpulla, 2001), rendering the overall regulation system tremendously complex.

3.3. MtDNA maintenance

As stated above, the replication of the mitochondrial genome, which is absolutely necessary for mtDNA maintenance, is independent from the replication of the nuclear DNA (Bogenhagen and Clayton, 1977). However, its initiation is dependent on mtDNA transcription having occurred before, as transcription is responsible for the generation of the RNA primer necessary for the initiation of replication.

MtDNA replication is catalysed by the mitochondrial-specific DNA polymerase gamma (POLG; Ropp and Copeland, 1996) and, according to the generally accepted model (Clayton, 1982), occurs asynchronously from two independent unidirectional origins. Essentially, replication starts at the origin of replication of the H-strand (O_H, which is localized in the D-loop, directly downstream of the LSP; Fig. 4) and proceeds in a clockwise-manner along the parental L-strand, to produce a new H-strand molecule. When approximately two-thirds of this new strand is produced, the origin of replication of the L-strand (O_L; Fig. 4) is exposed, and the synthesis of a new L-strand is initiated, in an anticlockwise manner (Wong and Clayton, 1985; Hixson *et al.*, 1986). The initiation of

replication at O_H is primed by a fragment of RNA that is generated by transcription from the initiation site L (Fig. 5). This transcript is processed by mitochondrial RNA processing endoribonuclease (RNase MRP; Lee and Clayton, 1997) and is then extended by POLG via DNA polymerisation. Transition from RNA to DNA synthesis is mediated through three conserved sequence blocks located in the D-loop (CSBI, CSBII and CSBIII; Xu and Clayton, 1996). Replication from O_H often arrests prematurely at short (15 bp) termination-associated sequences (TAS), thus generating the triplex-structure characteristic of the D-loop (Clayton, 1982; Shadel and Clayton, 1997). When replication from the O_H proceeds, O_L is eventually exposed and both strands are replicated. The initiation of replication from O_L requires the action of a mitochondrial specific primase (Wong and Clayton, 1985). In addition, POLG requires a number of other factors to perform the replication of both H- and L-strands. These include: a mitochondrial helicase (Twinkle; Hehman and Hauswirth, 1992; Korhonen *et al.*, 2003) mitochondrial single-strand binding protein (mtSSB; Hoke *et al.*, 1990; Tiranti *et al.*, 1993; Farr *et al.*, 1999) and mitochondrial-associated topoisomerases (Castora *et al.*, 1985; Kosovsky and Soslau, 1991). Once replication of both strands is terminated, the newly-synthesised molecules are separated, the RNA primers are removed, the remaining gaps are filled and the new molecules adopt their usual tertiary structure (Shadel and Clayton, 1997).

More recently, the analysis of replication intermediates through two-dimensional (2D) agarose gel electrophoresis led to the proposal of an additional mechanism for mtDNA replication. According to this mechanism, replication of both strands begins at or near O_H, proceeding synchronously around the entire circular molecule (Holt *et al.*, 2000). Importantly, these authors suggested that both mechanisms of replication might exist, and reported a shift, from the predominant strand-asynchronous mechanism to the strand-coupled one, in cells recovering from transient mtDNA depletion. However, since this report, the precise mechanism of mtDNA replication has been the centre of a heated discussion with followers of

the "orthodox", strand-asymmetric replication mode (Bogenhagen and Clayton, 2003; Brown *et al.*, 2005) on one side, and strand-coupled mechanism supporters (Yang *et al.*, 2002; Bowmaker *et al.*, 2003; Yasukawa *et al.*, 2005; 2006) on the other.

Unlike nuclear genes, and consistent with the fact that a cell's mtDNA copy number is variable, the expression of mitochondrial-encoded genes can be regulated at the level of gene dose, implying that cells are able to adjust mtDNA replication according to its mtDNA content. While it is well established that a number of factors are required for mtDNA maintenance (namely the factors involved in replication and transcription), it is not clear how mtDNA levels are controlled. Such a control may be achieved by the levels of proteins involved in mtDNA replication, by the existing nucleotide pools, and ultimately, by any factors controlling these two (Moraes, 2001). To this extent, and using mice models, it has been shown that mtDNA copy number is directly proportional to TFAM levels, suggesting that this protein is a key regulator of mtDNA content (Ekstrand *et al.*, 2004). Other factors that seem to be involved in this regulation include Twinkle, the mitochondrial helicase (Tyynismaa *et al.*, 2004), as well as different proteins involved in cellular proliferation (Trinei *et al.*, 2006; Kasashima *et al.*, 2008).

Studies with cultured mammalian cells presenting decreased mtDNA content, accomplished either by long-time incubation with ethidium bromide (Desjardins *et al.*, 1985) or siRNA of *TFAM* (Jeng *et al.*, 2008), have clearly settled that maintenance of mtDNA copy number and expression are crucial for mitochondrial function. Accordingly, mtDNA depletion is associated with a number of pathological conditions (see section 3.5).

The maintenance of the mitochondrial genome in a cell depends not only on the replication process, ensuring that adequate amounts of mtDNA are present, but also on the existence of repair mechanisms to emend possible errors introduced during the replication process, as well as to remove damaged DNA. Importantly, the catalytic subunit of POLG,

apart from its polymerase activity, also functions as 3' to 5' exonuclease, which largely contributes to the fidelity of mtDNA replication (Johnson and Johnson, 2001). Consistent with this, the expression of an exonuclease-deficient POLG fusion protein led to a major accumulation of mtDNA point mutations in cultured human cells (Spelbrink *et al.*, 2000). Likewise, homozygous knockin mice expressing a proofreading-deficient POLG presented increased levels of mtDNA point mutations and deletions (Trifunovic *et al.*, 2004). Furthermore, the POLG catalytic subunit is also implicated in base excision repair (BER) processes that correct oxidative damage to mtDNA, as it also possesses 5'-deoxyribose phosphate lyase activity (Longley *et al.*, 1998). Other enzymes participating in these repair processes have been described in mammalian mitochondria, including glycosylases, endonucleases and DNA ligases (reviewed in Graziewicz *et al.*, 2006). Hence, and contradicting previous assumptions, it is now well established that mitochondria do possess DNA repair systems, but these seem to be scarcer that the ones operating in nuclear DNA (for reviews see Croteau *et al.*, 1999; Larsen *et al.*, 2005). Failure of any of these mitochondrial repair mechanisms can result in the development of mtDNA mutations and ultimately, in mitochondrial diseases (see section 3.5).

3.4. MtDNA inheritance

Contradicting a established idea found in a number of textbooks, in most mammals, including humans, the entire sperm enters the oocyte at fertilisation (Ankel-Simons and Cummins, 1996). However, and also like in other mammals, human mitochondrial genome tends to be exclusively maternally inherited (Giles *et al.*, 1980). This entails that the mtDNA of an embryo is only transmitted from the oocyte, and that sperm mtDNA (which is present in the midpiece that enters the oocyte at fertilisation) is diluted or destroyed. Definitely, and while there is some variability, oocytes contain the largest cellular mtDNA content - about 10^5 copies/cell (Reynier *et al.*, 2001), and individual oocyte content seems to be indicative of its

quality (Almeida-Santos *et al.*, 2006). Although oocyte mtDNA copy number is high, mtDNA sequence variants are rapidly segregated between generations. This observation suggested the existence of a genetic bottleneck for the transmission of the mitochondrial genome (Hauswirth and Laipis, 1982). Accordingly, a tight restriction event occurs during early oogenesis ensuring that only a small pool of molecules of mtDNA is present in primordial germ cells for transmission to the next generation (Jansen and de Boer, 1998). During oocyte maturation, these molecules are clonally amplified, so that each oocyte will contain multiple copies of a single population of mtDNA (Marchington *et al.*, 1997).

The basis for the strictly maternally inheritance of the mitochondrial genome are not completely understood. Mammalian sperm presents the lowest cellular mtDNA copy number - roughly 10^2 copies/cell (Hecht *et al.*, 1984; May-Panloup *et al.*, 2003), three orders of magnitude lower than the oocyte's counterpart. Therefore, the simple dilution of paternal mtDNA after fertilisation guarantees that most of the embryo's mtDNA have a maternal origin. Furthermore, paternal mtDNA has been shown to be eliminated during early embryogenesis in intraspecific crosses in mice (Kaneda *et al.*, 1995; Cummins *et al.*, 1997), bovine (Sutovsky *et al.*, 1996b) and non-human primates (Sutovsky *et al.*, 1999b). On the other hand, paternal mtDNA leakage takes place in mice interspecific crosses (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995). The leaked paternal mtDNA is not, however, distributed to all tissues in hybrid individuals, nor transmitted to subsequent generations (Shitara *et al.*, 1998). Paternal mtDNA inheritance has also been reported in sheep (Zhao *et al.*, 2004) and in nonhuman primates produced by nuclear transfer (St John and Schatten, 2004). These leakage events, which barely happen in interspecific crosses (infrequent in nature), may result from a failure in the mechanism(s) of elimination of paternal mitochondria. Certainly, the selective destruction of mammalian sperm mitochondria (and hence, mtDNA) seems to be species-specific, involving an ubiquitination process (Sutovsky *et al.*, 1999a; 2000). Specifically,

mitochondria are ubiquitinated during spermatogenesis, and the ubiquitin tags are then masked by disulphide-bond cross-linking throughout epididymal transit. One of the ubiquitinated substrates appears to be prohibitin, a highly conserved mitochondrial protein (Thompson *et al.*, 2003). Once inside the egg, disulphide-bonds are reduced and ubiquitin is exposed, leading to paternal mitochondria degradation by embryo proteasomes and lysosomes. Additionally and more recently, active digestion of sperm mtDNA upon fertilisation has been shown in the Japanese medaka fish, even before mitochondrial destruction (Nishimura *et al.*, 2006).

Studies of mtDNA transmission using good-quality human embryos are certainly restricted by ethical issues. Regardless, it seems that paternal mtDNA may persist until the blastocyst stage in some abnormal embryos (St John *et al.*, 2000). Furthermore, paternal inheritance has been reported in a patient with a mitochondrial myopathy due to a pathogenic mtDNA 2 bp deletion (Schwartz and Vissing, 2002). Such a deletion was, however, only present in the patient's muscle, and not in other tissues. This case has certainly motivated the search for parallel outcomes in similar patients, but transmission of paternal mitochondrial genome failed to be proven in every single case (Filosto *et al.*, 2003b; Taylor *et al.*, 2003). Taken together, these results suggest that in humans, like in other mammals, paternal mtDNA leakage is an uncommon event, and such occasions may reflect a failure in the active destruction of sperm mitochondria/mtDNA in the developing embryo.

3.5. Mitochondrial disease

The term "mitochondrial disease" is generally used to describe any disorder associated with anomalies of the mitochondrial OXPHOS, leading to inadequate levels of ATP (Zeviani and Carelli, 2003). As already mentioned, ATP production through OXPHOS implies the correct assembly and function of a number of mitochondrial proteins. As anticipated, mutation of any of the genes encoding these proteins can lead to ETC defects and thus,

mitochondrial disease. Since the ETC is the only cellular apparatus encoded by both the nuclear and the mitochondrial genomes, such disorders are unusual, as inheritance can either be Mendelian or mitochondrial.

Importantly, mtDNA genetics presents some distinctive traits when compared to that of nuclear DNA. For instance and as previously mentioned, the mitochondrial genome tends to be maternally inherited. Moreover, this genome is polyploid (a single cell may contain hundreds of copies of mtDNA) and the diverse molecules are randomly distributed during cell division, a phenomenon called mitotic segregation (Wallace, 1986; Matthews *et al.*, 1995). In a normal situation, all mtDNA molecules present in a cell, tissue or organism are identical, a condition termed homoplasmy. However, mtDNA alterations can occur, and in this case, both wild-type and mutated mtDNA molecules will co-exist (heteroplasmy). The phenotypic expression of a certain mtDNA mutation is dependent on the relative proportion of the mutated molecule, and in fact, clinical symptoms will only emerge when a critical threshold is reached (Chinnery *et al.*, 1997). Such a threshold is different for each mutation, and may also vary between individuals and from tissue to tissue, according to the energetic demands, with the brain, heart and skeletal muscle being the most affected tissues (Wallace, 1999). These facts account for the existence of diverse clinical manifestations, confounding the diagnosis of a mtDNA disease (Chinnery *et al.*, 1999).

3.5.1. MtDNA mutations

Since the first reports stating an association between human mtDNA mutations and mitochondrial diseases were published (Holt *et al.*, 1988; Wallace *et al.*, 1988), the number of mutations described and their relationship with human disorders has been expanding (the commonest mtDNA mutations are depicted in Fig. 4; MITOMAP, 2008). These mutations include large-scale rearrangements (deletions and duplications; generally heteroplasmic and sporadic) and point mutations (that can be present in either homoplasmy or heteroplasmy, and

are usually maternally inherited). Any of these can affect one or more mtDNA-encoded ETC protein in particular, or change an rRNA or tRNA, with potential consequences in the expression of any mtDNA-encoded protein. Large-scale rearrangements include several genes and usually delete at least one tRNA, thus causing protein synthesis defects (Wallace, 2001).

MtDNA large-scale rearrangements are thought to result from a clonal amplification of a mutation that has occurred in the maternal oocyte or during early embryo development (Chen *et al.*, 1995). Because of the mtDNA mitotic segregation, different tissues may acquire distinct levels of rearranged molecules, and the eventual onset and severity of the disease will be dependent on the relative amount and tissue distribution of the mutation (Zeviani and Di Donato, 2004). Single large-scale deletions and duplications have been observed in sporadic conditions such as Kearns-Sayre Syndrome (KSS), a multisystem disorder with early onset, characterized by neuropathy, retinopathy and cardiomyopathy (Zeviani *et al.*, 1988); Pearson's syndrome (PS), a fatal disease in childhood, characterized by severe anaemia (Rotig *et al.*, 1990); and Progressive External Ophthalmoplegia (PEO), characterized by late-onset bilateral ptosis, muscle weakness and exercise intolerance (Shoffner *et al.*, 1989). The most common deletion is approximately 5 kb long (the so called "common deletion"), comprising both protein-coding genes and tRNAs (as shown in Fig. 4).

A vast number of maternally inherited point mutations have been described (some of which are illustrated in Fig. 4) that are associated with a range of clinical manifestations, particularly with neurodegenerative and neuromuscular disorders. For instance, heteroplasmic mutations are associated with Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes (MELAS; Goto *et al.*, 1990), Myoclonic Epilepsy and Ragged Red Fibres (MERRF; Shoffner *et al.*, 1990) and also Neuropathy, Ataxia and Retinis Pigmentosa (NARP; Holt *et al.*, 1990). Homoplasmic point mutations, which are generally limited to a single tissue, have been described in Leber Hereditary Optic Neuropathy (LHON), a disorder

that typically affects young adults, leading to loss of vision (Wallace *et al.*, 1988), and also in Sensorineural Hearing Loss (SNHL; Prezant *et al.*, 1993).

The existence of a vast number of mtDNA mutations mirror a particular feature of the mitochondrial genome, namely its high mutation rate, which seems to be around 20 to 100 times higher than that of nDNA (Wallace *et al.*, 1987; Pesole *et al.*, 1999). Several aspects may account for this: a) mtDNA is exposed to high levels of ROS generated by the ETC, which accumulate in the mitochondrial matrix and are highly mutagenic; b) the absence of protective histones; c) the existence of less effective repair mechanisms. Mutations can, therefore, result both from exogenous DNA injury and unrepaired replication errors (reviewed in Copeland *et al.*, 2003). As a result, the mitochondrial genome acquires somatic mutations during normal life span, puzzling the distinction between neutral polymorphisms and pathologic mutations.

While the disorders presented so far are relatively rare, the involvement of mtDNA mutations in more common diseases, such as in the neurodegenerative Parkinson's disease (PD) and Alzheimer's disease (AD), as well as in diabetes and cancer has also been proposed (reviewed in McFarland *et al.*, 2007). Moreover, an accumulation of somatically acquired mtDNA mutations have been shown in a number of tissues throughout life, suggesting a key role for these mutations in the aging process (Corral-Debrinski *et al.*, 1992; Hayakawa *et al.*, 1992; Zhang *et al.*, 1998, just to give a few examples).

3.5.2. Defects in nuclear-encoded genes

The increased interest in mitochondrial diseases has been expanding our understanding of this issue, and it is now well established that diverse genes can be implicated. Importantly, there are certain mitochondrial diseases whose clinically manifestations are similar to those caused by mtDNA mutations, but follow a Mendelian pattern of inheritance. These are caused by defects on nuclear genes directly or indirectly involved in OXPHOS function, including

genes encoding structural components of the ETC complexes; assembly factors of the ETC complexes; proteins implicated in mitochondrial biogenesis; and, finally, mtDNA maintenance factors (for reviews see Zeviani and Carelli, 2003; DiMauro, 2004; Schapira, 2006).

Defects in any protein involved in the maintenance of the mitochondrial genome can potentially affect mtDNA stability either qualitatively or quantitatively, promoting the arising of multiple mtDNA mutations and/or depletion. In any case, it is a primary nuclear gene defect that is in the origin of a secondary mtDNA deficiency (Suomalainen and Kaukonen, 2001). Most of these nuclear genes encode proteins mainly involved in mtDNA replication, either with a role in replication initiation, such as TFAM, or functioning at the replication fork, such as POLG and TWINKLE, or involved in mitochondrial nucleotide metabolism, such as Adenine Nucleotide Translocator 1 (ANT1), Thymidine Kinase 2 (TK2) and Deoxyguanosine Kinase (dGK) (reviewed in Copeland, 2008). Some of the information regarding the role of two of these proteins (POLG and TFAM) in mitochondrial disorders is listed below.

3.5.2.1. POLG

As mentioned before, POLG is the sole DNA polymerase present in mitochondria, responsible for both mtDNA replication and repair. Human POLG comprises three subunits (Yakubovskaya *et al.*, 2006): a 140 KDa catalytic subunit (encoded by *POLG*), and two 55 KDa accessory subunits (encoded by *POLG2*). The catalytic subunit (Fig. 6), whose gene is located in chromosome 15q24-15q26 (Ropp and Copeland, 1996; Walker *et al.*, 1997; Zullo *et al.*, 1997), possesses both polymerase and 3'to 5' exonuclease domains, the first catalyzing mtDNA replication and the second involved in proofreading and repair (Graves *et al.*, 1998). It also comprises a linker region (located in between the exonuclease and the polymerase domains) that may be involved in subunit interaction, in DNA binding, and in functional

combination of the polymerase and the exonuclease activities (Graziewicz *et al.*, 2006). The accessory subunit enhances DNA binding and promotes processivity and fidelity (Lim *et al.*, 1999; Longley *et al.*, 2001) and is also probably involved in primer recognition (Fan *et al.*, 1999).

As anticipated giving its role in mtDNA maintenance, POLG dysfunction may impair mitochondrial function and indeed, *POLG* is the main nuclear gene implicated in mitochondrial disorders. To date, more that 90 *POLG* mutations have been associated with a wide range of clinical phenotypes concurrent with mtDNA depletion and/or accumulation of mtDNA mutations and deletions (Fig. 7; Human Polymerase Gamma Mutation Database: http://tools.niehs.nih.gov/polg/index.cfm).

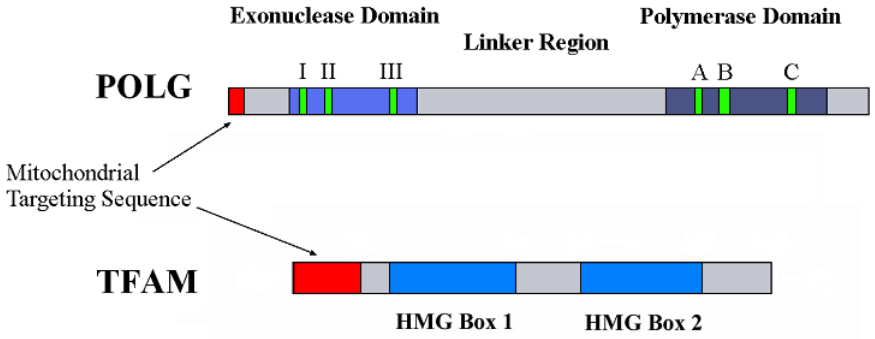


Figure 6. Schematic representation of DNA polymerase gamma (POLG) and mitochondrial transcription factor A (TFAM). These proteins possess a mitochondrial targeting sequence, allowing their translocation to the mitochondria. POLG posses both polymerase and exonuclease activities. TFAM is a member of the High Mobility Group (HMG) family of proteins. *Adapted from St. John et al. (2007).*

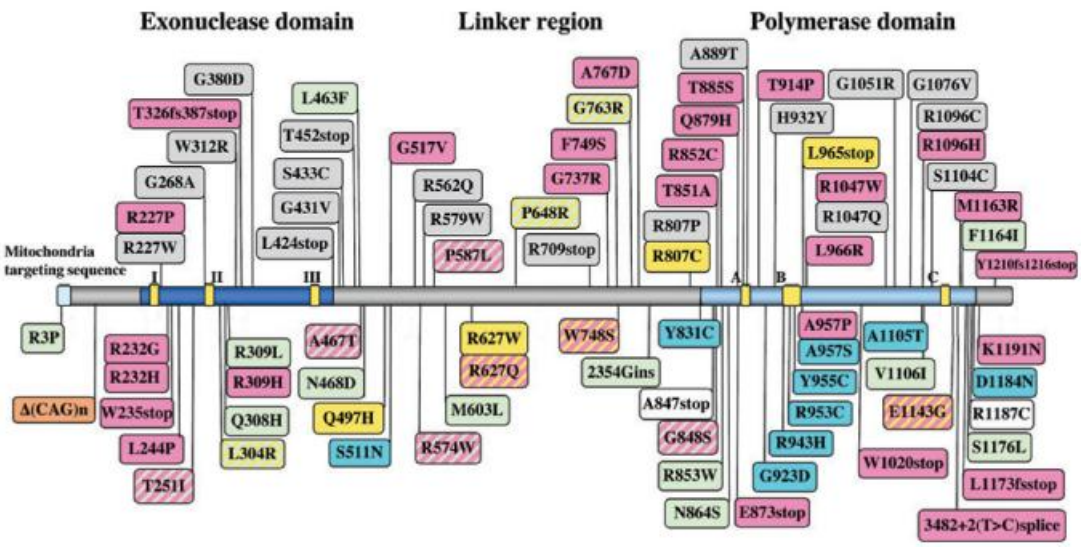


Figure 7. A vast number of POLG amino acids substitutions, caused by gene mutations, are associated with a range of human disease. These include: PEO, which can be autosomal dominant (blue boxes), autosomal recessive (green boxes) or sporadic (grey boxes); sensory ataxic neuropathies (yellow boxes); Alpers syndrome (pink boxes) and possible male infertility (orange box). Striped boxes represent substitutions associated with more than one disease. *Adapted from Copeland (2008).*

The majority of these mutations are linked with PEO, either autosomal dominant (adPEO) or autosomal recessive (arPEO), both of which presenting large mtDNA deletions and respiratory chain dysfunction. The onset of this disease is in adulthood, and the clinical manifestations, while heterogeneous, include progressive weakening of the external eye muscle, ptosis, skeletal muscle weakness and exercise intolerance. The association between POLG missense mutations and PEO was first described by Van Goethem and colleagues (2001) and latter confirmed by several other studies (Lamantea *et al.*, 2002; Agostino *et al.*, 2003; Di Fonzo *et al.*, 2003; Filosto *et al.*, 2003a; Van Goethem *et al.*, 2003a; Mancuso *et al.*, 2004). Most *POLG* mutations are associated with arPEO and patients are often compound heterozygotes, *i.e.*, with two different mutant *POLG* alleles. On the other hand, adPEO patients can be heterozygotes for the mutation and, in this case, mutations might produce a protein that competes with the wild-type POLG in a dominant negative way (Longley *et al.*, 2005). Interestingly, most adPEO mutations localise to the POLG polymerase domain and

change highly conserved aa residues, such as Arg943, Tyr955 and Ala957, that seem to be crucial for dNTP binding. *In vitro* experiments showed that such POLG alterations lead to decreased polymerase activity and reduced processivity and fidelity of the enzyme (Ponamarev *et al.*, 2002; Graziewicz *et al.*, 2004). More recently, transgenic mice with cardiac-targeted Y955C human POLG (a prevalent mutation where tyrosine is substituted with cisteine at codon 955) resulted in loss of heart mtDNA, cardiomyopathy and early death, clearly linking this mutation with cardiac dysfunction (Lewis *et al.*, 2007). POLG presenting another common mutation, A467T (causing an alanine to threonine aa substitution at codon 467), which co-segregates with arPEO, has decreased polymerase activity and fails to interact with the accessory subunit, at least *in vitro* (Chan *et al.*, 2005).

The diverse *POLG* mutations and their different effects on the enzyme may explain the heterogeneous phenotypic manifestations reported. Attesting to this diversity, mutations in *POLG* have been described in patients with sensory ataxic neuropathies (Van Goethem *et al.*, 2003a; 2003b; 2004; Gago *et al.*, 2006), Parkinsonism and premature menopause (Luoma *et al.*, 2004; Pagnamenta *et al.*, 2006; Hudson *et al.*, 2007), and Charcot-Marie-Tooth disease (CMT, Harrower *et al.*, 2008), a motor and sensory neuropathy characterized by loss of muscle tissue and touch sensation. *POLG* mutations are also responsible for Alpers syndrome, a rare but severe autossomal mtDNA depletion condition that affects young children, and is characterized by progressive cerebral degeneration leading to mental deterioration, spasms, blindness, deafness, liver failure and eventually death (Naviaux and Nguyen, 2004; Davidzon *et al.*, 2005; Naviaux and Nguyen, 2005; Nguyen *et al.*, 2005; 2006). In addition, polymorphisms in the *POLG* CAG-repeat region, encoding a polyglutamine tract localised near the amino-terminal of the protein, are possibly associated with male infertility (see Chapter III).

3.5.2.2. TFAM

TFAM is a member of the High Mobility Group (HMG) family of DNA-binding proteins and its human gene is located to chromosome 10q21 (Tiranti *et al.*, 1995). The protein (Fig. 6) consists of two HMG boxes and a mitochondrial targeting sequence that allows its translocation to the mitochondria (Parisi and Clayton, 1991). Here, and as mentioned previously, it activates mtDNA transcription (Garstka *et al.*, 1994), and it is also implied in replication, since transcription generates the RNA primer necessary for replication initiation (Chang *et al.*, 1985; Xu and Clayton, 1996). TFAM has also been proposed to package mtDNA (Alam *et al.*, 2003) and to be a key regulator of mtDNA copy number (Ekstrand *et al.*, 2004). Loss of TFAM function has been correlated with distinct diseases, which is not surprising giving its roles in mtDNA maintenance and expression. To this extent, low levels of TFAM expression have been associated with mtDNA-depletion/mutations syndromes, such as infantile mitochondrial myopathy and encephalomyophathy disorders (Larsson *et al.*, 1994; Poulton *et al.*, 1994; Siciliano *et al.*, 2000). Likewise, mice with disrupted TFAM in heart and muscle developed cardiac progressive respiratory chain deficiency caused by impaired mtDNA expression, and cardiomyopathy, mimicking diseases involving mtDNA mutations (Wang *et al.*, 1999). In a similar work, disruption of TFAM in dopamine neurons lead to reduced mtDNA expression and respiratory chain deficiency in these cells and ultimately to a Parkinsonism phenotype (Ekstrand *et al.*, 2007). In addition, certain *TFAM* variants are possibly associated with late-onset AD (Gunther *et al.*, 2004; Belin *et al.*, 2007; Alvarez *et al.*, 2008).

3.6. MtDNA and male infertility

Human sperm function is dependent on mitochondrial OXPHOS-derived ATP, and hence, on the mitochondrial genome and its integrity. Several studies have been performed to determine if sperm mtDNA mutations or deletions can cause loss of sperm function and,

consequently, male infertility. Two main approaches have been used: while some studies have compared the incidence of mtDNA mutations and/or deletions in distinctive quality sperm samples (judged according to the WHO standards), others have focused on subpopulations of sperm within a same sample. In the last case, sperm is usually fractionated by a discontinuous Percoll (or equivalent) gradient, and poorer quality sperm, with low motility, is collected in the lower density fraction (Gorus and Pipeleers, 1981; Pousette *et al.*, 1986).

The first indications that mtDNA point mutations could be associated with male infertility came from case reports of patients harbouring the A3243G point mutation, with mitochondrial encephalomyopathies, who also presented decreased sperm motility (Folgero *et al.*, 1993) or impaired reproductive function (Huang *et al.*, 1994). The first was particularly informative, as it has been shown that incubation of the patient's sperm with metabolic supplements to bypass the ETC complex I (which presented reduced activity, most likely as a result of the mtDNA point mutation) increased sperm motility three-fold. These founder studies, while suggestive, were not entirely conclusive, as the mtDNA point mutation was found in somatic tissues, and sperm mtDNA were not accessed. Clear evidence came from the analysis of sperm of another patient harbouring the same point mutation, where a greater mutation load was identified in lower density Percoll fractions, and was also associated with reduced sperm motility (Spiropoulos *et al.*, 2002). Less definitive, but equally interesting were the two reports of mutations in nucleotides 9055 (ATPase 6 gene) and 11719 and 11994 (both in ND4 gene), the occurrence of which was significantly higher in sperm samples of men with poor semen parameters when compared to normozoospermic individuals (Holyoake *et al.*, 2001; Selvi Rani *et al.*, 2006). It is however worth mentioning that these two works were questioned by others, who claimed that there is no evidence that these particularly point mutations can influence sperm motility (Bandelt, 2007; Pereira *et al.*, 2008).

The association between sperm mtDNA single large-scale deletions and male infertility has also been made, but, again, the results are conflicting. To this extent, in addition to the common 4977 bp deletion, two novel large deletions (7345 and 7599 bp) have been described in sperm. Analysis of fractionated different-quality sperm samples identified lower density fractions as presenting the highest frequency of occurrence of the deletions, along with a lower incidence of occurrence in sperm donor samples when compared to infertility patients (Kao *et al.*, 1995; 1998). More importantly, a high negative correlation was found between mtDNA deletion incidence and sperm motility. It should however be pointed out that the relative proportion of deleted molecule compared to the wild-type mtDNA was very low (< 0.08% and < 0.3% for the common deletion and for the 7345 and the 7599 bp deletions, respectively). Furthermore, these works were not verified by others, either analysing only sperm (St John *et al.*, 2001) or semen samples (Cummins *et al.*, 1998). However, in a more recent study comparing sperm samples from fertile individuals and OAT patients, the mtDNA common deletion was suggested to be a frequent alteration in infertility (Dhillon *et al.*, 2007), thus re-opening the debate.

While the association of a particular large-scale deletion with male infertility is somewhat conflicting, the same is not true for the occurrence of multiple mtDNA rearrangements, that, when in high numbers, seem to be related with loss of sperm function. Interestingly, multiple deletions identified in the skeletal muscle of a patient with a mitochondrial disease were also identified in his sperm (Lestienne *et al.*, 1997), again showing that OXPHOS dysfunction affecting one tissue was also characteristic of low sperm motility. Additionally, a latter study identified a high incidence of multiple deletions in sperm from a range of patients, irrespectively of sperm quality, suggesting that most sperm mitochondria present multiple DNA deletions (Reynier *et al.*, 1998). The persistence of multiple deletions in normozoospermic samples were confirmed by others (St John *et al.*,

2001), although this study also demonstrated an accumulation of multiple deletions in poorer quality sperm samples, with a greater number being observed in OATs. In accordance, analysis of high- and low-density sperm fractions showed that the former have a greater proportion of wild-type mtDNA molecules, along with a higher incidence of single and small deletions, while lower density fractions presented mainly double or multiple larger sized deletions (O'Connell *et al.*, 2003). Significantly, both the number and size of mtDNA deletions in either testicular or ejaculated sperm are negatively correlated with ICSI pregnancy outcomes (Lewis *et al.*, 2004), clearly showing the deleterious effect of mtDNA rearrangements in sperm function, even in those cases where the low motility is bypassed (by the introduction of sperm in the oocyte). These outcomes have been supported by transmitochondrial mice models carrying different proportions of the common deletion, where the accumulation of pathogenic mtDNA-derived ETC defects was responsible for male infertility (Nakada *et al.*, 2006).

Analysis of testicular and epididymal sperm of men with obstructive azoospermia (*i.e.* with normal spermatogenesis, but presenting no sperm in the ejaculate) has also provided some interesting results. It seems that testicular sperm possess more wild-type mtDNA molecules and lower incidence of multiple deletions (which are also smaller) than epididymal sperm (O'Connell *et al.*, 2002b), which most probably accounts for the higher fertilisation rates reported with testicular when compared to epididymal sperm from patients with obstructions (Palermo *et al.*, 1999). Additionally, fertile men also present mtDNA deletions in testicular sperm, but the mean size of these deletions is lower than in those men with obstructive azoospermia (O'Connell *et al.*, 2002a).

The influence of the mitochondrial genome in sperm is also typified by the suggestion that certain mtDNA haplogroups (groups of specific mtDNA types) are predisposed to reduced sperm motility in Caucasians. These variants are indicative of population lineages

and seem to be also associated with the susceptibility for certain diseases (Hofmann *et al.*, 1997), on one hand, and with longevity (De Benedictis *et al.*, 1998), on the other. Specifically related to male infertility, it seems that haplogroup T is more abundant in asthenozoospermic patients, while haplogroup H is overrepresented in nonasthenozoospermic men (Ruiz-Pesini *et al.*, 2000a). Supporting this, the two haplogroups showed differences in motility scores and in the enzymatic activities of mitochondrial complexes I and IV. It has also been revealed that there is apparently no link between mtDNA haplogroups and oligozospermia. A latter study by the same group showed differences in both sperm motility and vitality in sublineages of haplogroup U, which may be related to conserved missense mutations in COXIII and cytochrome c genes (Montiel-Sosa *et al.*, 2006). However, others have contradicted these findings and argued that there is not enough evidence for a link between mtDNA variants and sperm concentration and motility (Pereira *et al.*, 2005; 2007).

Overall, and despite the controversies on the exact effect of a particular mtDNA point mutation, deletion or haplogroup in human sperm, there are clear evidences that alterations in the mitochondrial genome can compromise sperm motility and function.

4. Aims

In the present work, distinct aspects of mitochondrial function were assessed in human sperm in order to understand their biological and clinical significances. Specifically, the aims were:

1) To determine if POLG and TFAM were expressed in mature human sperm, and understand if the expression of these and other mitochondrial proteins (COXI and COXVIc) were associated with sperm quality [both in distinct sperm samples (Chapter II) and different sperm subpopulations (Chapter IV)];

2) To establish if mtDNA copy number was related with sperm quality, and study the possible role of POLG and TFAM in regulating mtDNA content in mature sperm (Chapter II);
3) To ascertain if specific *POLG* polymorphisms were associated with sperm quality, and assess if these could affect sperm mtDNA copy number and the expression of mtDNA-related proteins (Chapter III);
4) To compare distinct mitochondrial probes and clarify their ability to monitor sperm MMP (Chapter IV);
5) To understand if POLG and COXI expressions were related with sperm MMP and sperm motility in subpopulations of human sperm (Chapter IV);
6) To find out the clinical value of the assessment of sperm MMP and understand if and how this was related with other functional parameters (Chapter V).

CHAPTER II

THE EXPRESSION OF POLYMERASE GAMMA AND MITOCHONDRIAL TRANSCRIPTION FACTOR A AND THE REGULATION OF MITOCHONDRIAL DNA CONTENT IN MATURE HUMAN SPERM

The results presented in this Chapter were originally published in:

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Part of the methodology described in this Chapter has also been published in:

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Abstract

Human mitochondrial DNA (mtDNA) encodes 13 polypeptides of the electron transfer chain (ETC). A series of mtDNA rearrangements are associated with reduced sperm quality and, consequently, with male infertility. Moreover, low quality sperm seem to present abnormal mtDNA content. The replication of the mitochondrial genome is dependent on the nuclear-encoded DNA polymerase gamma (POLG) and mitochondrial transcription factor A (TFAM). However, the expression of these factors has never been accessed in mature sperm. In the present work, whether POLG and TFAM could have functional roles in post-ejaculatory human sperm mtDNA has been determined.

Sperm samples were categorized as: normozoospermic, samples with one or two abnormal sperm parameters and oligoasthenoteratozoospermics (OATs). These samples were analysed by real-time PCR for mtDNA copy number and immunocytochemistry and western blotting for patterns of expression for POLG, TFAM and two cytochrome c oxidase (COX; the Complex IV of the ETC) subunits: the mtDNA encoded COXI and the nuclear DNA encoded COXVIc.

Paradoxically, good-quality sperm had fewer mtDNA copies but significantly more sperm expressing POLG, TFAM, COXI and COXVIc. On the other hand, the OAT group presented significantly higher mtDNA content and a lower percentage of sperm expressing POLG, TFAM, COXI and COXVIc.

The reduction in mtDNA content in normal samples likely reflects normal spermiogenesis, whereas increases in POLG and TFAM expression possibly compensate for the low mtDNA content, maintaining mitochondrial homeostasis.

1. Introduction

Mitochondria are dynamic organelles that provide eukaryotic cells with energy. The number of mitochondria per cell is variable, depending on the cell's requirement for ATP (Moyes *et al.*, 1998). In mature mammalian sperm, there are between 22 and 75 mitochondria per sperm localised to the midpiece (Otani *et al.*, 1988), where they provide energy for several proposes, including flagellar propulsion, and thus sperm motility. Mitochondria generate ATP through oxidative phosphorylation (OXPHOS) which takes place within the electron transfer chain (ETC) and is far more productive than anaerobic generation of ATP (Pfeiffer *et al.*, 2001). The importance of OXPHOS-derived ATP for sperm motility has been demonstrated through classical mitochondrial inhibitor experiments (see Chapter I).

The ETC is the only cellular apparatus that is encoded by both the chromosomal and mitochondrial genomes. Human mtDNA, which tends to be maternally inherited (Giles *et al.*, 1980, see Chapter I), is approximately 16.6 kb in size and encodes 13 of the polypeptides contributing to the ETC along with 22 tRNAs and 2 rRNAs (Anderson *et al.*, 1981). Mutation, deletion or depletion to any of these genes is associated with a range of mitochondrial diseases which can both impair cellular function and/or can be lethal (see Chapter I).

A series of mtDNA rearrangements are associated with male infertility. For instance, the maternally inherited A3243G point mutation has been identified as the cause of poor sperm motility (asthenozoospermia) in a male presenting mtDNA disease (Spiropoulos *et al.*, 2002). In a similar case, addition of metabolic supplements to bypass a point mutation causing reduced activity of the ETC Complex I increased sperm motility three-fold (Folgero *et al.*, 1993). Single large-scale mtDNA deletions have also been associated with male infertility (Kao *et al.*, 1995; 1998), although this association was not verified by others (Cummins *et al.*, 1998; Reynier *et al.*, 1998; St John *et al.*, 2001). However, an accumulation of multiple

deletions in poorer quality sperm samples has also been demonstrated (St John *et al.*, 2001), with a greater proportion of wild-type molecules being present in higher density gradient fractions (corresponding to better quality subpopulations) than in lower density ones (O'Connell *et al.*, 2003).

In addition to other events, mammalian spermatogenesis is characterized by morphological modifications of mitochondria, reflecting the permanent change in the testicular microenvironment (De Martino *et al.*, 1979). Concurrently, testis-specific mitochondrial proteins are expressed (reviewed in Meinhardt *et al.*, 1999), including, for example, cytochrome C_T (Goldberg et al., 1977) and COXVIb-2 (Huttemann *et al.*, 2003). Moreover, during spermiogenesis, some mitochondria are lost in the residual bodies, while the remaining undergo subcellular reorganization (see Chapter I). Likewise, the number of mitochondrial molecules per haploid genome decreases 8- to 10-fold between meiosis and the end of spermiogenesis in mice (Hecht *et al.*, 1984). This mtDNA down-regulation has been confirmed by others, not only in mice (Larsson *et al.*, 1996), but also in human (Larsson *et al.*, 1997) and rat (Rantanen *et al.*, 2001) spermatogenesis. Despite this, and at least in mice, testicular mtDNA seems to be synthesised during meiosis and early spermiogenesis (Hecht and Liem, 1984). As mtDNA synthesis terminates in mid-spermiogenesis, the mtDNA found in mature sperm may have been synthesised in spermatids or earlier.

It is evident that there is variability in the number of mtDNA molecules in different populations of human sperm with a tendency towards a greater proportion being present in poorer quality samples (Diez-Sanchez *et al.*, 2003a; May-Panloup *et al.*, 2003; Song and Lewis, 2008). The vast range in mtDNA content between these reports tends to be technique dependent although one other study contradicts this trend but still reports significant differences between good and poor quality samples (Kao *et al.*, 2004). However, these

outcomes suggest that an association may exist between mtDNA copy number, sperm quality and regulation of mtDNA replication.

The replication of mtDNA is controlled by nuclear-encoded replication factors that are translocated to the mitochondria (see Chapter I). Both the mitochondrial-specific DNA polymerase gamma (POLG; Ropp and Copeland, 1996) and mitochondrial transcription factor A (TFAM; Fisher and Clayton, 1985; 1988) are required.

POLG comprises two subunits: a catalytic subunit (POLG), with both polymerase and 3'to 5'exonuclease activity, and an accessory subunit (POLG2), which confers processivity. The importance of POLG has certainly been documented using mice models. To this extent, homozygous knockin mice expressing a proof-reading deficient POLG presented increased levels of mtDNA point mutations and deletions, and showed reduced lifespan and premature onset of aging-related phenotypes, including reduced fertility (Trifunovic *et al.*, 2004). Furthermore, POLG homozygous knockout mice died during early embryonic development with severe mtDNA depletion (Hance *et al.*, 2005). In humans, mutations in *POLG* are associated with the accumulation of mtDNA deletions in diseases such as progressive external ophtalmoplegia and Parkinsonism and premature menopause; and with mtDNA depletion syndromes such as Alper's disease (see Chapter I). Polymorphisms in the CAG-repeat region of *POLG* are possible associated with male infertility (see Chapter III). However, the expression of POLG in the testis or in mature sperm has never been determined.

TFAM is a high mobility group protein that activates mtDNA transcription in mammals (Parisi and Clayton, 1991; Garstka *et al.*, 1994) and, consequently, the generation of the RNA primer necessary for the initiation of mtDNA replication (see Chapter I). Mice knockout studies have suggested that TFAM is a regulator of mtDNA copy number with heterozygous knockouts displaying decreased mtDNA content and OXPHOS deficiency of the heart. Homozygous knockout embryos exhibited severe mtDNA depletion, loss of

OXPHOS, and died post-gastrulation (Larsson *et al.*, 1998). In the human, low levels of TFAM expression have been associated with mtDNA-depletion syndromes, such as infantile mitochondrial myopathy and encephalomyophathy disorders (see Chapter I). In human, mouse and rat, testis-specific *TFAM* transcripts result in down-regulation of TFAM protein levels in mitochondria, as they either encode a nuclear protein isoform (mouse - Larsson *et al.*, 1996) or are not translated (human - Larsson *et al.*, 1997; rat - Rantanen *et al.*, 2001). This has been suggested to account for the reduction in mtDNA copy number that occurs during mammalian spermatogenesis. Consequently, one could speculate that TFAM is poorly regulated during spermatogenesis in patients presenting a greater number of sperm mtDNA molecules. Moreover, this could also be the case for POLG.

In the present work, it has been sought to determine whether mtDNA copy number is uniformly regulated in different quality sperm samples, and whether there is a relationship between mtDNA copy number and the expression of POLG and TFAM. These outcomes were then assessed in terms of frequency of two ETC proteins and its presence in varying populations of sperm.

2. Materials and methods

Chemicals

All chemicals were supplied by Sigma Chemical Company (St. Louis, MO, USA), unless stated otherwise.

Biological Material

All patients were recruited from the Fertility Clinic (University Hospitals of Coimbra, Portugal) and were undergoing routine semen analysis or fertility treatment involving both *in vitro* fertilisation (IVF) and intracytoplasmatic sperm injection (ICSI). They signed informed

consent forms, and all human material was used in accordance with the appropriate ethical and Internal Review Board (IRB) guidelines provided by the University Hospitals of Coimbra.

Fresh semen samples were obtained by masturbation after 3 to 5 days of sexual abstinence and routine seminal analysis was performed according to the World Health Organization Guidelines (WHO, 1999). In order to isolate sperm from both seminal plasma and round cells, semen samples were prepared by density gradient centrifugation using ISolate® Sperm Separation Medium (Irvine Scientific, Santa Ana, CA, USA), according to the manufacturer's protocol. Samples were categorized based on sperm concentration, motility and morphology. For morphology analysis semen smears were prepared on microscopy slides and then treated with the Diff–Quik stain set (Dade Behring Inc., Newark, USA). Sperm morphology was assessed using the strict criteria (Kruger *et al.*, 1986). Samples were categorised as: normal (normozoospermic samples, *i.e.* concentration $\geq 20 \times 10^6$ sperm/ml, $\geq$ 50% motile sperm, $\geq$ 15% normal forms); 1- or 2-defects (samples with one or two abnormal parameters) or OAT (oligoasthenoteratozoospermic samples, *i.e.* samples with low sperm concentration, motility and morphology), according to WHO (1999) criteria.

Extraction of total DNA

As samples were prepared using density gradient centrifugation, it was able to eliminate the possibility of contaminating somatic cells. However, before DNA extraction, the absence of contaminating cells was confirmed by light microscopy. DNA from isolated sperm samples was extracted according to the whole blood DNA isolation protocol using the Puregene DNA Isolation Kit (Flowgen, Nottingham, UK) supplemented with 1.5 µl of 20 mg/ml Proteinase K and 12 µl of 1 mol/l DTT and incubated overnight at 55˚C, as described previously (St John *et al.*, 2001). The addition of DTT, not generally used in DNA extraction

procedures for other cell types, is used in sperm, both to break nucleus disulphide bounds, and to dissociate mitochondria from the mitochondrial sheath (Sutovsky *et al.*, 1997).

mtDNA/Beta-Globin quantification

For mtDNA quantification, an external standard of 152 bp PCR product was generated, using primers D41 and D56 (Reynier *et al.*, 2001, see Table I). Reaction conditions were as follows: initial denaturation at 94˚C for 3 min, 35 cycles of 94˚C for 30 sec, 53˚C for 30 sec and 72˚C for 1 min, and final extension at 72˚C for 3 min. In order to determine the number of sperm in each sample, quantification of the housekeeping gene Beta-Globin was also carried out. An external standard of 268 bp PCR product was generated, using primers previously described (Wykes and Krawetz, 2003, see Table I) with the following reaction conditions: initial denaturation at 95˚C for 5 min, 35 cycles of 95˚C for 30 sec, 56.5˚C for 30 sec and 72˚C for 1 min, and final extension at 72˚C for 5 min. For both genes, the 25 µl final volume reaction mixture contained 50 ng of total DNA, 1 x PCR Buffer (BioLine, London, UK), 1.5 mM $MgCl_2$ (BioLine), 200 µM dNTPs (BioLine), 0.5 µM of each primer and 2.5 U BioTaq DNA polymerase (BioLine). PCR products were run on 1% agarose gels, and products size confirmed against a 100 bp DNA ladder (Bioron, Ludwigshafen, Germany). Standards were purified using the QIAquick Gel Extraction Kit (Qiagen, London, UK), as described in the manufacturer's protocol. After spectrophotometer quantification, a series of 10-fold dilutions were prepared to generate a standard curve for each gene. It was considered that the number of molecules of double-stranded DNA present in 1 ng of a 152 bp fragment (mtDNA) was 6×10^9 molecules, and that 1 ng of a 268 bp fragment (Beta-Globin) contained 3.4×10^9 double-stranded DNA molecules.

Real-time PCR was performed using a 72-well Rotorgene-3000™ 4 Channel Multiplexing System machine and analysed with version 6 software (Corbett Research, Mortlake, NSW, Australia). PCR mixtures were prepared using a CAS-1200 Robotic Liquid

Handling System (Corbett Robotics, Queensland, Australia). Reaction mixtures (15 µl final volume) contained 7.5 µl 2x SensiMix (2x SensiMix DNA Kit, Quantace, London, UK), 0.3 µl 50x SYBR® Green solution (2x SensiMix DNA Kit), 330 µM each primer (see Table I) and 2 µl of each standard (range: 2 x 10^{-2} to 2 x 10^{-8} ng/µl) or 2.5 ng/µl DNA samples solutions. Each reaction was run in triplicate both for standards and samples, as well as for negative controls. An aliquot from a positive control was used in triplicate on each occasion to determine the repeatability of each reaction. Each reaction was repeated at least once.

Table I. Primers used for mtDNA quantification.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing Temp (ºC)	Product size (bp)
mtDNA	cga aag gac aag aga aat aag g	ctg taa agt ttt aag ttt tat gcg	53	152
Beta-Globin	caa ctt cat cca cgt tca cc	gaa gag cca agg aca ggt ac	56.5	268

Accession numbers are as follows: J01415 for mtDNA, and V00499 for Beta-Globin.

For mtDNA, reactions were performed as follows: initial denaturation at 95˚C for 10 min, followed by 45 cycles with denaturation at 95˚C for 10 sec, annealing at 53˚C for 10 sec, and extension at 72˚C for 15 sec. For Beta-Globin, reactions consisted of an initial denaturation at 95˚C for 10 min, 45 cycles with denaturation at 95˚C for 15 sec, annealing at 56.5˚C for 15 sec, and extension at 72˚C for 20 sec. Data were acquired in the FAM/Sybr channel during the extension phase. For each of the 45 cycles, a step of 76˚C for 15 sec (for mtDNA) and 81˚C for 20 sec (for Beta-Globin) with data acquisition was added, in order to exclude fluorescence generated by potential primer-dimers. Melt curve analysis was performed by ramping from 65^{o}C to 99^{o}C at 1^{o}C intervals and data acquired from the FAM/Sybr channel.

Only reactions with high efficiency (> 88%) were considered, and only standard curves with a Pearson correlation coefficient of at least 0.99 were taken into account. As each

sample was run in triplicate on two separate occasions, six values were considered for each sample. To correct for pipetting errors, the highest and lowest values obtained were discarded, and the remaining four were averaged (Bustin, 2000). The standard deviation and coefficient of variation (CV) were also calculated and only values with low CVs (maximum 10%) were considered reliable.

POLG, TFAM, COXI and COXVIc detection

Immunocytochemistry (ICC)

Immunocytochemistry for individual proteins was performed separately using the following primary antibodies: rabbit anti-human POLG polyclonal antibody (ab2969, Abcam, Cambridge, UK), goat anti-human TFAM polyclonal antibody (sc-19050, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human COXI monoclonal antibody (A-6403, Molecular Probes, Eugene, OR, USA) and mouse anti-human COXVIc monoclonal antibody (A-6401, Molecular Probes). For secondary staining, Alexa Fluor 488 goat anti-rabbit IgG (A-11008, Molecular Probes), Alexa Fluor 488 donkey anti goat IgG (A-11055, Molecular Probes) and AlexaFluor 488 goat anti-mouse IgG (A-1101, Molecular Probes) antibodies were used. A general procedure was performed as described previously (Ramalho-Santos *et al.*, 2000), with minor modifications. Sperm were attached to coverslips, fixed for 40 min with 2% (v/v) formaldehyde in phosphate-buffered saline (PBS; pH 7.2) and permeabilised in 1% Triton X-100 (v/v) in PBS for 20 min at room temperature. Non-specific antibody reactions were blocked by 30 min incubation in PBS containing 1 mg/ml BSA and 100 mM glycine. Samples were then incubated with the primary antibody solubilised in the blocking solution at 1:200, 1:50, 1:500 and 1:200 dilutions for POLG, TFAM, COXI and COXVIc, respectively, overnight, at 37° C. Control experiments without primary antibodies were also performed. Coverslips were washed for 30 min in PBS containing 0.1% Triton X-

100 (v/v), and then incubated with the appropriate secondary antibody at a 1:200 dilution in blocking solution for 40 min at 37º C. After rinsing again, the coverslips were mounted on a microscope slide in a drop of Vectashield mounting medium containing the nucleic acid-stain 4',6 diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. Slides were examined using a Zeiss Axiophot II microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter and 200 sperm per coverslip were counted in at least four different fields, in order to determine the percentage of stained sperm.

Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Sperm pellets were ressuspended in lysis buffer (25 mM TRIS, 1 M NaCl, 1% Triton, 1 mM CLAP, 0.2 mM PMSF, and 0.01 mM DTT) and briefly sonicated. After 1 hour, the clear supernatants obtained following centrifugation were diluted in denaturing solution (1:2 dilution) and incubated at 95˚C for 5 min. Samples were run on sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Roche Diagnostics Corporation, IL, USA) overnight in transferring buffer [190 mM TRIS, 25 mM glycine, 20% (v/v) methanol]. Membranes were blocked with TRIS Buffer Saline also containing 0.1% (v/v) Tween 20 (TBST) and 5% skim dried milk for 45 min and incubated overnight at room temperature with the primary antibodies described above (*Immunocytochemistry* section), diluted in TBST. After washing 5 times for 5 min each in TBST, the blots were incubated for 1 h at room temperature with the appropriate (anti-rabbit, anti-goat or anti-mouse) alkaline phosphatase conjugated antibody (Amersham Biosciences UK Limited, Buckingamshire, UK), 1:20000 diluted in TBST. The bands were developed using the ECF-substrate system, according to the manufacturer's recommendations (Amersham).

Statistical analysis

Statistical analysis was carried out using SPSS for Windows (version 13, Chicago, IL, USA). All variables were checked for normal distribution using the one-sample Kolmogorov-Smirnov test. For ICC and mtDNA copy number analysis, the different groups were compared using the one-way ANOVA test (or the Kruskal-Walli's non parametric test when distributions were not normal). Post-Hoc analyses were done using Tukey's test or Dunnet's test, depending on whether equal variances were assumed or not. Pearson's and Spearman's tests, for parametric and non-parametric data, respectively, were applied to assess the correlations of the principal semen parameters (concentration, motility and morphology) with both ICC and mtDNA copy number results, as well as between ICC outcomes and mtDNA copy numbers. $P < 0.05$ was considered significant.

3. Results

Assessment of mtDNA copy number

In order to determine whether sperm mtDNA was distributed evenly amongst sperm of differing qualities, the number of copies of mtDNA per sperm was quantified in 42 samples comprising the three patient groups (normal group, 1- or 2-defects group, and OAT group). The mean number of mtDNA copies per haploid genome was determined by the ratio of mtDNA to Beta-Globin molecules through real-time PCR. The mean (± SEM) mtDNA copy number for all samples analysed was 21.6 ± 5.8 (range 0.6 - 206.1). Comparing the three groups individually (Table II; Fig. 1), it is evident that mtDNA copy number per sperm increases as sperm quality decreases. To this extent, the normal group possessed 6.8 ± 1.8 mtDNA copies per sperm and the 1- or 2-defects group 11.3 ± 4.5, whereas the OAT group possessed 46.7 ± 15.0. The OAT group was statistically different from both the normal group ($P = 0.001$) and the 1- or 2-defects group ($P < 0.05$). Moreover, there was a significant

negative correlation between the number of mtDNA molecules per sperm and sperm concentration (R = -0.561, $P < 0.001$) and morphology (R = -0.467, $P = 0.002$). The same was not verified for motility, although there was a tendency towards this (R = -0.285, $P = 0.067$).

Table II. Mean mtDNA copy number for the normal, 1- or 2-defects and OAT groups. SEMs and ranges are also indicated.

	Normal group (n=14)	**1- or 2-Defects group (n=14)**	**OAT group (n=14)**	**Total (n=42)**
Mean ± SEM	6.8 ± 1.8^a	11.3 ± 4.5^a	46.7 ± 15.0^b	21.6 ± 5.8
Range	0.2 – 22	0.6 – 63.8	0.7 – 206.1	0.2 – 206.1

The mean mtDNA copy number for all the samples analysed is indicated (Total). Superscripts *a* and *b* denote a significant difference between groups (normal *vs.* OAT, $P < 0.01$; 1- or 2-defects *vs.* OAT, $P < 0.05$).

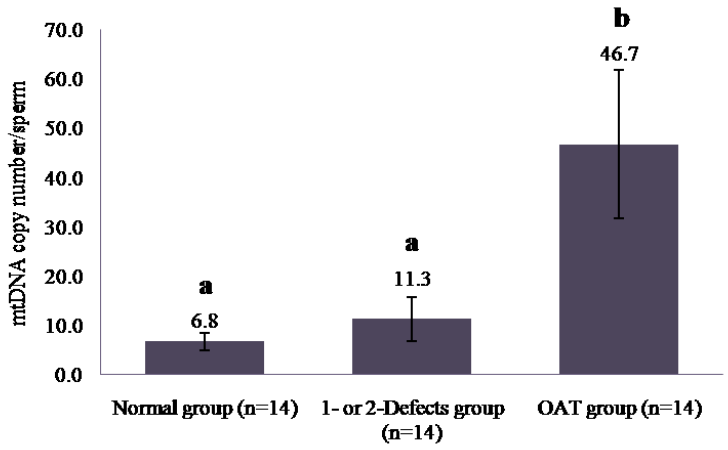

Figure 1. MtDNA copy number per sperm in the normal, 1- or 2-defects and OAT groups. The mtDNA copy number is significantly higher in the OAT group (*a* and *b* denote statistically significant differences: normal *vs.* OAT, $P < 0.01$; 1- or 2-defects *vs.* OAT, $P < 0.05$).

Expression and localisation of POLG

To determine whether POLG persisted in mature sperm having completed spermiogenesis and thus mtDNA replication, POLG expression was analysed using ICC, in 93 sperm samples, comprising the three patient groups. As anticipated, POLG localised to the

midpiece, the compartment housing the mitochondria (Fig. 2a), and POLG expression was confirmed by western blotting (Fig. 2c). ICC demonstrated that most of the sperm in each of the samples were stained in the midpiece (mean % ± SEM = 73.0 ± 1.6), whereas the remainder presented no staining. Interestingly, in almost all samples, some sperm were also stained in a ring-like structure localised between the head and the midpiece (referred here as the 'ring'; Fig. 2b; mean % ± SEM = 29.3 ± 2.5). The mean percentage of sperm stained in the midpiece (mean % ± SEM = 79.3 ± 2.3 for the normal group; 72.9 ± 2.3 for the 1- or 2-defects group; and 62.8 ± 2.8 for the OATs) was significantly lower in the OAT group when compared with the other 2 groups (normal *vs.* OAT, $P < 0.01$; 1- or 2-defects *vs.* OAT, $P < 0.05$; Fig. 2d). In relation to the mean percentage of sperm stained both in the midpiece and the ring (mean % ± SEM = 35.9 ± 4.5, 30.6 ± 3.5 and 14.9 ± 3.8 in the normal, 1- or 2-defects and OAT groups, respectively), the OAT group was also significantly different from the other groups (normal *vs.* OAT and 1-or 2-defects *vs.* OAT, $P < 0.05$; Fig. 2e).

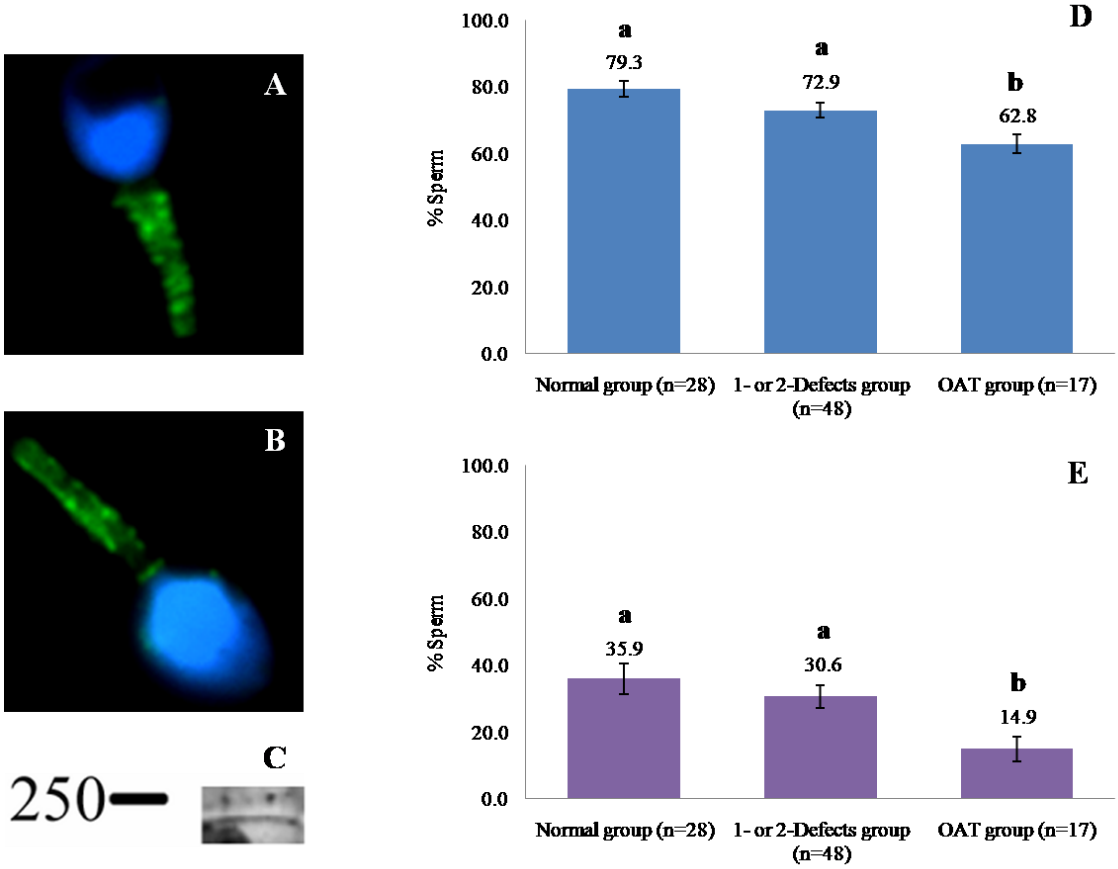

Figure 2. Expression and localisation of POLG in human sperm. **A, B)** POLG expression and localisation detected by immunocytochemistry (ICC). Human sperm express POLG in the midpiece (A, B - green) and in a ring structure localised between the head and the midpiece (B - green). Nuclear DNA is stained with DAPI (blue). **C)** POLG expression detected by western blotting. The molecular weight of human POLG is 140 kDa (right). A ladder band of 250 kDa is also shown (left). **D)** Percentage sperm stained in the midpiece using an anti-human POLG antibody (ICC) in the normal, 1- or 2-defects and OAT groups. Data are expressed as means ± SEM. The mean percentage of sperm stained in the midpiece is significantly lower in the OAT group. *a* and *b* denote significant differences (normal *vs.* OAT, $P < 0.01$; 1- or 2-defects *vs.* OAT, $P < 0.05$). **E)** Percentage sperm stained in the midpiece and the ring using an anti-human POLG antibody (ICC) in the normal, 1- or 2-defects and OAT groups. Data are expressed as means ± SEM. The mean percentage sperm stained in the midpiece and the ring is significantly lower in the OAT group. *a* and *b* denote significant differences (normal *vs.* OAT and 1- or 2-defects *vs.* OAT, $P < 0.05$).

Expression and localisation of TFAM

As the initiation of mtDNA replication depends on transcription having occurred previously, the expression and localisation of TFAM in mature human sperm was also investigated. In the 47 samples analysed, the majority of sperm from each sample were stained in the midpiece (mean % ± SEM = 65.6 ± 2.5; Fig. 3a), whereas the remaining all showed no staining. TFAM expression was confirmed by western blotting (Fig. 3b). Comparison between the three groups revealed that the percentage of sperm stained in the midpiece decreased from the normal group (mean % ± SEM = 72.8 ± 4.2; Fig. 3c) to the 1- or 2-defects group (64.2 ± 3.6) to the OATs (54.9 ± 4.4). The OAT group was significantly different from the normal group ($P < 0.05$).

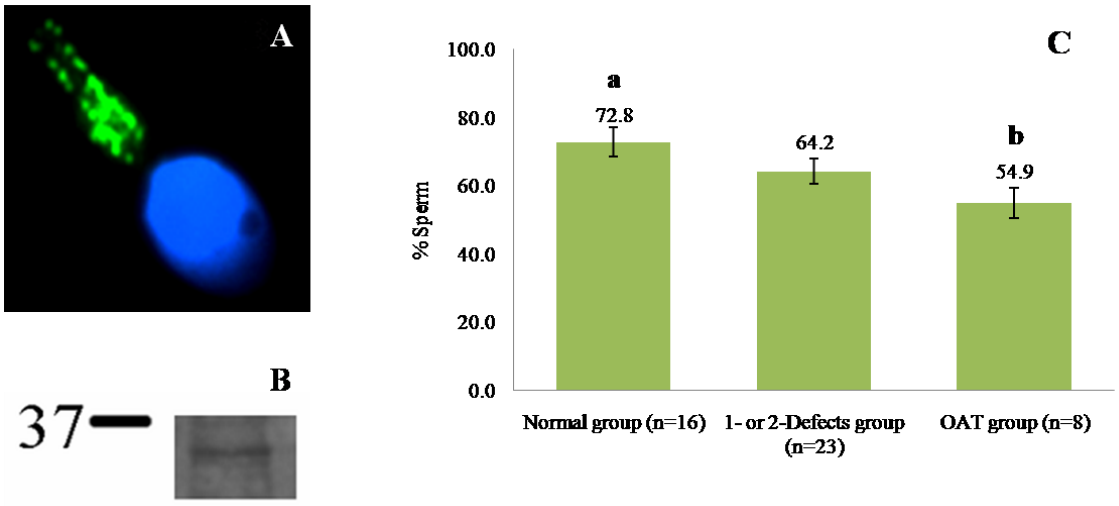

Figure 3. Expression and localisation of TFAM in human sperm. **A)** TFAM expression and localisation detected by immunocytochemistry (ICC). Human sperm express TFAM in the midpiece (A - green). Nuclear DNA is stained with DAPI (blue). **B)** TFAM expression detected by western blotting. The molecular weight of human TFAM is 25 kDa (right). A ladder band of 37 kDa is also shown (left). **C)** Percentage of sperm stained in the midpiece using an anti-human TFAM antibody (ICC) in the normal, 1- or 2-defects and OAT groups. Data is expressed as means ± SEM. The mean percentage of sperm stained in the midpiece is significantly lower in the OAT group. *a* and *b* denote significant differences (normal *vs.* OAT, $P < 0.05$).

Expression of mtDNA and nuclear-encoded genes of the ETC

To determine whether the expression of ETC proteins would be influenced by mtDNA copy number and by the expression of mtDNA replication factors, the expression of two cytochrome c oxidase (COX; the Complex IV of the ETC) subunits, COXI (mtDNA encoded) and COXVIc (nuclear-encoded) were examined. A total of 92 and 78 samples were analysed for COXI and COXVIc, respectively. Both antibodies stained the midpiece of the majority of sperm in each sample (mean % ± SEM = 79.0 ± 1.3 for COXI and 65.5 ± 2.5 for COXVIc; Figs. 4a, 4a'). The OAT group (mean % ± SEM = 68.5 ± 3.7) was distinct from the other two in the mean percentage of sperm expressing COXI in the midpiece (84.4 ± 1.7 for normals; 79.2 ± 1.7 for the 1- or 2-defects), representing significant differences to the normal ($P < 0.001$) and the 1- or 2-defects ($P < 0.01$) groups (Fig. 4c). On the other hand, the three groups were distinct from each other for COXVIc staining in the midpiece (mean % ± SEM = 75.3 ± 4.5 for the normal group; 67.0 ± 3.1 for the 1- or 2-defects; and 45.5 ± 5.2 for the OAT group; normal *vs.* OAT, $P < 0.001$; 1- or 2-defects *vs.* OAT, $P < 0.01$; normal *vs.* 1-or 2-defects, $P < 0.05$; Fig. 4c'). Differences in the expression of both COXI and COXVIc between the OAT and normal groups were also confirmed by western blotting (Figs 4b, 4b').

Correlation of individual sperm parameters with POLG, TFAM, COXI and COXVIc expression

To understand if the expression of POLG, TFAM, COXI and COXVIc were correlated with sperm quality, the three principal sperm parameters (concentration, motility and morphology) were analysed for the samples used to determine protein expression by ICC. To this extent, a positive correlation was found between the mean percentage of sperm expressing POLG, TFAM, COXI and COXVIc, as determined by ICC, and each of the three principal sperm parameters (Table III).

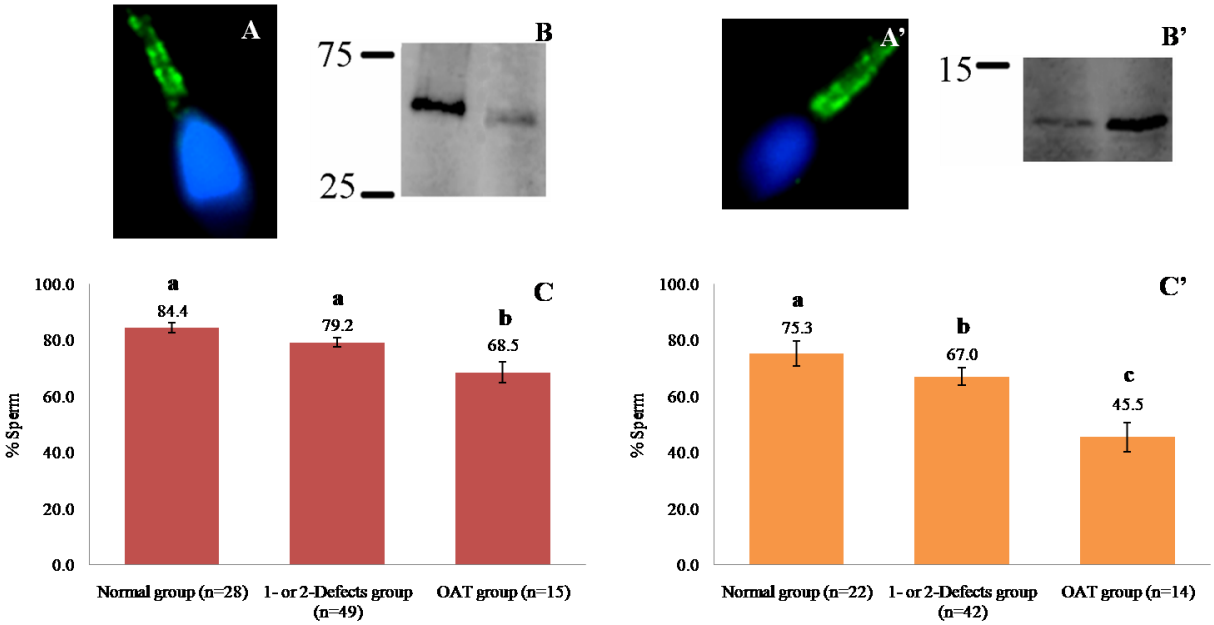

Figure 4. Expression and localisation of COXI and COXVIc in human sperm. **A, A')** COXI and COXVIc expression and localisation detected by immunocytochemistry (ICC). Human sperm express COXI (A - green) and COXVIc (A' – green) in the midpiece. Nuclear DNA is stained with DAPI (blue). **B, B')** COXI and COXVIc expression in a normal and an OAT sample detected by western blotting. B) The molecular weight of human COX 1 is 57 kDa. Two ladder bands of 25 and 75 kDa are shown. The normal sample (left band) presents a higher level of expression of COXI than the OAT sample (right band). B') The molecular weight of human COXVIc is 8.8 kDa. A ladder band of 15 kDa is also shown. The normal sample (right band) presents a higher level of expression of COXVIc than the OAT sample (left band). **C, C')** Percentage sperm stained in the midpiece using anti-human COXI and anti-human COXVIc antibodies (ICC) in the normal, 1- or 2-defects and OAT groups. Data are expressed as means ± SEM. C – COXI) The mean percentage sperm stained in the midpiece is significantly lower in the OAT group. *a* and *b* denote significant differences (normal *vs.* OAT, $P < 0.001$, 1- or 2-defects *vs.* OAT, $P < 0.01$). C' – COXVIc) The mean percentage sperm stained in the midpiece is significantly lower in the 1- or 2-defects and OAT groups. *a*, *b* and *c* denote significant differences (normal *vs.* 1- or 2-defects, $P < 0.05$, normal *vs.* OAT, $P < 0.001$, 1- or 2-defects *vs.* OAT, $P < 0.01$).

Table III. Values for the correlations (R) between the percentages of sperm stained with POLG, TFAM, COXI and COXVIc antibodies and the three principal sperm parameters.

	POLG (n= 93)	**TFAM (n=47)**	**COXI (n=92)**	**COXVIc (n=78)**
Sperm concentration	R = 0.318 ($P < 0.01$)	R = 0.335 ($P < 0.05$)	R = 0.322 ($P < 0.01$)	R = 0.388 ($P < 0.001$)
Sperm motility	R = 0.304 ($P < 0.01$)	R= 0.323 ($P < 0.05$)	R = 0.294 ($P < 0.01$)	R = 0.343 ($P < 0.05$)
Sperm morphology	R = 0.436 ($P < 0.001$)	R = 0.302 ($P < 0.05$)	R = 0.430 ($P < 0.001$)	R = 0.403 ($P < 0.001$)

POLG and TFAM expression and mtDNA copy number

As both POLG and TFAM are involved in mtDNA replication, and TFAM is believed to be a regulator of mtDNA copy number, the relationship between the expression of these proteins and mtDNA copy number was determined. Paradoxically, a negative correlation was found between the percentage of sperm expressing POLG and mtDNA content (R = -0.557, $P = 0.01$). However, and unexpectedly, no correlation was found between TFAM expression and mtDNA copy number ($P = 0.548$).

mtDNA copy number and COXI and COXVIc expression

As COXI is mtDNA encoded, it could be expected that cells with higher mtDNA content would express more COXI. However, a negative correlation was found between mtDNA copy number and the percentage of cells expressing COXI (R = -0.566, $P = 0.01$). As COXI and COXVIc are two subunits of the same complex, mtDNA content could influence COXVIc expression. However, no correlation was observed between mtDNA copy number and the percentage sperm expressing COXVIc ($P = 0.098$).

4. Discussion

The long standing debate concerning the nature of the ATP generated for sperm function, *i.e.* on the relative importance of glycolysis *versus* OXPHOS, has been often confounded by possible changes during the male gamete lifetime, as well as by species-specific differences (for recent reviews see Ford, 2006; Ruiz-Pesini *et al.*, 2007). However, in humans it has been well established that sperm require mitochondrial ATP generated through OXPHOS (Ruiz-Pesini *et al.*, 1998; 2000a; 2000b). It would thus be expected that good quality sperm express a significant amount of ETC proteins, when compared to low quality (OAT) samples, and indeed, the present study has shown that this is the case, both for mitochondrial (COXI) and nuclear-encoded proteins (COXVIc). In accordance, the expression of each of the two COX subunits was found to positively correlate with each of the three principal sperm parameters. These results imply the existence of a functional mtDNA complement in normal sperm, and, indeed, the analysis of samples from men with maternally inherited mtDNA mutations revealed decreased sperm motility (Folgero *et al.*, 1993; Spiropoulos *et al.*, 2002). Likewise, a patient with maternally inherited large-scale mtDNA deletions presented oligozoospermia (Lestienne *et al.*, 1997), whereas OAT patients appear to have acquired a higher number of spontaneously derived large-scale deletions (St John *et al.*, 2001; O'Connell *et al.*, 2003). These outcomes have been supported by the recent finding that a pathological maternally inherited mtDNA deletion was responsible for male infertility in a transmitochondrial mouse model (Nakada *et al.*, 2006).

Paradoxically, the expression of a mtDNA-encoded ETC protein (COXI) was inversely correlated with mtDNA content, while no correlation was found for a nuclear-encoded ETC protein (COXVIc). This suggests that other factors are affecting COXVIc expression, that, at least in rat, seems to be regulated by nuclear respiratory factors 1 and 2 (NRF1- Evans and Scarpulla, 1990; NRF2- Ongwijitwat *et al.*, 2006). However, it has also

been shown that the expression of ETC proteins (both mitochondrial- and nuclear-encoded) increases as sperm quality increases, while the opposite pattern occurred with mtDNA content. Thus, while human sperm possess a range of mtDNA molecules, OAT samples have a significantly higher content compared to either the normal group or the 1- or 2-defects group. This is in accordance with previous work demonstrating that sperm from poorer quality semen samples are also indicative of higher numbers of mtDNA copy (Diez-Sanchez *et al.*, 2003a; May-Panloup *et al.*, 2003; Song and Lewis, 2008). In agreement, a negative correlation seems to exist between mtDNA content and sperm concentration (present study; Song and Lewis, 2008), motility (Diez-Sanchez *et al.*, 2003a) and morphology (this study). These outcomes imply that there are errors in the regulation of mtDNA replication in men who will ultimately present poor quality sperm. The high mtDNA content in OAT sperm may therefore be an indicator of defective spermiogenesis, and may also reflect problems in energy metabolism in both testicular cells and mature male gametes.

TFAM not only functions as a transcription factor but, as it activates the generation of the primer necessary for mtDNA replication, is thought to also regulate mtDNA copy number (Ekstrand *et al.*, 2004). To this extent, a number of investigations indicate a positive correlation between mtDNA content and TFAM levels in various mammalian cells (Larsson *et al.*, 1994; Poulton *et al.*, 1994; Davis *et al.*, 1996; Li *et al.*, 2000; Seidel-Rogol and Shadel, 2002; Joseph *et al.*, 2004; Kanki *et al.*, 2004). This is perhaps best exemplified during the final stages of murine spermatogenesis. Here, as meiosis II is completed, creating a haploid chromosomal complement, mtDNA content is reduced (Hecht *et al.*, 1984). This is concurrent with the expression of an isoform of TFAM lacking the mitochondrial targeting sequence and thus preventing its translocation to the mitochondrion (Larsson *et al.*, 1996). In humans, TFAM is simply down-regulated in testicular sperm (Larsson *et al.*, 1997), most likely to match the fewer copies of mtDNA present. However, the present work shows that,

not only is TFAM present in mature ejaculated human sperm, but, unlike events that take place in other cell types, more TFAM is present in high quality sperm samples, which have lower mtDNA content. This is also supported by the abundance of TFAM transcripts in mature ejaculated sperm from fertile men (Zhao *et al.*, 2006). Likewise, overexpression of TFAM in cultured human cells resulted in outcomes similar to TFAM knockdown, namely in mtDNA depletion (Pohjoismaki *et al.*, 2006). The results presented here would indicate a high transcription rate per mtDNA molecule in normal sperm, to ensure that mtDNA-encoded proteins (such as COXI) are produced in sufficient amounts, overcoming the low mtDNA content. The consistent expression of COXVIc, a nuclear-encoded ETC protein, in theses same samples, would suggest that this is a highly regulated process. Alternatively, sperm TFAM, COXI and COXVIc may have been produced during spermatogenesis, prior to the mtDNA copy number decrease. It should however be mentioned that there are currently no data to support any of these hypotheses. Another interesting possibility is that TFAM could also be packaging sperm mtDNA, similar to what happens in somatic cells (Alam *et al.*, 2003). This, in good-quality sperm, would ensure protection from oxidative stress induced DNA damage, which sperm mtDNA has a higher susceptibility for than the highly condensed chromosomal DNA (Sawyer *et al.*, 2003; Bennetts and Aitken, 2005).

Although TFAM has a role in the initiation of mtDNA replication by activating the synthesis of the primer for extension by POLG, mtDNA replication is highly dependent on this sole mtDNA polymerase being present. The role of POLG as a regulator of mtDNA copy number tends to be cell-type specific. For example, in some cultured human cells subjected to mtDNA depletion, the levels of POLG transcripts and protein have been shown to be similar to non-depleted cells, suggesting that POLG expression is constitutive, even in the complete absence of mtDNA (Davis *et al.*, 1996). Accordingly, in a cell line derived from a patient triploid for the *POLG* locus (SA15q-3), the increased levels of POLG transcripts, compared to

control cells, were not accompanied by an increase in mtDNA levels (Schultz *et al.*, 1998). On the other hand, inducible expression of a dominant negative POLG in HEK293 cells resulted in mtDNA depletion, whereas this was reversible following suppression of the mutator gene (Jazayeri *et al.*, 2003). Furthermore, POLG heterozygous knockout mice developed normally, despite a small reduction in mtDNA levels, while homozygous knockout embryos died between embryonic days 7.5 and 8.5 through severe mtDNA depletion (Hance *et al.*, 2005). Interestingly, overexpression of TFAM in POLG heterozygous knockout mice resulted in elevated mtDNA levels and increasing amounts of POLG transcripts, suggesting that POLG expression can be induced when up-regulation of mtDNA replication is required (Hance *et al.*, 2005). To this extent, the results of the present study show a negative correlation between mtDNA copy number and the percentage of sperm expressing POLG, even in the presence of TFAM. Consequently, these outcomes could be explained by the increased expression of POLG attempting to compensate for the low mtDNA content, as occurs in mtDNA depleted cells (Lloyd *et al.*, 2006). A similar mechanism takes place in early pre-implantation development of *in vitro* fertilised incompetent oocytes, where up-regulation of POLG and TFAM transcripts and proteins occurs in an attempt to rescue the low mtDNA levels (Spikings *et al.*, 2007). This rescue mechanism does not seem to be effective in mature sperm, possibly due to the intervention of other, as yet unidentified, regulatory factors. On the other hand, and as speculated for TFAM, COXI and COXVIc, sperm POLG may have been produced during spermatogenesis, at a stage prior to the reduction of mtDNA content.

Smaller mtDNA molecules seem to have a replicative advantage (Moraes, 2001) and this could be the case in spermatogenesis giving rise to OAT sperm, as OAT samples often present multiple large-scale mtDNA deletions (St John *et al.*, 2001). Consistent with this, there is a strong negative correlation between human sperm mtDNA copy number and mtDNA integrity (Song and Lewis, 2008). On the other hand, the high amount of mtDNA in

OAT sperm could be due to either: a) failure to reduce copy number; or b) continued replication. Either option would result in the down-regulation of POLG expression. Alternatively, POLG would simply not be up-regulated due to an excessive initial amount of mtDNA, explaining why POLG expression is lower in the OAT group. In any case, the low levels of TFAM, COXI and COXVIc in OAT samples suggest that spermatogenesis is severely impaired at the mitochondrial level.

In conclusion, it is evident that high quality sperm have very low levels of mtDNA compared to other fully differentiated somatic cells (Miller *et al.*, 2003). The possible biological significance of this finding could be to facilitate the elimination of paternal mtDNA following fertilisation and thus ensure maternal inheritance of this genome (Larsson *et al.*, 1997). This work also suggests that POLG, possibly in conjunction with other factors, may play a role in regulating mtDNA copy in human sperm: unlike TFAM, the presence of POLG seems to (negatively) predict mtDNA copy number. However, the mechanisms for this possible regulation remain undetermined. Furthermore, it became clear that the expression of mtDNA maintenance factors and of components of the ETC (either mtDNA- or nuclear DNA-encoded) is associated with sperm quality, which indirectly illustrates the importance of both mitochondria and its genome in human sperm function.

CHAPTER III

THE RELEVANCE OF POLG CAG REPEATS POLIMORPHISM IN HUMAN SPERM QUALITY

The results presented in this Chapter were originally published in:
Amaral, A., Ramalho-Santos, J. and St John, J. C. (2007) The expression of polymerase gamma and mitochondrial transcription factor A and the regulation of mitochondrial DNA content in mature human sperm. *Hum Reprod* **22** (6): 1585-1596.

Part of the methodology described in this Chapter has also been published in:
St John, J. C., Amaral, A., Bowles, E., Oliveira, J. F., Lloyd, R., Freitas, M., Gray, H.L., Navara, C.S., Oliveira, G., Schatten, G.P., Spikings, E. and Ramalho-Santos, J. (2006) The analysis of mitochondria and mitochondrial DNA in human embryonic stem cells. *Methods Mol Biol* **331**: 347-374.

Abstract

The gene for the catalytic subunit of human DNA polymerase gamma (POLG), the sole DNA polymerase present in mitochondria, possesses a CAG-repeat region of variable length, which encodes a polyglutamine tract. The potential effect of the absence of the common 10 CAG-repeat allele in sperm quality, and male infertility, has been the subject of a recent debate, with different studies presenting conflicting results. On the other hand, the functional significance of this polymorphism in human sperm has not been defined. In the present study, whether *POLG* CAG-repeat polymorphisms are associated with sperm quality, and also whether these can affect sperm mtDNA maintenance, have been investigated.

Sperm samples were categorised as: normozoospermics; samples with one or two abnormal sperm parameters; and oligoasthenoteratozoospermics (OATs). These samples were analysed by fluorescent PCR to determine the *POLG* CAG-repeat genotype; real-time PCR for mtDNA copy number; and immunocytochemistry for POLG expression.

The OAT group presented a significantly higher incidence of heterozygosity for the *POLG* CAG-repeat genotype. However, this polymorphism did not seem to affect each of the three sperm principal parameters, nor mtDNA copy number or POLG expression.

These results support, to some extent, the original findings that an association between sperm quality and *POLG* CAG repeats does exist. However, the biological significance of these variants in male infertility remains unclear, as these do not seem to affect mtDNA maintenance.

1. Introduction

In recent years, a number of studies have considered the possible association between certain genetic polymorphisms (gene sequence variants) and male infertility (for a recent review see Tuttelmann *et al.*, 2007). These include genes on the Y chromosome, as well as genes involved in endocrine regulation of spermatogenesis, just to give some examples. The role of a particular polymorphism in human sperm quality is often difficult to establish seeing that different outcomes are frequently obtained in independent studies. This is especially so because, in contrast with certain gene mutations that are definitely associated with spermatogenesis impairments, polymorphisms are only considered predisposing factors to infertility (Krausz and Giachini, 2007). This implies that their effect on sperm (dys)function most probably depends on other genetic and/or environmental factors. Despite this, understanding the role of gene polymorphisms in sperm quality is particularly important, since it is anticipated that most of the idiopathic infertility cases (*i.e.*, infertility with unknown causes) have a genetic origin.

Human sperm motility seem to depend on OXPHOS-derived ATP and hence, on mitochondria and mtDNA integrity. Indeed, mtDNA haplogroups, mutations and deletions have been associated with male infertility (see Chapter I). The integrity of the mitochondrial genome depends, among other factors, on the activity of DNA polymerase gamma (POLG, the sole DNA polymerase present in mitochondria). The catalytic subunit of POLG is expressed in the midpiece of ejaculated human sperm (where mitochondria are localised) and its expression seems to be indicative of sperm quality (see Chapter II, Amaral *et al.*, 2007).

Unlike other species, human *POLG* comprises a CAG-repeat region at its 5' end (see Fig. 7 in Chapter I) that gives rise to a polyglutamine tract (Ropp and Copeland, 1996). This region, usually 10 codons long (constituting the 'common allele'), is variable in length, with reports of alleles containing between 5 and 15 repeats (Rovio *et al.*, 1999; 2001; 2004). The

frequency of the 10 CAG-repeat allele varies slightly in the populations analysed so far, but is always higher than 80% (Rovio *et al.*, 2004; Malyarchuk *et al.*, 2005). The second most common allele contains 11 CAG repeats and, in most cases, is present in combination with the 10 CAG-repeat allele, *i.e.* in heterozygosity. Heterozygosity is also the rule for the other ≠10 alleles. The absence of the common allele, either in heterozygosity (10/≠10) or in homozygosity (≠10/≠10) is then sporadic, suggesting that it may have a deleterious effect in POLG function. However, it does not seem to play a role in mitochondrial diseases with multiple mtDNA deletions or depletion (Rovio *et al.*, 1999). On the other hand, the POLG CAG-repeat polymorphism is probably a risk factor for testicular cancer (Nowak *et al.*, 2005; Jensen *et al.*, 2008).

The effect of *POLG* CAG-repeat polymorphisms in sperm quality and function is unclear, as a series of contradictory results have been documented. In a wide-ranging study, a significant increase in the homozygous mutant genotype (≠10/≠10), as well as in the heterozygous genotype (10/≠10), has been described in infertile men when compared with a fertile cohort (Rovio *et al.*, 2001). Furthermore, a correlation between the lack of the common allele and idiopathic infertility (undefined fertility disorders, but presenting normal semen parameters) was identified in a Danish population (Jensen *et al.*, 2004). However, analysis of an Italian (Krausz *et al.*, 2004) and a French (Aknin-Seifer *et al.*, 2005) cohort determined the frequency of the homozygous mutant genotype to be similar in both infertile and normozoospermic fertile men. Likewise, no significant differences in the frequency of each genotype were found in Italian men with low sperm counts (oligozoospermia) when compared to normozoospermics (Brusco *et al.*, 2006). In a more recent study, the combined frequency of heterozygous and homozygous mutant *POLG* genotypes was actually higher in normozoospermic than non-normozoospermic men, contrary to what had been anticipated (Harris *et al.*, 2006). These authors hypothesised that the results obtained by Rovio *et al.* and

Jensen *et al.* could be caused by the presence of mutations somewhere else in the *POLG* gene. To test this possibility, they analysed the three exonuclease motifs of *POLG*, but no mutations were found in all the samples tested. The most recent study (Westerveld *et al.*, 2008) analysed a large cohort of individuals with different ethnicities and concluded that the absence of the common allele is not associated with a clinically significant decrease in sperm quality.

However, and despite the variability in the distinct studies, the functional significance of any of these genetic correlations has not been defined in sperm. In the present study, whether *POLG* CAG-repeat polymorphisms are associated with sperm quality in a series of patients classified according to the WHO (1999) criteria was investigated. Importantly, a group of oligoasthenoteratozoospermic (OAT) patients, that present all three principal sperm parameters below the defined cut-offs, were included. This group has not been included in the studies performed so far, or at least has never been analysed in isolation. Moreover, it has also been assessed whether polyglutamine-repeat length can affect sperm mtDNA copy number, as well as POLG expression (and, as a consequence, the expression of TFAM, COXI and COXVIc – see Chapter II).

2. Materials and methods

Chemicals

All chemicals were supplied by Sigma Chemical Company (St. Louis, MO, USA), unless stated otherwise.

Biological Material

Patients were recruited from the Fertility Clinic and fresh semen samples (n=135) were processed and categorized as described previously (Chapter II, Materials and methods section).

Extraction of total DNA

DNA from isolated sperm samples was extracted according to the whole blood DNA isolation protocol using the Puregene DNA Isolation Kit (Flowgen, Nottingham, UK) with minor modifications, as formerly described (Chapter II, Materials and methods section).

Molecular analysis of POLG CAG repeats

Fluorescent PCR amplification of 287 bp of the POLG gene (accession number X98093) was performed in 50 µl reactions using primers (forward: ggt ccc tgc acc aac cat ga; reverse: ctt gcc cga aga ttt gct cgt) and conditions as described previously (Jensen *et al.*, 2004). The forward primer was labelled with a fluorescent tag. Each reaction contained 5 ng of total DNA, 1 x PCR Buffer (BioLine, London, UK), 1.5 mM $MgCl_2$ (BioLine), 200 µM dNTPs (BioLine), 125 nM of each primer and 2.5 U BioTaq DNA Polymerase (BioLine). Negative controls, with H_2O instead of DNA, were performed in each run. Reaction conditions were as follows: initial denaturation at 98ºC for 5 min, 40 cycles of 98ºC for 30 sec, 63ºC for 1 min and 72ºC for 1 min 45 sec and final extension at 72ºC for 5 min. The size of the DNA fragments obtained was determined using a Capillary Electrophoretic Genetic Analysis System (CEQ^{TM} 8000, Beckman Coulter Inc, Fullerton, CA, USA). Mixtures containing 0.2 µl PCR products, 30 µl CEQ sample loading solution (Beckman Coulter Inc) and 0.8 µl CEQ 400-bp DNA size standards (Beckman Coulter Inc) were analysed.

For conventional PCR, non-labelled primers were used. Reaction mixtures were similar to the ones used for fluorescent PCR, except for the amount of DNA (200 ng) and the concentration of primers (0.5 µM each). PCR products were resolved on 3% agarose gels and product size confirmed against a 100 bp DNA ladder (Bioron, Ludwigshafen, Germany). The PCR products were excised from the agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, London, UK) as described in the manufacturer's protocol. The purified DNA was then sequenced using the automated direct sequencing protocol (Hopgood

et al., 1992) using an ABI PRISM BigDye terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Determination of mtDNA content

mtDNA content was determined by the ratio of copies of mtDNA to Beta-Globin, as described before (Chapter II, Materials and methods section).

POLG, TFAM, COXI and COXVIc expression

The expression of these proteins was detected by immunocytochemistry (ICC), as previously explained (Chapter II, Materials and methods section).

Statistical analysis

Statistical analysis was carried out using SPSS for Windows (version 13, Chicago, IL, USA). Pearson Chi-square test was used to compare *POLG* CAG variants (allele and genotype frequencies) in the different groups defined. All numerical variables were checked for normal distribution using the one-sample Kolmogorov-Smirnov test. For sperm parameters, ICC and mtDNA copy number analysis, the two different-genotype groups were compared using Independent-samples t-test (or the non parametric Mann-Whitney U test when distributions were not normal). $P < 0.05$ was considered significant.

3. Results

POLG CAG repeats polymorphisms

The CAG-repeat region of the *POLG* gene have been analysed in groups of patients attending an infertility clinic, according to the WHO criteria (1999). These patients were categorised as normal (sperm with normal parameters); patients possessing 1- or 2-defects (sperm with one or two abnormal parameters); and oligoasthenoteratozoospermic patients

(OAT; samples with three abnormal parameters). Samples were examined by fluorescent PCR followed by fragment analysis. These outcomes were verified by conventional PCR and DNA-sequencing (Fig. 1). CAG repeats ranging from 9 to 13 codons were identified. After the 'common' 10 repeat allele, the most frequent allele consisted of 11 codons (Table I). The frequency of the 11 CAG repeats allele was significantly higher ($P < 0.05$; Table I) in the OAT group (21.4%) compared with both the normal group (12.5%) and the 1- or 2-defects group (8.2%). Likewise, the combined frequency of ≠10 alleles (*i.e.*, 9, 11, 12 and 13) was also higher ($P < 0.05$; Table I) in the OAT group (23.2%) compared with the others (16.2% and 9.7% in the normal and in the 1- or 2-defects groups, respectively).

Table I. Allelic frequency of the *POLG* CAG repeats for the normal, 1- or 2-defects and oligoasthenoteratozoospermic (OAT) groups.

Allele (no. of repeats)	Normal group (n=80 alleles)	1- or 2-Defects group (n=134 alleles)	OAT group (n=56 alleles)	Total (n=270 alleles)
9	0.012	0.000	0.000	0.003
10	0.838	0.903	0.768*	0.856
11	0.125	0.082	0.214*	0.122
12	0.025	0.015	0.000	0.015
13	0.000	0.000	0.018	0.004
≠10	0.162	0.097	0.232*	0.144

The allelic frequencies of the POLG CAG repeats for all the samples are also indicated (Total). ≠10 denotes the addition of the alleles 9, 11, 12 and 13. * $P < 0.05$.

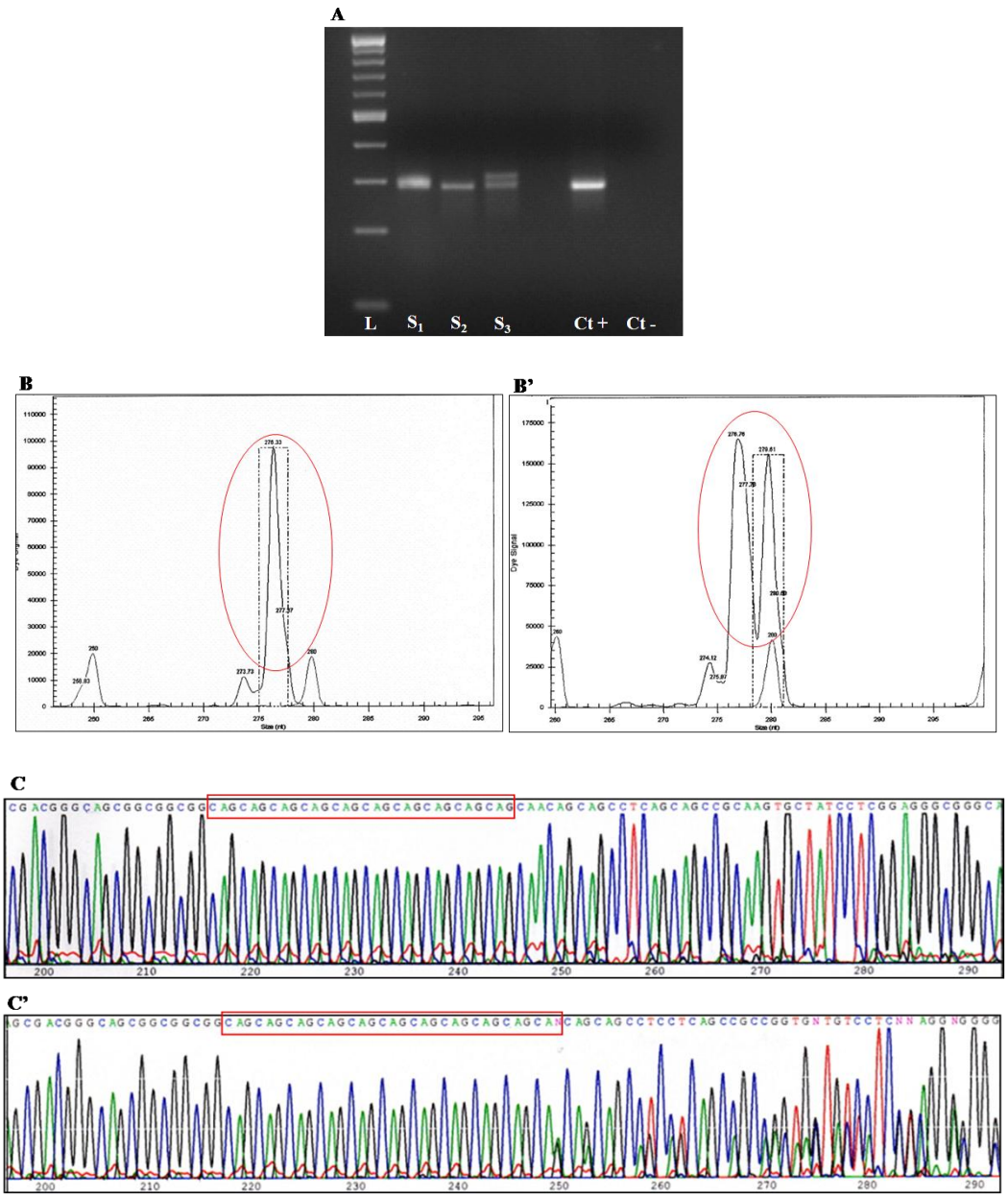

Figure 1. Analysis of the CAG-repeat region of the *POLG* gene in human sperm samples. **A)** Agarose gel of PCR products of a 287 bp region of POLG containing the CAG-repeat tract. Legend: L- DNA ladder; S1, S2, S3 – Samples 1 (heterozygous 10/11), 2 (homozygous 10/10) and 3 (heterozygous 10/13), respectively; Ct+ - Positive control (blood sample); Ct- - Negative control (without DNA). **B, B')** Fragment analysis of two samples: B (homozygous 10/10), B' (heterozygous 10/11). **C, C')** DNA sequencing of two samples: C (homozygous 10/10), C' (heterozygous 10/11).

Combining the two alleles for each man allowed patients to be categorized into one of the three possible genotypes: homozygous wild-type (10/10), heterozygous (10/≠10) and homozygous mutant (≠10/≠10). Of 135 men, 96 (71.1%) were homozygous wild-type and 39 (28.9 %) were heterozygous. A different distribution of the genotypes was observed in the three groups analysed (Fig. 2), with heterozygosity being higher in the OAT group (46.4%) than in the normal and in the 1- or 2-defects groups (32.5% and 19.4 %, respectively; $P < 0.05$).

However, by comparing each of the three principal sperm parameters (concentration, motility and morphology) individually, no differences were found between the genotypes ($P = 0.298$ for concentration and $P = 0.417$ for motility and morphology; Table II).

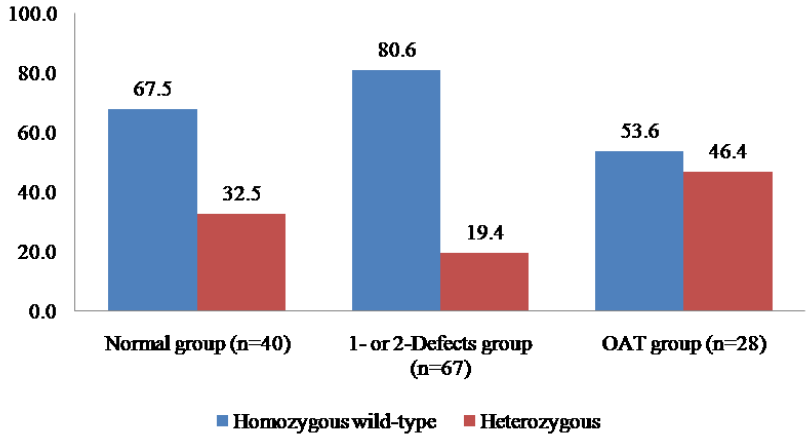

Figure 2. POLG CAG-repeat genotype distribution in the normal, 1- or 2-defects and OAT groups. The values represent the percentage of homozygous wild-type and heterozygous individuals found in each of the three groups. The distribution of the genotypes is different in the OAT group ($P < 0.05$).

Table II. Sperm principal parameters for the homozygous wild-type (10/10) and the heterozygous (10/≠10) groups. Values indicate means ± SEM. $P > 0.05$ for the three parameters.

Sperm parameter	Homozygous wild-type group (n=96)	Heterozygous group (n=39)
Concentration (millions sperm/ml)	54.9 ± 6.4	58.4 ± 13.9
Motility (% progressive motile sperm)	45.0 ± 2.1	41.7 ± 3.9
Morphology (% normal sperm)	13.7 ± 0.6	12.7 ± 1.2

MtDNA copy number and POLG CAG-repeat genotype

To determine whether the *POLG* CAG-repeat genotype influences sperm mtDNA content, the mtDNA copy number of 38 samples (22 homozygous wild-type and 16 heterozygous for *POLG* CAG-repeat variants) were compared. The mean mtDNA copy number was higher in the heterozygous men compared to the homozygous wild-type group (Table III). However, this difference was not statistically significant ($P = 0.261$).

Table III. Sperm mtDNA copy number and expression of POLG, TFAM, COXI and COXVIc for the homozygous wild-type (10/10) and the heterozygous (10/≠10) groups. Values indicate means ± SEM. For mtDNA copy number ranges are also indicated. $P > 0.05$ for each parameter.

Parameter	Homozygous wild-type group	Heterozygous group
mtDNA copy number	16.8 ± 6.0 (0.2 – 100.1)	27.0 ± 12.6 (0.7 - 206.1)
% Sperm expressing POLG	72.2 ± 2.4	72.7 ± 3.2
% Sperm expressing TFAM	64.9 ± 4.1	73.7 ± 3.7
% Sperm expressing COXI	79.6 ± 2.1	79.4 ± 2.8
% Sperm expressing COXVIc	71.1 ± 2.7	63.0 ± 7.6

The effect of POLG CAG repeats in POLG, TFAM, COXI and COXVIc proteins expression

In order to determine whether specific *POLG* CAG-repeat genotypes would affect the expression of POLG, TFAM, COXI and COXVIc, the ICC outcomes between the distinct *POLG* CAG repeats genotypes (homozygous wild-type: n= 40 for POLG, n= 25 for TFAM, n= 41 for COXI, and n=33 for COXVIc; heterozygous: n=17 for POLG, n=8 for TFAM, n=18 for COXI, and n=12 for COXVIc) have been compared. Similar to the results obtained for sperm parameters and mtDNA copy number, the two genotypes showed no significant differences in the mean percentage sperm expressing each of the proteins (*P* values range from 0.226-0.956; Table III).

4. Discussion

The gene for the catalytic subunit of POLG possesses a series of CAG repeats which can vary in number (Rovio *et al.*, 1999), thus encoding a mtDNA polymerase with distinct numbers of glutamines. The occurrence of proteins with CAG repeats-encoded polyglutamine tracts is widespread, and these are recurrently of variable length. While the functions of CAG repeats are often unclear, the association between the expansion of CAG repeats in different genes and certain neurodegenerative disorders is well documented (for reviews see Ross *et al.*, 1999; Zoghbi and Orr, 2000; Orr, 2001; Shao and Diamond, 2007). These are usually called polyglutamine diseases, and typically involve neuronal cell death. The classical example is Huntington disease, where expansion of a polyglutamine tract in the protein huntingtin is at the origin of neuronal dysfunction, leading to progressive neurological symptoms, and ultimately to death.

On the other hand, the role of certain CAG-repeat variants in male infertility is not completely understood as different studies reporting on the same gene have often presented

discordant outcomes. This is certainly the case for the length variation of the CAG-repeat region of the androgen-receptor (AR) gene, that has been positively (Tut *et al.*, 1997; Komori *et al.*, 1999; Mifsud *et al.*, 2001; Mengual *et al.*, 2003) and negatively (Giwercman *et al.*, 1998; Dadze *et al.*, 2000; Ferlin *et al.*, 2004; Lavery *et al.*, 2005) associated with infertility. The results seem to depend on the geographic origin of the populations studied, suggesting that the genetic background may possibly play a role in the expression of the AR defects (Yong *et al.*, 2003).

Such disparity is also certainly typical for the *POLG* CAG-repeat region. To this extent, two studies have demonstrated an association between the absence of the common allele and male infertility, either typified by a range of sperm quality defects (Rovio *et al.*, 2001), or by idiopathic infertility (Jensen *et al.*, 2004). However, this association was not identified in populations of Italian (Krausz *et al.*, 2004; Brusco *et al.*, 2006) and French (Aknin-Seifer *et al.*, 2005) infertile men. Likewise, a recent study in a large cohort of men of distinct ethnicities suggested that *POLG* variants have no influence on sperm quality (Westerveld *et al.*, 2008). The results of the present study do not show an association between each of the three principal sperm parameters, when assessed independently, and *POLG* genotypes, which is in agreement with Krausz *et al.* (2004), Aknin-Seifer *et al.* (2005), Brusco *et al.* (2006) and Westerveld *et al.* (2008). However, a higher frequency of the heterozygous genotype in OAT samples, when compared to the normal and 1- or 2- defects groups, was found in this study, which is similar to the original outcomes of Rovio *et al.* (2001).

Importantly, the functional meaning of the length variation of the *POLG* CAG-repeat region in sperm has not yet been elucidated. As *POLG* alleles are segregated during spermatogenesis, samples from heterozygous individuals will present sperm with 10 CAG repeats and sperm with ≠ 10 CAG repeats. If the absence of the common allele were to affect

the enzymatic properties of POLG, it is anticipated that an accumulation of mtDNA mutations and/or an alteration in mtDNA copy number would occur in heterozygous and homozygous mutant men, due to the presence of sperm with ≠ 10 repeats. However, and while in the present study all samples with a mutant allele were heterozygous, no relation between mtDNA copy number and *POLG* CAG-repeat variants was found. Furthermore, it is also apparent that the incidence of mtDNA nucleotide substitutions is similar for each of the *POLG* genotypes (Harris *et al.*, 2006).

Therefore, and at least in human sperm, it seems that the length of the POLG polyglutamine tract does not affect the main enzymatic activities of the polymerase, namely the replication and repair of the mitochondrial genome. In accordance, mutant *POLG* alleles seem not to be involved in phenotypic defects in other tissues. For instance, the allelic frequencies of the POLG trinucleotide repeat have been shown to be similar in patients with mitochondrial diseases when compared to healthy controls (Rovio *et al.*, 1999). Similar outcomes were described for patients with Parkinson disease (Taanman and Schapira, 2005), while this has been contradicted by others (Luoma *et al.*, 2007).

Perhaps most striking of all is that the deletion of the *POLG* CAG-repeat region in cultured human cells resulted in the continued unaffected enzymatic properties of POLG, although the expression of the protein was moderately up-regulated (Spelbrink *et al.*, 2000). Thus, one could hypothesise that the expression of POLG would be affected in sperm possessing a mutant *POLG* CAG-repeat allele. However, this study has shown that the percentage of sperm expressing POLG was not significantly different in samples from different *POLG* genotypes. This is equally so for TFAM and for two COX subunits: COXI (mtDNA encoded) and COXVIc (nuclear DNA encoded), which also validates that the less frequent *POLG* CAG-repeat variants do not impair the expression of sperm mitochondrial proteins.

Consequently, these cumulative outcomes suggest that there is currently no direct functional association between *POLG* CAG-repeat variants and male infertility, with a possible exception in men with very poor sperm quality (OATs). This suggests that the absence of the *POLG* common allele in sperm may affect, in certain circumstances (not yet identified, but probably operating only in very poor sperm samples) POLG function. As polyglutamine tracts are generally considered as interfaces for protein-protein interactions, Rovio and colleagues (2001) have proposed that a sperm-specific protein could interact with the polyglutamine region of POLG and that this interaction would be affected when the tract length is altered. This interaction could be deleterious in OAT samples, if such a protein would also be mildly impaired in these samples.

In fact, gene polymorphisms cannot explain fertility impairments *per se*, since they are also present in normozoospermic fertile men, although at a lower frequency. Thus, they should be viewed as predisposing factors, that would only have clinical significance in the presence of specific environmental factors or in association with certain genetic background, infections, altered hormonal status, etc (Tuttelmann *et al.*, 2007). These factors might contribute to the different outcomes described in the literature for the effects of distinct gene polymorphisms in male infertility, of which POLG is only one example. Recently, it has been revealed that the frequency of the three *POLG* CAG-repeat genotypes is dependent on ethnic background (Westerveld *et al.*, 2008). Furthermore, these authors have also shown that *POLG* genotypes can have distinct effects on sperm concentration and motility in cohorts of men of different ethnic and geographic origin.

In conclusion, while the biological significance of *POLG* CAG-repeat variants in male infertility remains unclear, the heterozygous genotype seems to be associated with very poor quality sperm. In fact, this study, to an extent, supports the original findings that an

association between sperm quality and *POLG* CAG repeats does exist. However, these variants do not directly affect POLG expression, nor mtDNA maintenance.

CHAPTER IV

THE ASSESSEMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL AND THE EXPRESSION OF MITOCHONDRIAL PROTEINS IN SUBPOPULATIONS OF HUMAN SPERM

The results presented in this Chapter are in preparation for submission.

Part of the methodology described in this Chapter has also been published in:
Ramalho-Santos, J., Amaral, A., Sousa, A. P., Rodrigues, A. S., Martins, L., Baptista, M., Mota, P. C., Tavares, R., Amaral, S. and Gamboa, S. (2007) Probing the structure and function of mammalian sperm using optical and fluorescence microscopy. In: Méndez-Vilas, A. and Díaz, J. (Eds) *Modern Research and Educational Topics in Microscopy*, **1**: 394 - 402.

Abstract

The production of ATP through oxidative phosphorylation depends upon the existence of a mitochondrial membrane potential (MMP), which relies on the correct function of the mitochondrial electron transfer chain, and hence, on its proteins. Mounting evidences have shown that MMP, assessed using specific mitochondrial probes, is correlated with human sperm motility and fertilisation ability. However, the capacity of distinct probes to dynamically evaluate sperm MMP has not been determined. In the present study, sperm samples were independently incubated with MitoTracker Green (MT-G), MitoTracker Red (MT-R) and JC-1 and the results were analysed and compared by fluorescence microscopy. The ability of each probe to monitor MMP was determined by incubating sperm with MMP disruptors, either before or after the incubation with the probes. Moreover, subpopulations of sperm with distinct MMP were isolated using MT-G and a cell-sorter. These subpopulations were analysed for the expression of two mitochondrial proteins: DNA polymerase gamma (POLG) and cytochrome c oxidase I (COXI). A similar analysis was also performed in subpopulations of sperm isolated by the classical swim-up procedure.

The three mitochondrial probes provided similar results, and were able to monitor changes in MMP when sperm were previously incubated with MMP-disruptor drugs. However, only JC-1 could, to a small extent, mirror MMP alterations when the drugs were added after the incubation with the probes. The expression of both POLG and COXI was significantly higher in subpopulations of MT-G positive sperm, when compared to subpopulations of MT-G negative sperm. Similar outcomes were obtained for the comparison between the swim-up migrated and non-migrated fractions of sperm.

1. Introduction

Cellular mitochondrial membrane potential (MMP) and its changes are commonly monitored using permeant cationic fluorescent probes, such as carbocyanine, rhodamine and rosamine derivatives, which accumulate in either isolated or cellular mitochondria depending on MMP. Different methods can be used to evaluate fluorescence intensities after staining cells with these probes, including fluorescence microscopy, spectrofluorometry and flow cytometry (reviewed in Solaini *et al.*, 2007).

The importance of MMP to mammalian sperm motility, and hence, function, has been recognized for a long time. Initial studies relied on the use of flow cytometry to measure fluorescence intensities of sperm samples stained with both the classical rhodamine 123 dye (Rh123, to assess sperm MMP) and either ethidium or propidium iodide (to determine sperm viability). Using this approach, it has been determined that both human sperm motility and viability are correlated with MMP (Evenson *et al.*, 1982; Auger *et al.*, 1989). Similar outcomes were obtained in cryopreserved bovine sperm using not only Rh123, but also 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and MitoTracker Green, a carbocyanine-based probe from Molecular Probes (Garner *et al.*, 1997).

Similarly to Rh123, other probes such as MitoTrackers and 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] accumulate in mitochondria according to MMP, fluorescing either green or red, depending on the probe. On the other hand, JC-1 is known by its unique property of discriminating between high and low MMP. This molecule exists in two distinct forms: monomeric, fluorescing green, and the so-called J-aggregate, emitting red-orange fluorescence. In the presence of a high MMP, J-aggregates are formed, and fluorescence changes reversibly from green to red-orange. Thus, mitochondria with high MMP fluoresce orange, while mitochondria with low MMP fluoresce green (Smiley *et al.*, 1991).

JC-1 has been extensively used to study sperm function, not only in men (Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Kasai *et al.*, 2002; Marchetti *et al.*, 2004), but also in other mammalian species including equines (Gravance *et al.*, 2000; Love *et al.*, 2003), rats (Gravance *et al.*, 2001), boars (Huo *et al.*, 2002; Spinaci *et al.*, 2005) and rams (Martinez-Pastor *et al.*, 2004). These reports confirmed the original findings that mammalian sperm motility is related with mitochondrial functional status. Moreover, and at least for human sperm, it has also been shown that sperm fertilisation potential correlates with MMP assessed using either JC-1 (Kasai *et al.*, 2002; Marchetti *et al.*, 2004) or $DiOC_6(3)$ (Marchetti *et al.*, 2002; 2004). Studies using $DiOC_6(3)$, which has recently become popular, have also shown a negative correlation between sperm MMP and both DNA fragmentation and the generation of ROS (Marchetti *et al.*, 2002; Wang *et al.*, 2003). Taken together, these cumulative outcomes strongly suggest that MMP is an indicator of the sperm functional status, and may have an important clinical value.

The efficacy of the various mitochondrial probes to monitor MMP is usually established by using specific drugs that: a) inhibit the ETC (such as antimycin, rotenone and cyanide); b) eliminate the mandatory link between the respiratory chain and the phosphorylation system (OXPHOS uncouplers); c) make the inner mitochondrial membrane permeable to specific ions (ionophores, such as valinomycin and nigericin), which also uncouple electron transfer from phosphorylation. In either case, MMP is disrupted and the mitochondrial production of ATP is diminished. This kind of experiments should be performed in every study using mitochondrial probes, as differences may exist between probes batches, equipments, concentrations and incubation times, and, most likely, cell types. For example, when studying sperm, it is important to consider that sperm mitochondria are distinct from the somatic cell counterparts, as they are enclosed in the so-called mitochondrial capsule (see Chapter I).

In most of the studies performed so far, the ability of distinct mitochondrial probes to monitor human sperm MMP has been shown in control experiments using uncouplers, either before or during the incubation of sperm with the probe. To this extent, JC-1, DiOC6(3), Chloromethyl-X-rosamine (MitoTracker Red CMX-Ros) and tetramethylrhodamine ethyl ester (TMRE), coupled to flow cytometry, have been shown to accurately monitor human sperm MMP (Marchetti *et al.*, 2004). Importantly, the results obtained were similar for the four probes, suggesting that a variety of probes can be used to study human sperm MMP.

However, none of the studies have asked the question of whether any of these probes are dynamic, *i.e.*, if once inside mitochondria, the probes are able to mirror MMP alterations. In the first part of the present study, fluorescence microscopy was used to study and compare the ability of two carbocyanines (JC-1 and MitoTracker Green) and of a rosamine (chloromethyl-dihydro-X-rosamine; MitoTracker Red CM-H_2XRos) to react to human sperm MMP alterations, occurring either before or after the incubation of the cells with the probes. Three distinct drugs were used: potassium cyanide (KCN; which blocks cytochrome c oxidase, the complex IV of the ETC); p-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP; a weak acid that carries protons across the inner mitochondrial membrane, thus abolishing the proton gradient and hence, the electrochemical potential); and valinomycin (a potassium ionophore that dissipates the electrical potential).

The maintenance of sperm MMP requires an accurate function of the ETC which, in turn, depends on the proper assembly and communication between distinct nuclear and mitochondrial proteins (see Chapter I). These comprise all the subunits that constitute the ETC complexes (which are encoded by both chromosomal and mitochondrial genes), as well as the factors that control the expression of these subunits. It has been previously shown that the expressions of two mitochondrial specific proteins, DNA polymerase gamma (POLG), and cytochrome c oxidase I (COXI) are correlated with sperm quality (see Chapter II, Amaral

et al., 2007). Specifically, it was shown that human samples present a number of sperm expressing these proteins in the midpiece, and that this number is higher for better quality samples. On the other hand, it is well established that human sperm samples are very heterogeneous, presenting subpopulations with distinct biochemical and physiological characteristics. For this reason, only a small subpopulation of sperm in a sample is able to fertilise an oocyte (Holt and Van Look, 2004). Thus, one of the major challenges in modern andrology is to better characterise, distinguish and isolate this particular subpopulation. Would "better quality" subpopulations have improved mitochondrial parameters?

The classical swim-up procedure (Lopata *et al.*, 1976) is routinely used to obtain a population of highly motile sperm for many ARTs. This very efficient technique is based on the movement of sperm from a pre-washed pellet in to an overlaying medium, and is characterized by the recovery of > 90% motile sperm (reviewed in Henkel and Schill, 2003). In the present study, this procedure was used to isolate and compare subpopulations of motile and immotile sperm, in terms of the expression of both POLG and COXI. Similarly, and to determine if the expressions of both POLG and COXI are correlated with sperm MMP, subpopulations of sperm with polarised and non-polarised mitochondria were also isolated and compared. For this purpose, the mitochondrial probe MitoTracker Green and a cell-sorter were used to separate stained and unstained sperm from each sample. Similar approaches have been used by others to isolate sperm with better fertilisation potential, based on mitochondrial function (Auger *et al.*, 1993; Gallon *et al.*, 2006).

Hence, the aims of the present work were dual: to compare distinct mitochondrial probes and clarify their ability to truly monitor MMP in human sperm; to understand if POLG and COXI expressions are related with both sperm MMP and sperm motility in subpopulations of human sperm.

2. Material and Methods

Chemicals

All chemicals were supplied by Sigma Chemical Company (St. Louis, MO, USA), unless stated otherwise.

Biological Material

Patients were recruited from the Fertility Clinic and fresh semen samples were processed and categorized as described previously (Chapter II, Material and Methods section).

Determination of sperm mitochondrial and membrane integrity status

1) MitoTracker Green (MT-G), MitoTracker Red (MT-R) and JC-1 labelling

MitoTracker® Green FM (M-7514), MitoTracker® Red CM-H_2XRos (M-7513) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; T-3168) were purchased from Molecular Probes (Eugene, Oregon, USA). Primary stock solutions of 1 mM (both MitoTrackers) and 3.8 mM (JC-1) were prepared in DMSO, stored at -20ºC and protected from light. Secondary stock solutions were prepared in phosphate-buffered saline (PBS; pH 7.2), immediately prior using. For labelling, live sperm suspensions (10 millions of sperm/ml PBS) were separately incubated with a) 20 nM MitoTracker Green; b) 150 nM MitoTracker Red; c) 2 μM JC-1, for 10 min at 37 ºC, in the dark.

2) LIVE/DEAD Sperm Viability Kit

The LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes) consists of two DNA-binding fluorescent dyes: SYBR® 14 (which is membrane-permeant and thus stains all sperm nuclei, fluorescing bright green) and propidium iodide (PI; that only penetrates sperm nuclei with compromised membrane integrity, fluorescing red, which overwhelms the green

fluorescence exhibited by SYBR-14). Primary stock solutions of 1 mM SYBR and 2.4 mM PI were prepared in DMSO (SYBR) and water (PI), and stored at -20º C, protected from light. Secondary stock solutions were prepared in PBS, immediately before using and sperm samples (already incubated with MT-G, MT-R or JC-1, as described) were simultaneously incubated with SYBR (100 nM) and PI (240 nM), for an additional 10 min, at 37 ºC, in the dark.

3) Sperm categorization and counting

After the 20 min total incubation, sperm suspensions were mounted on a microscope slide and observed using a Zeiss Axiophot II microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter. Sperm were categorized according to patterns of staining for both the mitochondria and the nucleus. For each slide, at least 200 sperm in at least four different fields were observed and categorized.

Incubation with mitochondrial inhibitors and uncouplers

In order to study and compare the response of each of the mitochondrial probes (MT-G, MT-R and JC-1) to altered mitochondrial status, live sperm suspensions were incubated in either the presence or absence (controls) of a) 5 mM potassium cyanide (KCN; blocks cytochrome c oxidase) for 30 min; b) 1 μM p-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP; a mitochondrial uncoupling agent) for 2 h; c) 1 μM valinomycin (a potassium ionophore that dissipates the mitochondrial electrical gradient) for 2 h. The concentrations used are in the ranges described in the literature for KCN (Ruiz-Pesini *et al.*, 2000a; St John *et al.*, 2005) and FCCP and valinomycin (Keij *et al.*, 2000; Kalbacova *et al.*, 2003). As FCCP and valinomycin stock solutions were dissolved in ethanol, control experiments for these chemicals included similar amounts of ethanol. The incubation with the drugs KCN, FCCP and valinomycin was performed in two distinct experimental conditions:

a) before incubation with the mitochondrial probes; b) after incubation with the mitochondrial probes.

Isolation of subpopulations of sperm

a) With distinct mitochondrial membrane potential

To separate subpopulations of sperm with distinct mitochondrial membrane potential, live sperm suspensions were incubated with MT-G for 20 min at 37ºC, as previously described. Sperm were then sorted with a BD FACSAria^{TM} cell-sorting system (BD Biosciences, Becton, Dickinson and Company, NJ, USA) at a 488 nm wavelength, leading to the isolation of two subpopulations of sperm for each sample: MT-G positive sperm and MT-G negative sperm.

b) With distinct progressive motility (Swim-up)

To separate subpopulations of sperm with distinct progressive motility, samples were processed by the routine swim-up procedure using Sperm Preparation medium (Medicult, Jyllinge, Denmark). Briefly, sperm pellets were gently covered with the medium and incubated for 30 min at 37º C, allowing the motile sperm to swim-up to the medium. For each sample, two subpopulations were isolated: sperm that migrated to the medium (referred here as "migrated sperm"); sperm that did not migrate (referred herein as "non-migrated sperm").

POLG and COXI expression

The expression of these proteins was detected by immunocytochemistry (ICC), as previously explained (Chapter II, Materials and Methods section), except for the secondary antibodies: Alexa Fluor 594 goat anti-rabbit IgG (A-11012, Molecular Probes) and Alexa Fluor goat anti-mouse IgG (A-11005, Molecular Probes) were used for POLG and COXI, respectively.

Statistical analysis

Statistical analysis was carried out using SPSS for Windows (version 13, Chicago, IL, USA). All variables had a normal distribution, as established by the one-sample Kolmogorov-Smirnov test. Paired-samples T-test was used to compare the results obtained with the 3 mitochondrial probes. Comparison between controls and a) KCN; b) FCCP; c) valinomycin; were performed using independent-samples T-test, as the requisites for paired-samples T-test were not fulfilled. Comparisons between ICC outcomes in distinct subpopulations of sperm were also carried out using independent-samples T-test. Pearson's test was applied to assess the correlations between the three mitochondrial probes and between these and sperm motility. $P < 0.05$ was considered significant.

3. Results

Comparison between distinct mitochondrial probes

To compare MitoTracker Green (MT-G), MitoTracker Red (MT-R) and JC-1 staining in human sperm, 40 sperm samples were incubated with each of the probes, individually, along with the LIVE/DEAD Sperm Viability Kit. The use of the viability kit had two purposes: to stain sperm heads, so that all sperm, even those not stained with the mitochondrial probes, could be visualized; to assess sperm membrane integrity, which also evaluates sperm viability. As anticipated, the three mitochondrial probes (MT-G, MT-R and JC-1) stained the sperm midpiece, the compartment housing the mitochondria (Fig.1). Samples incubated with MT-G and MT-R presented both midpiece-stained sperm (green and red, respectively; referred here as "Mito +") and unstained sperm (referred herein as "Mito – "). On the other hand, sperm incubated with JC-1 presented three midpiece staining patterns: orange (almost always accompanied with green; Fig.1); green (together constituting "Mito+"); and unstained ("Mito-"). Unsurprisingly, the viability kit stained all sperm heads

(where the nucleus is localized) green ("Live") or red ("Dead"). Combining the head and mitochondria staining patterns allowed sperm to be categorized in the following categories: 1) "Live, Mito+"; 2) "Dead, Mito-"; 3) "Live, Mito-"; 4) "Dead, Mito+". As expected, the majority of sperm in each sample belonged to the categories 1 and 2 (Table I).

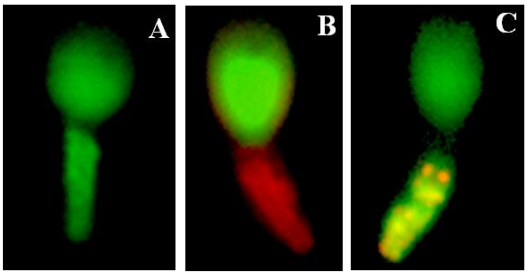

Figure 1. Human sperm labelled with mitochondrial probes. **A)** MitoTracker Green (green midpiece). **B)** MitoTracker Red (red midpiece). **C)** JC-1 (orange and green midpiece). Nuclear DNA is stained with SYBR-14 (green heads).

Table I. Distribution of data for the patterns of staining of sperm samples (n=40) incubated with the Live/Dead sperm viability kit along with a) MitoTracker Green (MT-G); b) MitoTracker Red (MT-R); c) JC-1. Data are expressed as mean percentages ± SEM.

A	**Live/Mito+**	**Dead/Mito-**	**Live/Mito-**	**Dead/Mito+**
MT-G	73.3 ± 2.0	11.1 ± 1.8	2.5 ± 0.7	13.2 ± 1.6
MT-R	74.9 ± 1.7	13.4 ± 1.5	2.0 ± 0.5	9.8 ± 1.1
JC-1	72.5 ± 1.9	16.8 ± 1.4	3.3 ± 0.7	7.4 ± 1.4

B	**Live/Orange**	**Live/Green**	**Dead/Mito-**	**Live/Mito-**	**Dead/Orange**	**Dead/Green**
JC-1	44.8 ± 4.0	27.7 ± 4.3	16.8 ± 1.4	3.3 ± 0.7	0.7 ± 0.3	6.7 ± 1.4

A) "Live" denotes sperm head stained green; "Dead" denotes sperm head stained red; "Mito+" and "Mito-" denote stained and unstained midpieces, respectively. **B)** For JC-1, "Orange" and "Green" midpieces are discriminated. See text for details.

For simplification, to compare the distinct mitochondrial probes, results were analysed as percentage of sperm with viability ("Live"; sum of the categories 1 and 3), and as percentage of sperm with stained mitochondria ("Mito+"; category 1 plus category 4) only. Comparing the outcomes obtained with the viability kit, it was observed that the mean

percentage of "Live" sperm was, as expected, independent from the mitochondrial probe used (mean ± SEM = 75.7 ± 1.7 for MT-G; 76.8 ± 1.5 for MT-R; 75.8 ± 1.6 for JC-1; *Ps* > 0.05; Fig.2). This indicated not only that the viability kit provided very consistent results, but also that the mitochondrial probes used did not interfere with the kit. On the other hand, and while the results for the distinct mitochondrial probes were highly correlated (Table II), the mean percentage of sperm with stained mitochondria was different for JC-1 (79.9 ± 1.7) compared with both MT-G (86.5 ± 2.1; *P* <0.001) and MT-R (84.6 ± 1.7; *P* < 0.001; Fig.2). Moreover, there were significant differences between viability and mitochondrial staining with each of the mitochondrial probes (*Ps* < 0.001 for both MT-G and MT-R; *P* < 0.01 for JC-1; Fig. 2), evidencing that the viability kit and the mitochondrial probes stained different cellular features. Nevertheless, for each probe, the viability and mitochondrial values were highly correlated (Table II).

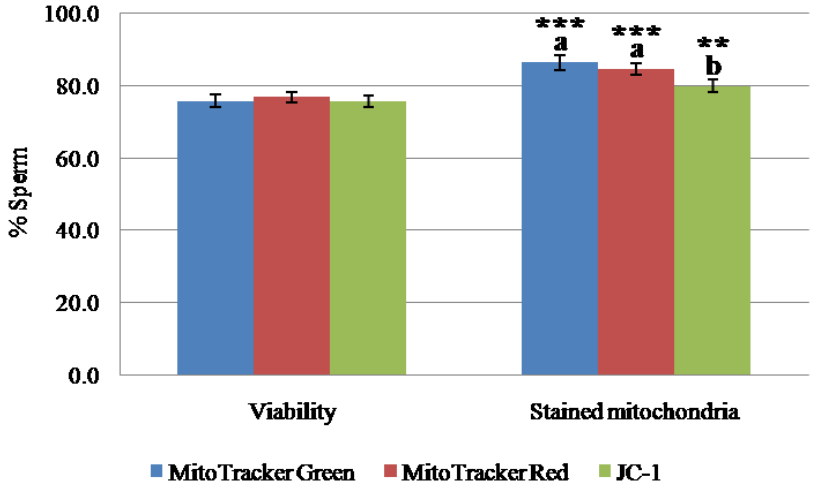

Figure 2. Comparison between the percentage of sperm viability and of sperm with stained mitochondria of samples (n=40) incubated with the Live/Dead sperm viability kit along with a) MitoTracker Green (MT-G); b) MitoTracker Red (MT-R); c) JC-1. Data are expressed as mean percentages ± SEM. *a* and *b* denote significant differences between JC-1 and MT-G and MT-R (*P* < 0.001 for both). Asterisks denote, for each mitochondrial probe, differences between sperm viability and sperm with stained mitochondria (***P* < 0.01; ****P* < 0.001).

Table II. Values for the correlations (R) between a) viability values (blue blackground); b) mitochondrial staining using MitoTracker Green (MT-G), MitoTracker Red (MT-R) and JC-1 (pink background); c) Viability and mitochondrial staining (green background). $P < 0.001$ in all cases. See text for details.

	Viability MT-G	Viability MT-R	Viability JC-1	MT-G staining	MT-R staining
Viability MT-R	0.726				
Viability JC-1	0.788	0.628			
MT-G staining	0.585				
MT-R staining		0.712		0.833	
JC-1 staining			0.591	0.643	0.764

Effect of KCN, FCCP and Valinomycin

To evaluate whether each of the mitochondrial probes stains sperm mitochondria according to MMP, samples were previously incubated in either the presence or absence (control) of a) the ETC-inhibitor KCN; b) the mitochondrial uncoupling agent FCCP; c) the potassium ionophore valinomycin. Additionally, and to evaluate the capacity of the dyes to respond to MMP alterations after entering mitochondria, a second set of experiments were done in which the samples were first incubated with the mitochondrial probes and then with the drugs.

KCN

The pre-incubation of sperm with 5 mM KCN for 30 min resulted in significant decreases in both sperm viability (as assessed by the Live/Dead sperm viability kit) and mitochondrial staining with the three mitochondrial probes (Fig. 3a). Importantly, the decrease in mitochondrial staining was more accentuated than the decrease in the percentage of viable sperm, showing that, at least in some sperm, the loss of mitochondrial staining was not related with sperm dying. Indeed, the mean percentage of "Live, Mito-" sperm was significantly higher in samples pre-incubated with KCN (means ± SEM = 26.4 ± 2.9; 34.6 ± 8.7 and 28.6 ± 2.0 for MT-G, MT-R and JC-1, respectively) compared to controls (4.8 ± 3.0

for MT-G, $P < 0.01$; 2.0 ± 1.8 for MT-R, $P < 0.01$; 3.2 ± 1.2 for JC-1, $P < 0.001$). Thus, the incubation with KCN resulted in a decrease in mitochondrial staining, irrespectively of the mitochondrial probe used. Moreover, and while the decrease in mitochondrial staining seemed more accentuated in JC-1, no significant differences were found when comparing the threes probes. Noteworthy, for JC-1 staining, the KCN pre-incubation resulted in significant decreases in both the "orange" staining (% relative to control = 0.6 ± 0.6; $P < 0.001$) and the "green" staining (% relative to control = 19.2 ± 14.3; $P < 0.05$).

Incubation with KCN after staining with the probes (n=5) resulted in a similar decrease in sperm viability (Fig. 3b). On the other hand, the mean percentage of sperm stained in the midpiece decreased only slightly for JC-1. In accordance, JC-1 staining was significantly different from that of both MT-G and MT-R. Importantly, such decrease in JC-1 staining occurred particularly for the "orange" pattern (% relative to control = 35.1 ± 13.2; $P < 0.05$). Conversely, the mean percentage of sperm with green midpiece increased, but this was not significant (% relative to control = 148.6 ± 25.5; $P > 0.05$).

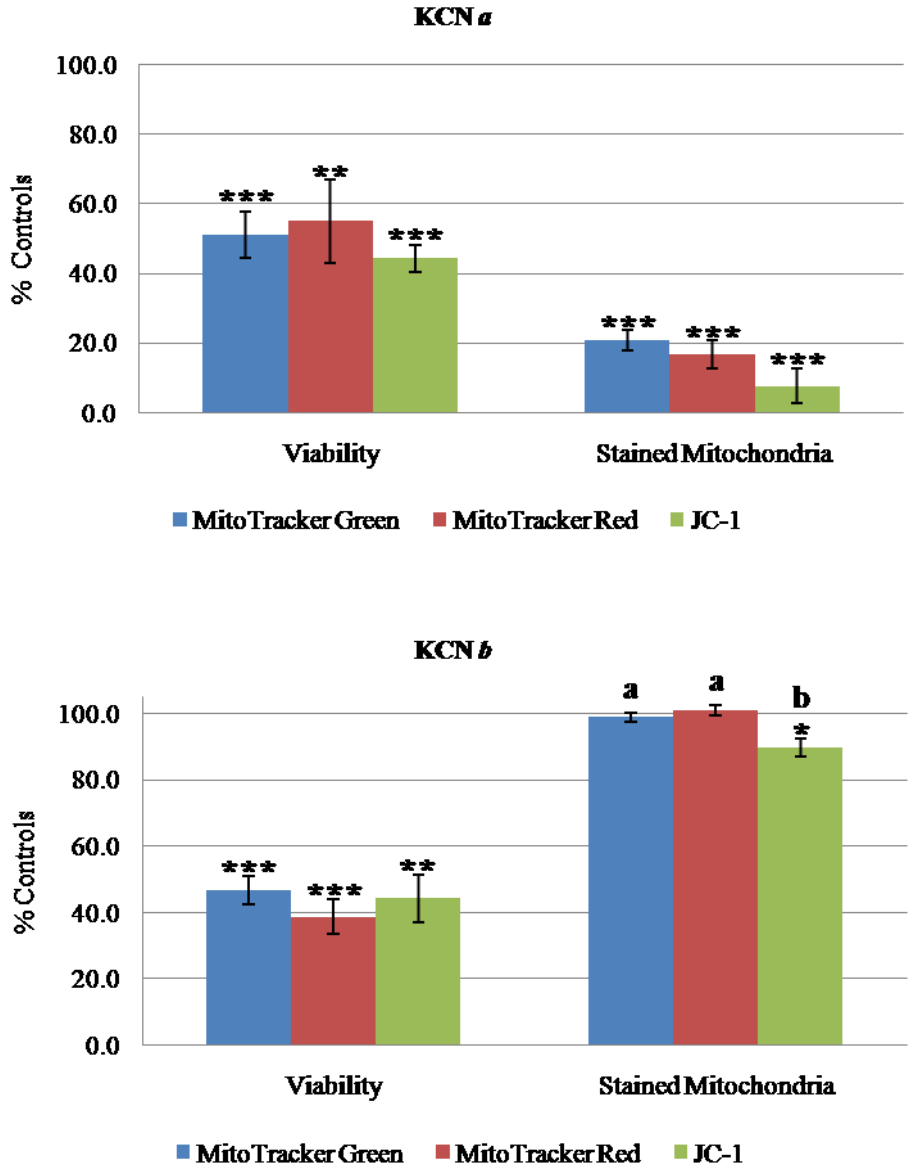

Figure 3. Effect of KCN in sperm viability and mitochondrial staining. Sperm was stained with the Live/Dead Sperm viability kit and 1) MitoTracker Green; 2) MitoTracker Red; 3) JC-1. **KCN *a*)** Incubation with KCN before staining with the nuclear and mitochondrial probes (n=5). **KCN *b*)** Incubation with KCN after staining with the nuclear and mitochondrial probes (n=5). For each case, the percentages of viable sperm and of sperm with stained mitochondria were determined. Data are expressed as means ± SEM of percentages relative to controls. Asterisks denote significant differences relative to controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). *a* and *b* denote significant differences between the mitochondrial probes (JC-1 *vs.* MT-G, $P < 0.05$; JC-1 *vs.* MT-R, $P < 0.01$).

FCCP

Incubation of sperm with 1 μM FCCP for 2 h, either before or after incubation with the dyes, resulted in a slight decrease in sperm viability, but this was not statisticaly significant (*Ps* > 0.05; Fig. 4). Similarly to the results with KCN, when sperm samples were pre-incubated with FCCP, the mean percentage of sperm stained in the midpiece decreased for the three mitochondrial probes (Fig. 4a). Thus, in the experimental conditions used here, it was possible to alter mitochondrial staining without affecting sperm viability. Moreover, the results were similar ($P > 0.05$) for the three mitochondrial probes. The incubation of sperm with FCCP after incubation with the probes resulted in a decrease in the mean percentage of sperm stained in the midpiece for JC-1 only (Fig. 4b). However, when comparing the three probes, no differences were found ($P > 0.05$). Remarkably, the decrease in JC-1 stained sperm was only significant when considering the two patterns of staining together ("orange" and "green"), for both experimental conditions (*i.e.*, incubation with FCCP before and after staining with the mitochondrial probes).

Valinomycin

Similarly to the outcomes obtained with KCN and FCCP, the pre-incubation of sperm with 1 μM valinomycin for 2 h resulted in a significant decrease in the mean percentage of sperm stained in the midpiece for the three mitochondrial probes (Fig. 5a). For JC-1, there was a significant decrease in the "orange" staining (% relative to control = 8.2 ± 2.4; $P < 0.001$) and a significant increase in the "green" staining (% relative to control = 390.2 ± 95.8; $P < 0.05$). Incubation with valinomycin after staining with the mitochondrial probes resulted in no significant differences relative to control (Fig.5b).

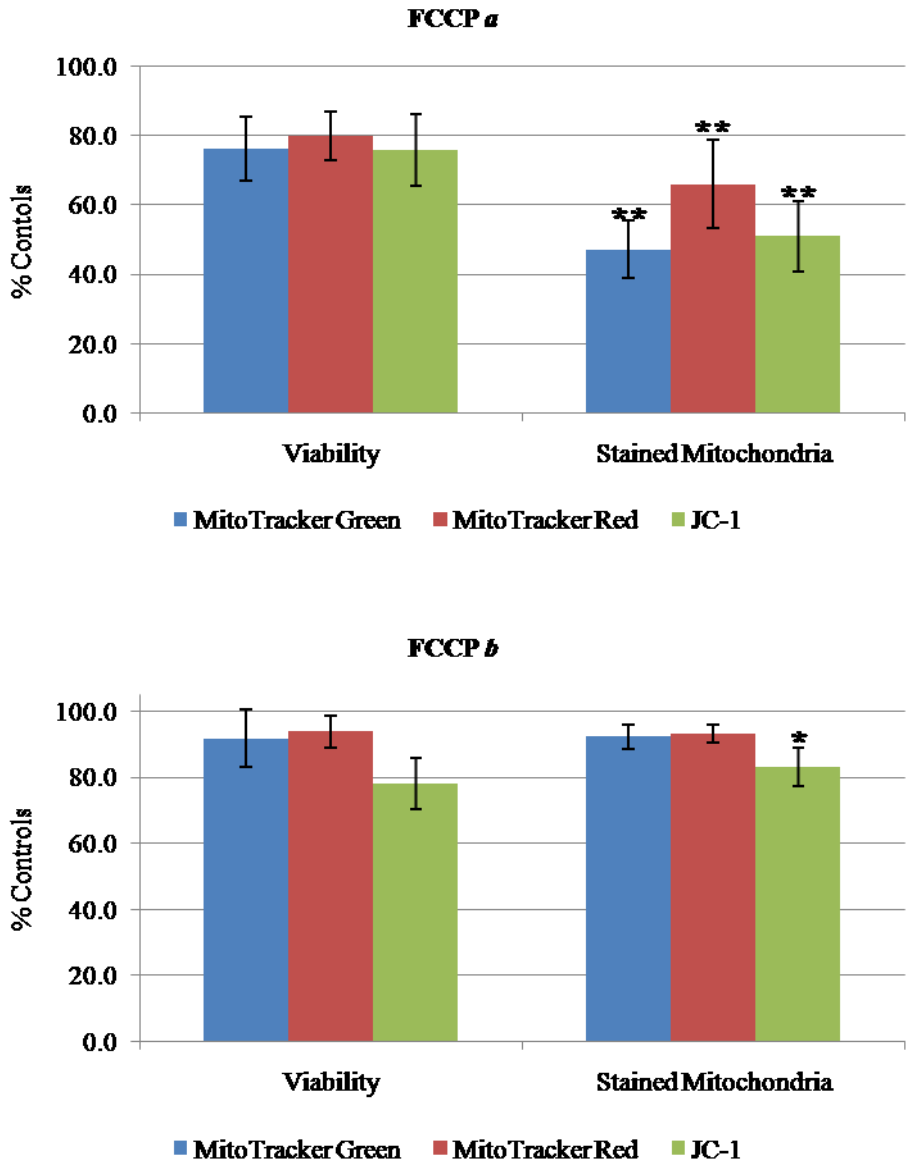

Figure 4. Effect of FCCP in sperm viability and mitochondrial staining. Sperm was stained with the Live/Dead Sperm viability kit and 1) MitoTracker Green; 2) MitoTracker Red; 3) JC-1. **FCCP a)** Incubation with FCCP before staining with the nuclear and mitochondrial probes (n=5). **FCCP b)** Incubation with FCCP after staining with the nuclear and mitochondrial probes (n=5). For each case, the percentages of viable sperm and of sperm with stained mitochondria were determined. Data are expressed as means ± SEM of percentages relative to controls. Asterisks denote significant differences relative to controls (*$P < 0.05$; **$P < 0.01$).

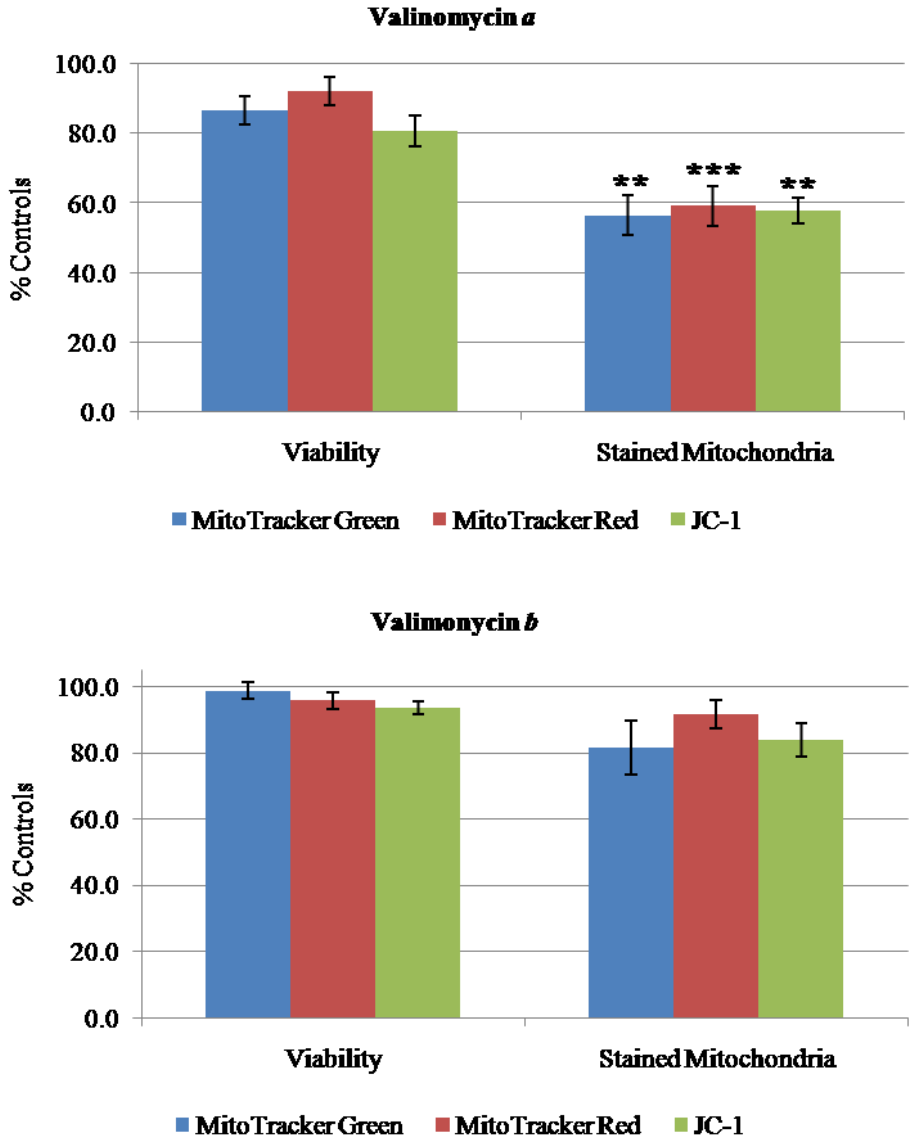

Figure 5. Effect of valinomycin in sperm viability and mitochondrial staining. Sperm was stained with the Live/Dead Sperm viability kit and 1) MitoTracker Green; 2) MitoTracker Red; 3) JC-1. **Valinomycin *a*)** Incubation with valinomycin before staining with the nuclear and mitochondrial probes (n=5). **Valinomycin *b*)** Incubation with valinomycin after staining with the nuclear and mitochondrial probes (n=5). For each case, the percentages of viable sperm and of sperm with stained mitochondria were determined. Data are expressed as means ± SEM of percentages relative to controls. Asterisks denote significant differences relative to controls (***P* < 0.01; ****P* < 0.001).

Mitochondrial probes and sperm motility

As the percentage of sperm stained with MT-G, MT-R and JC-1 seem to depend on MMP, the relationship between the staining with the three mitochondrial probes and sperm

motility has been determined, in the 40 samples analysed. To this extent, a positive correlation was found between the mean percentage of sperm stained with each of the three probes and the percentage of sperm with progressive motility (% a+b; Table III). Moreover, and as expected, there was a negative correlation between mitochondrial staining and the percentage of immotile sperm (% d). Noteworthy, the motility of the samples used was high (mean ± SEM = 60.7 ± 2.8) and almost all samples were normal for this parameter.

Table III. Values for the correlations (R) between the mean percentage of sperm stained with a) MitoTracker Green (MT-G); b) MitoTracker Red (MT-R); c) JC-1; and sperm motility assessed according to the World Health Organization guidelines (WHO, 1999). *P < 0.005; **P < 0.001.

	Sperm with progressive motility (% a+b)	**Immotile sperm (% d)**
MT-G	0.353*	- 0.368*
MT-R	0.426**	- 0. 429**
JC-1	0.318*	- 0.321*

Expression of POLG and COXI in subpopulations of sperm

a) MitoTracker positive versus MitoTracker negative

To understand if POLG and COXI expressions are correlated with sperm MMP, sperm samples were stained with MT-G, which, had been shown to stain human sperm according to the mitochondrial status at the time of incubation (above). After staining, a FACSaria sorter was used to separate two subpopulations from each sample (Fig. 6a): MitoTracker positive sperm (with polarised mitochondria) and MitoTracker negative sperm (with depolarised mitochondria). The distinct subpopulations were studied and compared for the expression of POLG and COXI evaluated by ICC (n=31). MitoTracker-positive subpopulations presented a higher percentage of sperm expressing POLG (65.3 ± 4.1) and COXI (63.2 ± 2.7) than MitoTracker-negative subpopulations (42.0 ± 4.9; P < 0.01, for POLG; and 38.2 ± 2.7; P < 0.001, for COX I; Fig.6a').

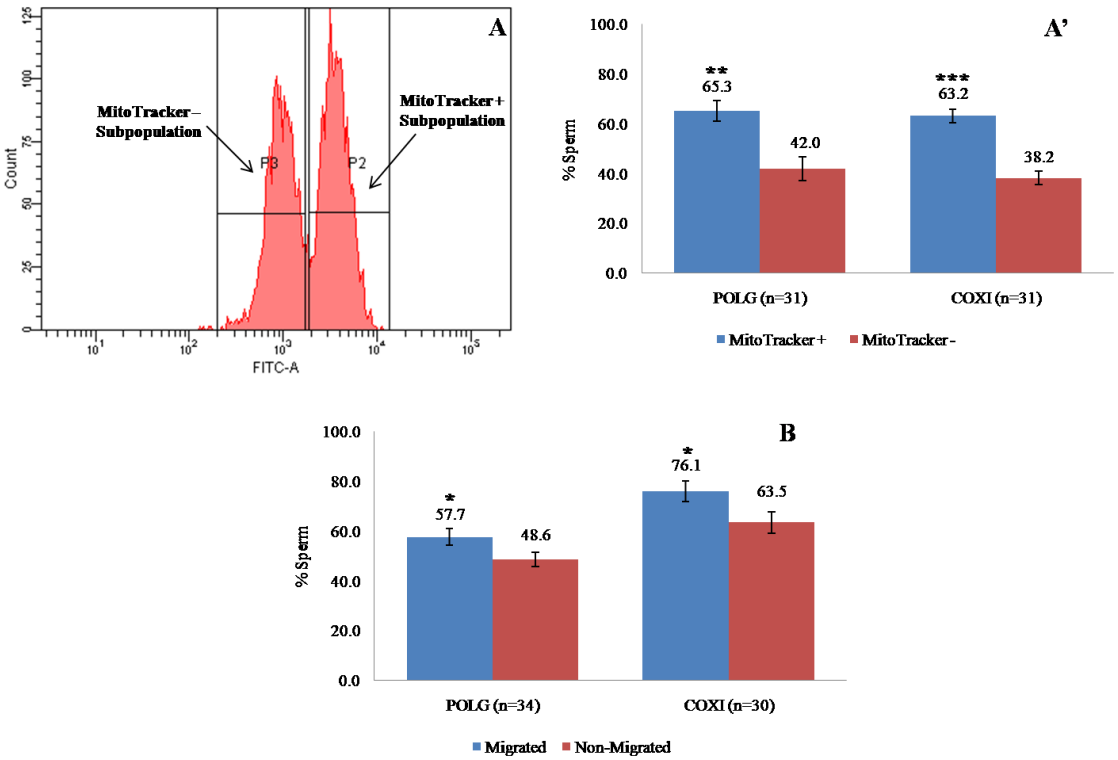

Figure 6. Subpopulations of human sperm present distinct mitochondrial-traits. **A)** Representative histogram of a sperm sample incubated with MitoTracker Green. Two subpopulations can be isolated using cell-sorting: MitoTracker positive (stained sperm) and MitoTracker negative (unstained sperm). **A', B)** Expression of POLG and COXI in subpopulations of human sperm with: A') distinct mitochondrial membrane potential; B) distinct motility. Sperm subpopulations, recovered after cell sorting (A') or the swim-up procedure (B) were independently stained with a) anti-human POLG antibody; b) anti-human COXI antibody. The percentage of stained sperm was determined. Data are expressed as means ± SEM. Asterisks denote significant differences between the subpopulations (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

b) Migrated versus non-migrated

To determine if the expression of the mitochondrial proteins POLG and COXI is related with the ability of sperm to swim, the classical swim-up procedure was used to separate sperm with distinct motility from a same sample. This procedure allowed the recovering of two subpopulations: the migrated subpopulation (containing motile sperm), and the non-migrated subpopulation (enriched in non-motile sperm). POLG (n=34) and COXI (n=30) expressions were analysed using ICC, as described for the sorted subpopulations. Comparison between the two subpopulations revealed that the percentage of sperm expressing

both POLG and COXI was significantly higher in migrated (mean % ± SEM = 57.7 ± 3.4 for POLG, and 76.1 ± 4.1 for COXI) than in non-migrated sperm (48.6 ± 2.9 and 63.5 ± 4.4 for POLG and COXI, respectively; *Ps* < 0.05; Fig.6b), but the difference was smaller than in the sorted subpopulations.

4. Discussion

The mitochondrial generation of ATP depends upon the existence of a transmembrane electrical gradient generated by the activity of the ETC. The maintenance of this mitochondrial membrane potential (MMP) is thus essential for mitochondrial function. This parameter can be monitored using a wide range of permeant cationic fluorochromes, that accumulate in the negatively charged mitochondrial matrix, depending on the magnitude of the transmembrane potential (Johnson *et al.*, 1981; Smiley *et al.*, 1991; Solaini *et al.*, 2007). These probes have been used in a variety of mammalian cells to study alterations in mitochondrial function during, for example, cell proliferation and differentiation (Darzynkiewicz *et al.*, 1981; 1982) and apoptosis (Metivier *et al.*, 1998; Poot and Pierce, 1999; Jayaraman, 2005).

A number of recent studies have shown a strong association between the percentage of human sperm exhibiting high MMP, determined by the extent of probe uptake [particularly JC-1 and $DiOC_6(3)$] assessed by flow cytometry, and both sperm motility and fertilisation competence (Troiano *et al.*, 1998; Kasai *et al.*, 2002; Marchetti *et al.*, 2002; 2004). In the present study, sperm MMP has been determined by fluorescence microscopy using two MitoTracker probes, Green FM (MT-G) and Red CM-H_2XRos (MT-R), and also JC-1, independently. The use of fluorescence microscopy, while having some disadvantages compared to flow cytometry, namely the impracticability of counting thousands of cells, presents the advantage of permitting the examination of the staining patterns. Notably, this

procedure allowed the observation that a sperm sample stained with JC-1 invariably presents three subpopulations: 1) sperm presenting an orange midpiece; 2) sperm with a green midpiece; 3) sperm with unstained midpiece. Although not generally reported in the literature, the existence of a percentage of sperm without JC-1 staining has also been reported in bulls (Garner *et al.*, 1997). These outcomes may imply that, at least in sperm, JC-1 stains mitochondria with high MMP orange (which seldom represent the entire population of mitochondria in a given sperm cell), and mitochondria with medium or low membrane potential green. Untained sperm may present depolarised mitochondria. This is supported by the results presented here, where sperm previously incubated with KCN, FCCP or valinomycin, showed a significant increase in the percentage of non-stained cells when compared to controls. The reliability of JC-1 to measure human sperm MMP has also been proven by others using mitochondrial uncouplers (Troiano *et al.*, 1998; Marchetti *et al.*, 2004).

As expected, sperm stained with either MT-G or MT-R presented only two subpopulations: sperm with stained midpiece and unstained sperm. The results were very similar for these two probes, and were also highly correlated. Furthermore, and similarly to JC-1, the MitoTrackers were able to respond to MMP alterations induced by either inhibition of the ETC (pre-incubation with KCN), or OXPHOS uncoupling (pre-incubation with FCCP or valinomycin). Thus, the two subpopulations might represent sperm with polarised mitochondria (stained midpiece) and sperm presenting depolarised mitochondria (unstained midpiece). These outcomes contradict the established notion that MT-G stains mitochondria independently of MMP. Indeed, MT-G has been used in somatic cells to reveal the amount of mitochondria per cell (Metivier *et al.*, 1998; Poot and Pierce, 1999). However, MT-G staining intensity has been proven to change following treatment with various MMP-altering drugs, in three different human cell lines (Keij *et al.*, 2000), although this has been contradicted by

others (Pendergrass *et al.*, 2004). Additionally, the response of MT-G to FCCP disturbances in rat cultures seems to depend on cell-type (Buckman *et al.*, 2001). Measurement of MT-G fluorescence intensity with a fluorometer has been used in human sperm to estimate mitochondrial activity (Meseguer *et al.*, 2004). However, no experiments were undertaken to prove the effectiveness of MT-G to monitor MMP in human sperm, so one cannot compare the results presented here with previous work at this level.

The use of either chloromethyl-X-rosamine (MitoTracker Red CMXRos) or of its reduced form chloromethyl-dihydro-X-rosamine (MitoTracker Red CMH_2XRos, used in this study) to monitor MMP in somatic cells is much more consensual (Macho *et al.*, 1996; Poot *et al.*, 1996; Poot and Pierce, 1999; Pendergrass *et al.*, 2004). Moreover, and in accordance with the results presented here, flow-cytometry analysis of human sperm has shown that MT-R CMXRos effectively responds to mitochondrial uncouplers (Marchetti *et al.*, 2004).

Unsurprisingly, and as has been described in bulls (Garner *et al.*, 1997), a high correlation was found between sperm MMP, monitored by each of the three mitochondrial probes, and sperm viability. Moreover, the results for the three mitochondrial probes also correlate well, which is in accordance with other reports using not only JC-1 and MitoTrackers, but also DiOC6(3) and TMRE (Garner *et al.*, 1997; Marchetti *et al.*, 2004). These cumulative outcomes suggest that, at least in mammalian sperm, the distinct mitochondrial probes provide comparable results.

However, the mean percentage of stained sperm has been shown here to be slightly lower for JC-1 when compared to both MitoTrackers. Furthermore, JC-1 was the only probe to be able to slightly respond to MMP alterations in the experiments where samples previously stained with the mitochondrial probes where incubated with KCN or FCCP. Thus, once inside mitochondria, none of the probes were able to clearly reveal modifications in MMP, although JC-1 seems to be, to some extent, more dynamic than both MT-G and MT-R.

This dynamism seems to be more pronounced in somatic cells, given that the incubation of human (Smiley *et al.*, 1991) and rat (Mathur *et al.*, 2000) cell cultures with mitochondrial uncouplers, after incubation with JC-1, resulted in a more significant decline in orange staining, accompanied by an increase in green staining. The inability of both MitoTrackers to respond to MMP alterations after entering mitochondria may be related with their structure. Indeed, both possess chloromethyl moieties, which can bind to thiol groups of proteins, enhancing the retention of the probes (Poot *et al.*, 1996).

In any case, MT-G, MT-R and JC-1 seem to be good enough to monitor sperm MMP at the time of incubation. In addition, the percentage of sperm presenting polarised mitochondria, determined by each of the mitochondrial probes, was shown to equally correlate with sperm motility. Moreover, and while this was not quantified, it was also observed that the incubation of sperm with the mitochondrial drugs resulted in reduced motility, particularly for KCN (data not shown). Thus, these outcomes, in accordance with others (Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Kasai *et al.*, 2002; Marchetti *et al.*, 2002; 2004), seem to confirm that, among other factors, human sperm motility depends on the presence of polarised mitochondria.

In the second part of this study, MT-G was used to separate and compare subpopulations of sperm with functional and non-functional mitochondria (presenting polarised and depolarised mitochondria, respectively). The choice of MitoTracker instead of JC-1 had two main practical reasons. First, it is simpler to separate and compare two subpopulations of sperm instead of three. Also, cell-sorting could not be done exactly at the same time after the incubation period for all samples, as would be ideal. Thus, the use a non-dynamic probe seemed better, since all samples were separated according to the mitochondrial status at the time of incubation. MT-R seemed less adequate to use, as some non-specific

staining was observed in most of the samples (not shown), which would compromise cell-sorting according to mitochondrial staining.

Noteworthy, sperm with polarised mitochondria seem to also present other important cellular attributes, and to constitute a more functional subpopulation of sperm, most likely one with a higher fertilisation potential. To this extent, when compared to MT-G negative sperm, subpopulations of MT-G positive sperm presented a) a lower level of nuclear DNA fragmentation; b) a higher percentage of capacitated sperm; c) a higher percentage of sperm with intact acrosomes (Sousa, A.P., Amaral, A. *et al., in preparation*). Likewise, using a similar approach, others have shown that subpopulations of sperm with polarised mitochondria, separated using either DiOC6(3) or CMX-Ros, also presented normal morphology, higher motility and higher capacity to respond to induced acrosome reaction (Gallon *et al.*, 2006). Consequently, these and other reports (Chapter V, Marchetti *et al.*, 2002) stress that sperm MMP is a very good indicator of sperm quality.

MMP maintenance depends on the activity of the ETC, the only cellular apparatus that is encoded by both the nuclear and the mitochondrial genomes. Moreover, the expression of mtDNA-encoded proteins, such as COXI, is controlled by nuclear-encoded factors, including POLG, the sole mtDNA polymerase. It has been demonstrated here that subpopulations of sperm with polarised mitochondria presented a higher expression of both POLG and COXI, when compared to subpopulations presenting depolarised mitochondria. This suggests that the expression of these mitochondrial proteins (among, of course, other factors) might contribute for the maintenance of MMP in human sperm, and thus for mitochondrial function and sperm motility.

Similar outcomes were obtained in subpopulations of sperm fractionated accordingly to the ability to swim; *i.e.*, migrated sperm subpopulations obtained by swim-up had a higher percentage of sperm expressing POLG and COXI than their non-migrated counterparts. This

confirms that the expression of these proteins is associated with sperm motility, as has been previously demonstrated, not only for motility, but also for sperm concentration and morphology, using sperm samples of patients with distinct aetiologies (see Chapter II, Amaral *et al.*, 2007). Interestingly, the results presented here for the expression of POLG and COXI seem to suggest that the separation of sperm using MT-G and a cell-sorter is more efficient than the classical swim-up method. Indeed, for POLG and COXI expressions, the distinction between MitoTracker positive and MitoTracker negative sperm was more pronounced (and also statistically more significant) than the one observed by comparing migrated and non-migrated sperm. This is in accordance with the results obtained for other sperm parameters (Sousa, A.P., Amaral, A. *et al., in preparation*) and suggests that fractionation of sperm according to MMP can be used as a tool to isolate the best sperm in a sample.

In conclusion, it is clear that human sperm contain subpopulations with distinct mitochondrial functionality, which can similarly be analysed using diverse mitochondrial probes, including MT-G, MT-R and JC-1. Analysis of subpopulations of sperm indicated that the expression of POLG and COXI seem to be related with sperm MMP and confirmed their association with sperm motility, and also validated the evaluation of MMP as a reliable indicator for sperm function.

CHAPTER V

THE CLINICAL RELEVANCE OF DISTINCT SPERM FUNCTIONAL PARAMETERS: MITOCHONDRIA, MEMBRANE, CHROMATIN AND ACROSOME STATUS – A PRELIMINARY STUDY

Part of the methodology described in this Chapter has also been published in:

Ramalho-Santos, J., Amaral, A., Sousa, A. P., Rodrigues, A. S., Martins, L., Baptista, M., Mota, P. C., Tavares, R., Amaral, S. and Gamboa, S. (2007) Probing the structure and function of mammalian sperm using optical and fluorescence microscopy. In: Méndez-Vilas, A. and Díaz, J. (Eds) *Modern Research and Educational Topics in Microscopy*, **1**: 394 - 402.

Abstract

The clinical value of classical semen evaluation is doubtful. For this reason, a number of tests have been developed to evaluate distinct sperm functional traits. However, most the studies have analysed these parameters individually.

In the present study, freeze-thawed sperm samples, from healthy donors and normozoospermic and oligoasthenozoospermic (OA) patients, were fractionated into high and low quality fractions through density gradient centrifugation. The two fractions and the three distinct quality groups of samples were compared for the following sperm functional parameters: mitochondrial membrane potential, membrane integrity, acrosomal status and chromatin status.

The distinct parameters significantly correlated with each other, as well as with sperm motility. The higher quality sperm fractions presented lower sperm counts, but more motile cells, as well as more sperm with polarised mitochondria, integral membrane, intact acrosome and normal chromatin. The distinctiveness of each group (donors, normozoospermic and OAs) was evident when comparing sperm motility, membrane integrity and MMP. The acrosome and chromatin status seemed less relevant, as only the OA samples had a lower incidence of intact acrosomes.

Although all the traits analysed seem to be significant, none of them appear to be superior to sperm motility in describing sperm quality. However, further analyses will need to be performed in order to determine the importance of these parameters in predicting fertilising potential.

1. Introduction

The diagnosis of male infertility is traditionally dependent on the descriptive evaluation of human semen, particularly on the assessment of sperm concentration, motility and morphology. Based on these criteria, the World Health Organization (WHO, 1999) has defined threshold values that characterize a normal semen profile. However, there are doubts on the absolute clinical value of a semen analysis. This is especially so for three main reasons: first, there is a lack of standardisation in semen analysis between laboratories (Keel, 2004); second, there are differences in the sperm characteristics in distinct ejaculates of the same man (Alvarez *et al.*, 2003); and third, sperm analysis outcomes cannot completely predict if a man can be a father or not (Kvist and Bjorndahl, 2002). This is best exemplified in the so-called idiopathic patients, who present a normal semen profile, and whose female partners are fertile, but are nevertheless unable to conceive.

In the last few years, a number of tests have been developed to evaluate distinct characteristics of sperm function (Aitken, 2006; Lewis, 2007), including survival, motility patterns, mitochondrial membrane potential (MMP), acrosome and chromatin status, among others. In addition, there are different *in vitro* tests to monitor sperm-oocyte interactions, from zona pelucida binding to oocyte penetration (Muller, 2000; Liu and Baker, 2002). The former are particularly useful in predicting sperm fertilising ability, but involve elaborate protocols, as well as the use of biological material, which precludes routine use in the clinics.

Sperm viability, an obvious essential functional feature, can be monitored by the assessment of sperm membrane integrity by either dye exclusion (using trypan blue or eosin Y) or the classical hypo-osmotic swelling test (HOS; based on the principal that only sperm tails with intact membrane will swell under hypo-osmotic conditions). Fluorescent DNA-binding dyes can also be used, as is the case for the LIVE/DEAD Sperm Viability Kit (see Chapter IV). Routinely, live sperm are selected from the whole population using sperm

preparation methods such as density gradient centrifugation, to separate sperm from both seminal plasma and round cells (Gorus and Pipeleers, 1981). Additionally, this method permits the isolation of a fraction of mature, motile, morphologically normal sperm, which is then used to perform ART (Pousette *et al.*, 1986; reviewed in Henkel and Schill, 2003).

The evaluation of sperm MMP seems to constitute an important sperm functional test. The relationship between human sperm MMP and sperm motility and fertilising potential has been clearly demonstrated (see Chapter IV).

The integrity of the sperm chromatin, which is highly compacted due to its unique composition, is essential for the correct transmission of paternal genetic information. However, sperm chromatin organization and/or DNA structure can be perturbed when defects in chromatin packaging occur, or when cells are subjected to oxidative stress or abortive apoptosis (reviewed in Aitken and Krausz, 2001; Erenpreiss *et al.*, 2006). Sperm chromatin status can be assessed using a variety of techniques (for reviews see Agarwal and Said, 2003; Shamsi *et al.*, 2008), such as the single cell gel electrophoresis assay (COMET), the terminal deoxynucleotidyl transferase-mediated transferase dUTP nick end labelling (TUNEL), the sperm chromatin structure assay (SCSA) and the acridine orange test (AOT). In addition, chromatin status can also be monitored in a very simple way, using any Diff-Quik-like stain (Sousa *et al.*, 2008). Mounting evidence has shown that sperm DNA damage and/or other sperm chromatin defects are negatively associated with fertility potential both *in vivo* and *in vitro* (Evenson *et al.*, 1999; Larson *et al.*, 2000; Spano *et al.*, 2000; Duran *et al.*, 2002, just to give a few examples). For this reason, the evaluation of sperm chromatin structure/DNA damage has been indicated as a valuable tool to assess male fertility potential and predict assisted reproduction outcomes (Bungum *et al.*, 2007; Erenpreiss *et al.*, 2008).

Another proposed sperm functional test consists on the analysis of acrosome integrity. The binding of capacitated sperm to the zona pellucida triggers the acrosome reaction, an

exocytotic process that involves the fusion of the acrosomal membrane with the sperm plasma membrane, ultimately resulting in the release of acrosomal contents, including hydrolytic enzymes that assist the penetration of sperm through the oocyte's protective layers (Yanagimachi, 1994). The occurrence of the acrosome reaction is an absolute condition for *in vivo* fertilisation, and it must occur at a precise moment: when sperm is bound to the zona pellucida. Sperm that undergo the acrosome reaction prematurely (or that do not possess an acrosome) lose their ability to fertilise *in vivo*. There are a variety of methods to assess sperm acrosomal status, including labelling with antibodies against acrosome components (Wolf *et al.*, 1985; Gallo *et al.*, 1991; Albert *et al.*, 1992) or with lectin conjugates (Kallajoki *et al.*, 1986; Mortimer *et al.*, 1987; Holden *et al.*, 1990). *Pisum sativum* agglutinin (PSA), for example, is a lectin that binds to the acrosome contents of permeabilised acrosome-intact sperm, particularly to glycoproteins present in the acrosomal matrix (Liu and Baker, 1988). This lectin, normally conjugated with fluorescein isothiocyanate (FITC-PSA) can thus be used to determine sperm acrosomal status. Importantly, using this and other methods, it has been shown that human samples containing a low proportion of sperm with intact acrosome also present low *in vitro* fertilising potential (Liu and Baker, 1988; Albert *et al.*, 1992; Chan *et al.*, 1999).

Therefore, besides being motile and morphological normal, sperm must possess other attributes to be functionally competent. It is however worth mentioning that most of the studies performed so far assessed these functional parameters individually, and each group tend to claim its "own selected attribute" as the one that better predicts sperm functionality. In the present study, the status of distinct cell components (plasma membrane, mitochondria, chromatin and acrosome) was determined in distinct sperm samples from both healthy donors and patients. The aim was to determine: 1) if, and how, these parameters are correlated with each other and with motility, a classical sperm quality parameter; 2) which (if any) of these

parameters is a better predictor of sperm fertilising potential. It is important to mention that the present study is a preliminary approach, and that a multicenter study, with access to a larger number of samples, will be required to fulfil the aims proposed.

2. Materials and methods

Chemicals

All chemicals were supplied by Sigma Chemical Company (St. Louis, MO, USA) unless stated otherwise.

Biological Material

Healthy donors and patients undergoing fertility treatment were recruited from the Fertility Clinic (Tambre Clinic, Madrid, Spain) and all signed informed consent forms. Fresh semen samples were obtained by masturbation after 3 to 5 days of sexual abstinence, and were used in accordance with the appropriate ethical and Institutional Review Board (IRB) of the Tambre Clinic. Routine seminal analysis was performed according to the World Health Organization Guidelines (WHO, 1999) and samples were categorised based on sperm concentration and motility.

Sample freezing

Samples were frozen in TEST buffer with 6% (w/v) glycerol and 10% (w/v) egg yolk as cryoprotectants, as previously described (Alvarez and Storey, 1992), and were kept in liquid nitrogen until further processing.

Sample thawing and pos-thawing processing

Semen samples were thawed by incubation in a water bath for 10 min at 37ºC. After determination of sperm concentration and motility, samples were prepared by density gradient

centrifugation using ISolate® Sperm Separation Medium (Irvine Scientific, Santa Ana, CA, USA) according to the manufacturer's protocol. The two sperm fractions (higher density, which corresponds to higher quality sperm, and lower density, corresponding to lower quality sperm) were separately recovered, washed and resuspended in Sperm Preparation medium (Medicult, Jyllinge, Denmark). For each fraction, sperm concentration and motility were determined.

Determination of mitochondrial membrane potential (MMP) and of membrane integrity

Sperm mitochondrial membrane potential and membrane integrity were evaluated using MitoTracker Green FM (M-7514; Molecular Probes, Eugene, OR, USA) and the LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes), respectively. A general procedure was performed as described previously (Chapter IV, Materials and methods section). However, as live sperm suspensions were prepared in Sperm Preparation medium, this medium was used to prepare secondary stock solutions of MitoTracker Green, SYBR-14 and propidium iodide.

Determination of acrosomal status

Acrosomal status was determined using the acrosome content marker *Pisum sativum* agglutinin, linked to fluorescein isothiocyanate (FITC-PSA). Sperm were attached to coverslips and were fixed, permeabilised and blocked as previously described (Chapter II, Materials and methods section). Samples were then incubated with FITC-PSA (diluted 1:200 in blocking solution) for 20 min at 37º C. After rinsing, coverslips were mounted as formerly described (Chapter II, Materials and methods section), and observed using a Zeiss Axiophot II microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band pass filter. Sperm showing green staining in the whole acrosomal region were considered to present an intact

acrosome. Unstained sperm, or sperm presenting green staining in the equatorial region or an irregular staining in the acrosome region, were considered acrosome-reacted. In order to determine the percentage of sperm presenting intact acrosome 200 sperm were counted, in at least four fields.

Determination of chromatin status

Chromatin status was evaluated using the Diff-Quik stain set (Dade Behring Inc., Newark, USA), as recently established (Sousa *et al.*, 2008). This protocol is based on the intensity of nuclei staining: normal sperm nuclei stain lightly, while abnormal nuclei (containing damaged chromatin, *i.e.*, either decondensed or presenting fragmented DNA) stain dark. Sperm smears were prepared in microscope slides and sequentially exposed to the three components of the stain set (10 sec each): 1) methanol (fixation); 2) eosin (stains basic proteins red); 3) thiazin (stains DNA blue). After rinsing in water, slides were allowed to air-dry and were then examined using an optical microscope. The percentage of sperm with dark nuclei was determined by counting 200 sperm, in at least four different fields.

Statistical analysis

Statistical analysis was carried out using SPSS for Windows (version 13, Chicago, IL, USA). All variables were checked for normal distribution using the one-sample Kolmogorov-Smirnov test. Pearson's and Spearman's tests, for parametric and non-parametric data, respectively, were applied to assess the correlations between the distinct variables. Independent-samples T-test (or the non-parametric Mann-Whitney test, when distributions were not normal) was used to compare the results for the distinct sperm quality fractions. The One-way ANOVA test was used to compare results for the three groups of samples. Post-Hoc analyses were done using either Tukey's or Dunnet's tests, depending on whether equal variances were assumed or not. $P < 0.05$ was considered significant.

3. Results

Spermiogram results in fresh and freeze-thawed samples

This study included a total of 48 semen samples: 21 from healthy donors and 27 from patients undergoing fertility treatment (Table I). Of these, 17 were normozoospermic (*i.e.* concentration ≥ 20 millions of sperm/ml, ≥ 50% motile sperm) and 10 were oligoasthenozoospermic (OA; samples with low concentration and motility). As expected, after freeze-thawing, both the concentration and motility of the samples had changed. However, and importantly, donors' samples preserved better characteristics when compared to both normozoospermic and OA samples (Table I).

Table I. Concentration and progressive motility of the sperm samples used in this study (n=48). Data are expressed as means ± SEM. Superscripts *a*, *b* and *c* denote significant differences between groups (*Ps* < 0.05).

	FRESH SAMPLES		**FREEZE-THAWED SAMPLES**	
	Concentration (10^6 sperm /ml)	**Motility (% a+b)**	**Concentration (10^6 sperm/ml)**	**Motility (% a+b)**
Donors (n=21)	96.0 ± 7.0^{a}	66.8 ± 2.6^{a}	51.2 ± 5.8^{a}	36.2 ± 3.2^{a}
Normo (n=17)	56.6 ± 4.9^{b}	55.4 ± 2.9^{b}	33.2 ± 5.7^{ab}	19.1 ± 3.3^{b}
OA (n=10)	12.5 ± 3.8^{c}	27.8 ± 3.4^{c}	20.9 ± 10.7^{b}	9.9 ± 2.8^{b}

Abbreviations: Normo – normozoospermic; OA – Oligoasthenozoospermic.

Correlations between the distinct sperm functional parameters

In order to understand the possible significance of different sperm functional parameters as novel tools to better evaluate sperm quality, distinct cellular features were analysed in all the samples included in the study. These were: sperm mitochondrial membrane potential (MMP; expressed as the percentage of sperm presenting MitoTracker Green staining in the midpiece; Fig. 1a), sperm membrane integrity (evaluated by means of the dual SYBR-14/PI probes, and expressed as the percentage of sperm stained with SYBR-14 only, i.e, those with intact membrane; Fig. 1a), acrosome status (percentage of sperm presenting an intact

acrosome, as judged by PSA-FITC staining; Fig. 1b) and chromatin status (percentage of sperm presenting dark nuclei, *i.e.*, those with abnormal chromatin, as assessed by a recently established method using the Diff-Quik stain; Figs. 1c,c'). The correlations between these parameters with each other and with both total (% a+b+c) and progressive (% a+b) motility were established (Table II). Essentially, all the parameters correlate positively and significantly with each other, except for chromatin status, which, as expected, presented negative correlations with the other parameters. Importantly, the highest correlations were found between sperm motility, MMP and membrane integrity.

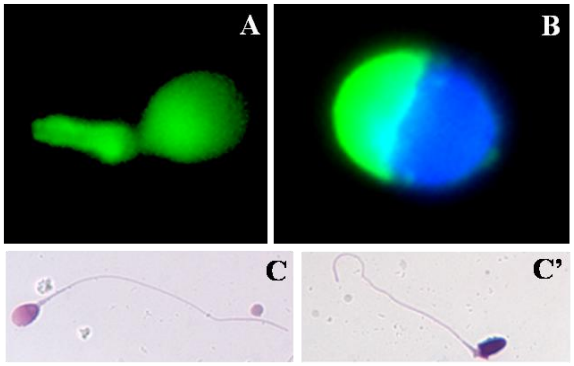

Figure 1. Human sperm functional characteristics can be monitored using distinct markers and observed under fluorescence (A and B) or light (C and C') microscopy. **A)** Sperm presenting polarised mitochondria and integral membrane after staining with both MitoTracker Green (green midpiece) and the Live/Dead sperm viability kit (green head). **B)** Sperm presenting an intact acrosome after staining with FITC-conjugated *Pisum sativum* agglutinin (green acrosome). Nuclear DNA was counterstained with DAPI (blue head). **C, C')** Sperm samples stained with the Diff-Quik set present both pale (C – normal chromatin) and dark (C' – abnormal chromatin) heads. Note that the scales of the distinct pictures are different.

Table II. Values for the significant correlations (*R*; upper numbers) between the distinct sperm parameters analysed. *P* values are also indicated (lower numbers).

	Progressive motility	**MMP**	**Membrane integrity**	**Acrosomal status**	**Chromatin status**
Total motility	0.982 *<0.001*	0.866 *<0.001*	0.860 *<0.001*	0.332 *0.001*	-0.339 *0.001*
Progressive Motility		0.840 *<0.001*	0.834 *<0.001*	0.345 *0.001*	-0.358 *<0.001*
MMP			0.986 *<0.001*	0.329 *0.001*	-0.343 *0.001*
Membrane integrity				0.328 *0.001*	-0.333 *0.001*

Details: Total motility - % a+b+c (WHO grades); Progressive motility - % a+b; Mitochondrial membrane potential (MMP) - sperm with polarised mitochondria; Membrane integrity - sperm with intact membrane; Acrosomal status - sperm presenting intact acrosome; Chromatin status - sperm presenting abnormal chromatin.

Comparison between different quality sperm fractions

In order to understand the relevance of the sperm parameters analysed, samples were fractionated using a density gradient centrifugation, and two subpopulations were recovered from each sample: sperm from the higher and the lower density fractions, corresponding to higher and lower quality sperm, respectively. For each sample, the percentage of sperm recovered in each fraction was determined ("recovered sperm"; calculated by dividing the concentration of sperm in each fraction by the total concentration of sperm in the non-fractionated sample). The percentage of sperm recovered in the higher quality fraction (33.7 ± 2.6) was significantly lower than the percentage of sperm in the lower quality fraction (66.3 ± 2.6; $P < 0.001$; Table III). On the other hand, and as expected, the higher quality sperm fraction presented a significantly higher percentage of motile sperm, as well as a higher percentage of sperm with progressive motility (*Ps* < 0.001; Table III, Fig. 2). Likewise, this

fraction showed higher percentage of sperm presenting a) polarised mitochondria; b) membrane integrity; c) intact acrosome; d) normal chromatin (*Ps* < 0.05; Table III, Fig. 2).

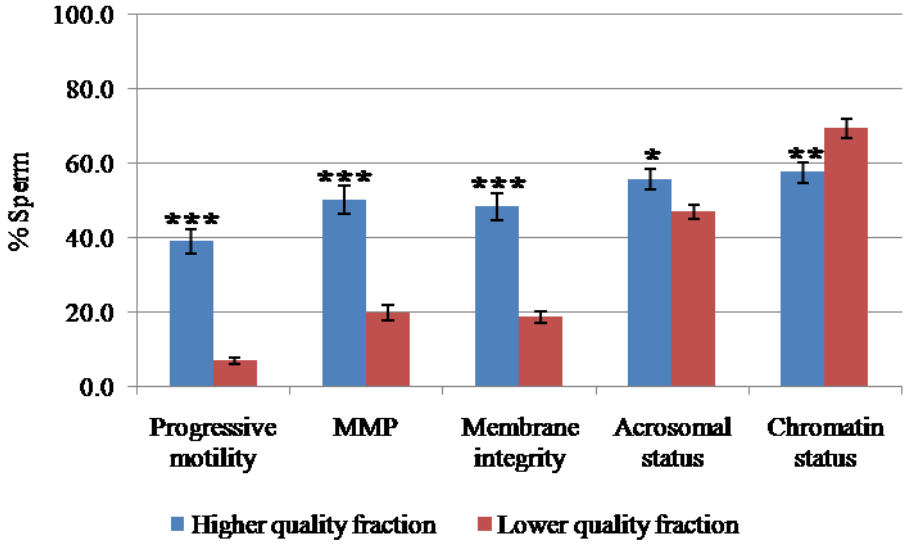

Figure 2. Comparison between higher- and lower quality sperm fractions (obtained by gradient centrifugation; n = 48) for the following parameters: progressive motility (% a+b), mitochondrial membrane potential (MMP; sperm with polarised mitochondria); membrane integrity (sperm with intact membrane); acrosomal status (sperm presenting intact acrosome); chromatin status (sperm presenting abnormal chromatin). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table III. Sperm functional parameters in distinct fractions/samples. Data are expressed as means percentage ± SEM. Comparison between higher and lower quality sperm fractions obtained through density centrifugation: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Comparison between different quality samples (donors; normozoospermic; oligoasthenozoospermic - OAs) for the higher quality fractions: superscripts *a*, *b* and *c* denote significant differences between groups (*Ps* < 0.05).

	Recovered sperm	Total motility	Progressive motility	MMP	Membrane integrity	Acrosomal status	Chromatin status
Higher-quality sperm fraction	33.7 ± 2.6***	42.5 ± 3.5***	38.9 ± 3.2***	50.0 ± 3.8***	48.2 ± 3.7***	55.5 ± 2.7*	57.4 ± 2.8**
Lower-quality sperm fraction	66.3 ± 2.6	9.7 ± 1.1	6.8 ± 0.9	19.8 ± 2.0	18.5 ± 1.7	46.8 ± 1.8	69.4 ± 2.6
Donors	40.8 ± 3.8^{a}	58.0 ± 3.4^{a}	53.8 ± 2.9^{a}	67.9 ± 3.3^{a}	64.6 ± 3.5^{a}	58.8 ± 4.5^{a}	54.7 ± 4.8
Normozoospermic	31.1 ± 4.1^{ab}	40.1 ± 5.2^{b}	36.0 ± 4.8^{b}	45.6 ± 5.5^{b}	44.9 ± 5.5^{b}	58.6 ± 3.6^{a}	53.6 ± 4.4
OAs	22.1 ± 5.3^{b}	14.3 ± 5.7^{c}	12.4 ± 4.7^{c}	19.7 ± 6.0^{c}	19.5 ± 6.5^{c}	40.8 ± 4.3^{b}	69.6 ± 3.4

Details: Recovered sperm - % sperm recovered in each fraction; Total motility - % a+b+c (WHO grades); Progressive motility- % a+b (WHO grades); Mitochondrial membrane potential (MMP) - % sperm with polarised mitochondria; Membrane integrity - % sperm with intact membrane; Acrosomal status - % sperm presenting intact acrosome; Chromatin status - % sperm presenting abnormal chromatin.

Comparison between different quality sperm samples

To further determine the relevance of the sperm functional parameters analysed in this study, the three groups of samples (donors, normozoospermic and OAs) were also compared. When comparing the higher quality sperm fractions, the ones used in ART, the three groups were found to be distinct from each other (*Ps* ≤ 0.01) for total and forward motility, MMP, and membrane integrity (Table III; Fig. 3); with donors presenting the best results and OAs presenting the worst. Furthermore, the percentage of sperm recovered in the higher quality fraction was significantly higher in the donors compared to the OA group (*P* < 0.05). Also, both the donors and the normozoospermics presented more sperm with intact acrosomes, when compared to the OAs (*Ps* < 0.05).

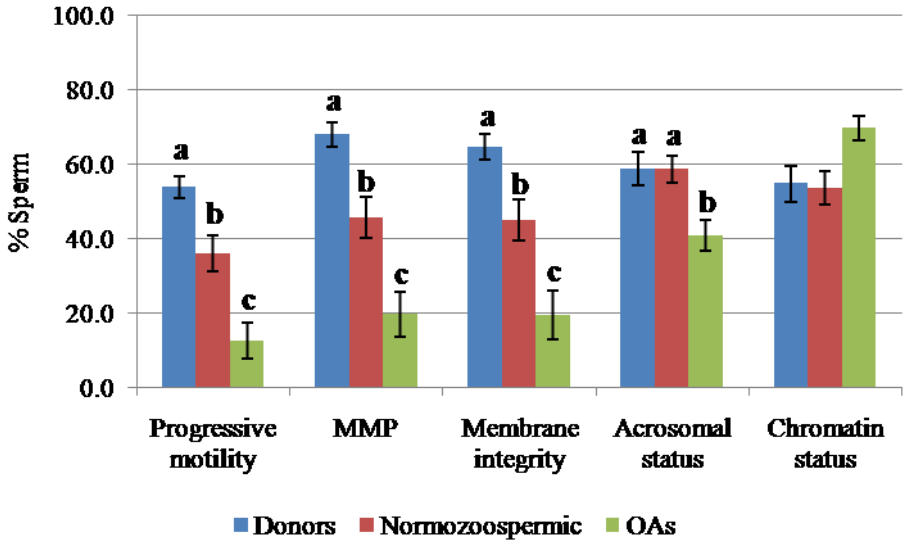

Figure 3. Comparison between the higher quality sperm fractions of distinct sperm samples: donors (n=21); normozoospermic (n=17); and oligoasthenozoospermic (OAs; n=10). Details: progressive motility (% a+b); mitochondrial membrane potential (MMP; sperm with polarised mitochondria); membrane integrity (sperm with intact membrane); acrosomal status (sperm presenting intact acrosome); chromatin status (sperm presenting abnormal chromatin). Superscripts *a*, *b* and *c* denote significant differences between groups for each of the parameters analysed (*Ps* < 0.05).

As anticipated, the differences between groups were less significant in the lower quality fractions, although donor samples still presented the best characteristics (data not shown).

4. Discussion

The standard semen parameters (sperm concentration, morphology and motility), while indicative of some degree of semen quality, do not assess sperm function, and their clinical significance in predicting fertility has been debated (Polansky and Lamb, 1988; Bonde *et al.*, 1998; Guzick *et al.*, 2001). To be able to fertilise an oocyte, a semen sample must contain functionally competent sperm. Understanding which sperm functional attributes are essential for fertilisation and establishing cut-off values for each parameter are among the most challenging subjects in modern andrology.

In the present study, a number of sperm functional parameters has been evaluated, not only in distinct quality sperm samples, but also in different fractions of each sample. Besides viability, a basic and essential cellular feature, the function of the three main components of sperm was analysed: the head (by assessing both chromatin and acrosomal status), the midpiece (mitochondrial membrane potential) and the tail (whose function was indirectly assessed through motility analysis).

The results for these distinct parameters were significantly correlated. Particularly, and as expected, sperm membrane integrity and MMP were highly correlated with each other, as well as with motility. Similar outcomes have been described by others, using similar techniques, not only in humans (Evenson *et al.*, 1982; Auger *et al.*, 1989; Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Marchetti *et al.*, 2002), but also in other mammals, including bovines (Garner *et al.*, 1997; Thomas *et al.*, 1997; 1998), stallions (Love *et al.*, 2003) and boars (Huo *et al.*, 2002). Moreover, although to a lesser extent, these parameters were also correlated with

both acrosomal and chromatin integrities. Similarly, others have described negative correlations between chromatin defects and sperm viability, motility and MMP (Donnelly *et al.*, 2000; Marchetti *et al.*, 2002; Varum *et al.*, 2007; Sousa *et al.*, 2008). Likewise, a relationship between sperm acrosomal integrity and motility and viability has been demonstrated both in humans (Liu and Baker, 1988) and bovines (Thomas *et al.*, 1997; 1998), where a correlation with MMP was also reported. Taken together, these results suggest that sperm have the property of maintaining a functional equilibrium in the distinct cellular compartments, and that when this homeostasis is perturbed, all the elements are affected.

The relevance of the sperm parameters analysed here was demonstrated by the comparison between distinct quality sperm fractions/samples. Fractionation of sperm using density gradient centrifugation is a worldwide technique used to select sperm for many ART procedures (reviewed in Henkel and Schill, 2003). This method permits the recovery and subsequent comparison of two sperm fractions from each sample: one enriched in motile, morphologically normal sperm (high density fraction), and another containing mainly dead sperm and immature cells (low density fraction). In conformity with this, the results presented here have shown that the two fractions were indeed significantly different, not only for motility and viability (membrane integrity), but also in terms of mitochondrial, chromatin and acrosomal status. Likewise, it has been shown that sperm from low density fractions present, not only a higher incidence of nuclear DNA fragmentation, but also more large-scale deletions in the mitochondrial genome (O'Connell *et al.*, 2003), as well as enhanced ROS formation (Aitken and West, 1990). These outcomes confirm the usefulness of the density gradient centrifugation as a method to select the more functional sperm of a sample, on one hand, and illustrate the significance of the various parameters analysed, on the other.

Interestingly, it has been shown that the majority of sperm in each sample belonged to the lower quality fraction, thus supporting the necessity of efficient sperm preparation

techniques. In addition, and as expected, the percentage of sperm recovered in the higher quality fraction was higher in donor samples when compared to OAs. The distinctiveness of each group (donors, normozoospermic and OAs) was also evident from the comparison between sperm motility, membrane integrity and MMP. The acrosome and chromatin status seemed less relevant, as only the OA group presented a lower percentage of sperm with intact acrosome.

Thus, apart from motility, conventionally evaluated in any semen analysis, sperm viability and MMP seem to constitute the more relevant functional parameters. Using fresh semen samples, others have suggested that analysis of MMP is, together with viability, nuclear DNA fragmentation and ROS production, the most sensitive test to determine sperm quality (Marchetti *et al.*, 2002). The results of the present work suggest, however, that sperm membrane integrity and mitochondrial function are almost coincident aspects, at least in frozen samples analysed immediately after thawing and processing. Whatever the case may be, MMP seems to be a good indicator of sperm quality, not only because better quality samples present more sperm with polarised mitochondria (present work, Marchetti *et al.*, 2002; Wang *et al.*, 2003; Liu *et al.*, 2004), but also because this functional parameter seems to be correlated with *in vitro* fertilising potential (Kasai *et al.*, 2002; Marchetti *et al.*, 2002; 2004). The high correlation found between sperm motility and MMP seems to validate the importance of mitochondrial-produced ATP for sperm movement.

The relevance of the sperm acrosome is best exemplified in those infertile men whose sperm present round acrosomeless heads and are not able to penetrate zona-free hamster oocytes (Weissenberg *et al.*, 1983; Jeyendran *et al.*, 1985; Lalonde *et al.*, 1988). Such a condition is, however, rare. Nevertheless, sperm samples with low percentages of intact acrosomes seem to be associated with *in vitro* failed fertilisations (Liu and Baker, 1988; Chan *et al.*, 1999). The outcomes presented here are in agreement with this idea, as both lower-

density sperm fractions and poorer-quality sperm samples (OAs) possessed the lowest proportion of sperm with intact acrosome.

Sperm DNA/chromatin integrity has been suggested to be one of the most sensitive tests to assess sperm quality (Bungum *et al.*, 2007; Erenpreiss *et al.*, 2008). In accordance with this notion, this parameter seems to be impaired in infertile men (Agarwal and Said, 2005; Shamsi *et al.*, 2008). Although the results presented here could not fully corroborate this idea (at least when comparing the three groups of samples), it has been shown that lower quality sperm fractions presented a higher prevalence of chromatin anomalies. Sperm chromatin abnormalities may be particularly relevant post-fertilisation, as the activation of the paternal genome only occurs between the four- to eight-cell stages (Braude *et al.*, 1988). Concomitantly, these anomalies seem to negatively correlate with embryo development rates and with the establishment of clinical pregnancies, but not with fertilisation rates (Sousa *et al.*, 2008).

To conclude, although all the sperm functional traits analysed in the present study were shown to be significant, none of them seem to be better in describing sperm quality than the conventional parameter "motility". This study is, however, incomplete, as fertilisation potentials have not been determined yet. The sperm samples evaluated here are being used to perform ART and, in the future, the results will be correlated with clinical outcomes. Moreover, to validate the usefulness of any of the parameters analysed in predicting sperm fertilising potential, a multicenter study will be carried out. Indeed, the involvement of various institutions in this kind of studies is critical, not only to increase the number and types of samples available, but also because distinct laboratories often use distinct technical protocols, both to evaluate and prepare the male and female gametes, and to perform distinct ARTs.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Although the relative significance of mammalian sperm OXPHOS *versus* glycolysis in terms of the production of the ATP necessary for sperm motility remains controversial, mounting evidences have supported the concept that the integrity of mitochondria and their genome are critical in human sperm function. In the present study, diverse mitochondrial traits were analysed in human sperm samples, and their possible biological and clinical significances were assessed. These included: mtDNA copy number; the expression of ETC proteins (COXI and COXVIc); the expression of nuclear factors involved in mtDNA maintenance (POLG and TFAM); POLG polymorphisms and MMP.

First, it has been shown that high quality sperm have very low levels of mtDNA compared to fully differentiated somatic cells (Chapter II). The possible biological significance of this could be to facilitate the elimination of paternal mtDNA following fertilisation and thus ensure maternal inheritance of this genome. At any rate, a minimum content of functional mtDNA molecules might be necessary to ensure the expression of mitochondrial-encoded proteins, such as COXI, which has been shown to be related with sperm quality. On the other hand, poorer quality sperm presented higher mtDNA copy numbers, which suggests that there are errors in the regulation of mtDNA replication in men who will ultimately present poor quality sperm. The high mtDNA content in poor quality samples may be an indicator of defective spermiogenesis, and may also reflect problems in energy metabolism in both testicular cells and mature male gametes.

The outcomes also suggested that POLG, possibly in conjunction with other factors, may play a role in regulating mtDNA copy in human sperm, although the exact mechanism remains unclear. To completely understand this regulation, the analysis of ejaculated sperm is not sufficient. Instead, it seems necessary to consider the distinct cellular stages of human spermatogenesis and spermiogenesis and, possibly, epididymal maturation. To this extent, both mtDNA content and POLG expression should be analysed in testicular/epididymal

biopsies or using *post-mortem* organs. The study of POLG at the RNA level should also be considered, to test the hypothesis of the existence of testis-specific transcripts isoforms.

Nevertheless, it became clear that the expression of mtDNA maintenance factors and of components of the ETC (either mtDNA- or nuclear DNA-encoded) is associated with sperm quality. The expression of other factors involved in mtDNA maintenance (such as POLG2 or mtSSB) or of components of other ETC complexes should also be examined.

The analysis of *POLG* CAG-repeat polymorphisms has shown an association between the heterozygous genotype and very poor quality sperm (Chapter III). However, the biological significance of these variants remained uncertain, as these do not seem to affect POLG expression, or to impair mtDNA maintenance, or the expression of sperm mitochondrial proteins. Moreover, no correlations were found between *POLG* genotypes and each of the three principal sperm parameters. The clinical relevance of these polymorphisms is thus doubtful. It should however be noticed that this does not exclude the involvement of *POLG* mutations in male infertility. Thus, analysis of the entire *POLG* coding region in distinct quality sperm samples is warranted. Moreover, genes for a) other components of the mtDNA replication machinery (such as Twinkle); b) factors involved in mitochondrial nucleotide metabolism (such as ANT1 or TK2); c) ETC complexes subunits; should also be regarded as candidates for involvement in male infertility.

Furthermore, it has been shown that human sperm samples are very heterogeneous, containing, for instance, subpopulations with better mitochondrial functionality (Chapters IV and V), which seem to be precisely the ones with higher fertilisation potential. Sperm MMP, an indicator of mitochondrial function, could be similarly monitored using MT-G, MT-R and JC-1 (Chapter IV). However, none of the mitochondrial probes seemed to be dynamic enough to reveal modifications in MMP occurring after cellular incubation. Analysis of subpopulations of sperm seemed to indicate that the expression of POLG and COXI is related

with sperm MMP and to confirm their association with sperm motility (Chapter IV). This study also showed that the evaluation of MMP is a reliable indicator of sperm function. It is tempting to indicate mitochondrial probes as possible tools to select the best sperm for ART: the detrimental effects of the use of sperm stained with these probes in ART are theoretical low, as sperm mitochondria are degraded upon fertilisation. However, the use of such an approach in humans is certainly unviable, at least without appropriate controls.

Lastly, the analysis of distinct-quality sperm samples/fractions validated the importance of MMP in sperm function (Chapter V). Unfortunately, the absolute clinical significance of this has not been determined, as fertilising potential could not be fully assessed. Efforts are currently underway to involve various institutions in these kinds of studies. Nevertheless, it has been shown that sperm MMP is a relevant functional parameter, which seems to reflect other important cellular traits. The high correlation found between MMP and sperm motility further suggests that OXPHOS produces at least some of the ATP used by the human sperm flagellum. To completely reveal the role of mitochondria in sperm motility, it would be interesting to analyse the male gametes in the female reproductive tract environment, where they can persist for some time until fertilisation takes place. It would thus be useful to mimic such conditions *in vitro*, *i.e.*, to maintain sperm "cultures" and analyse their behaviour.

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