Mitochondrial DNA involvement in frontotemporal lobar degeneration – analysis of sequence variants in *MTND* genes

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Abstract

Mitochondria dysfunction and oxidative damage have been suggested to have an important role in ageing-related neurodegenerative diseases. One of the possible mechanisms is related to mitochondrial DNA (mtDNA) alterations that may impair mitochondrial respiratory chain function. Contrary to other neurological disorders, frontotemporal lobar degeneration (FTLD) pathophysiology is still poorly understood, and the etiology of most cases remains unknown. Recently, two mtDNA alterations were reported in a patient with FTLD and another study proposed an association between FTLD and a specific mtDNA haplogroup.

To determine if mtDNA is involved in FTLD, we sequenced 4 mtDNA genes encoding subunits of NADH dehydrogenase from 17 patients. The alterations detected were submitted to *in silico* analysis for evaluating possible pathogenicity.

In 82% patients we found 29 different alterations, including polymorphisms (62.1%), mutations already associated to other diseases (27.1%) and unpublished variants (13.8%).

Many of the alterations detected (69%) were not associated to a change in the amino acid translated and so are not expected to cause mitochondrial dysfunction. The non-synonymous variants are predicted to be benign, according to the *in silico* analysis. However, even if these alterations are not primarily pathogenic, an interaction with other mutations may occur, leading to the disease, worsening its expression or influencing age of onset.

Our study is still preliminary, but the high number of mtDNA alterations identified suggests a possible role of this genome in FTLD. However, it is not yet possible to determine whether these variants are part of the etiology or an epiphenomenon.

Keywords

mitochondrial DNA; frontotemporal lobar degeneration; neurodegenerative diseases; dementia; mutations; NADH dehydrogenase

Abbreviations

FTLD - frontotemporal lobar degeneration; MRC – mitochondrial respiratory chain; MTND – mitochondrial NADH dehydrogenase; mtDNA - mitochondrial DNA; nDNA – nuclear DNA; OXPHOS – oxidative phosphorylation; ROS – reactive oxygen species

Introduction

Mitochondria are fundamental organelles in the cell, not only responsible for ATP production by OXPHOS pathway, occurring in the MRC system, but also intervening in many other cell mechanisms, namely apoptosis and signal transduction. Each cell contains about 10^3-10^4 molecules of mtDNA (Greaves *et al*., 2012), a circular double-strand molecule of 16,568 base pairs (Andrews *et al*., 1999), encoding 37 genes: 13 peptides of MRC (7 from complex I, 1 from complex III, 3 from complex IV, 2 from complex V), and also 22 tRNAs and 2 rRNAs, needed for mitochondrial protein synthesis.

mtDNA has specific characteristics, distinct from the ones of nDNA and that justify the particularities of mtDNA associated diseases. The more determinant are (1) maternal inheritance, (2) heteroplasmy, (3) random mitotic segregation during cell division, (4) replication "independently" of the cell cycle, occurring even in post-mitotic cells – relaxed replication (Greaves *et al*., 2012) - and (5) variable number of mitochondria in each cell, depending on the tissue (Greaves *et al*., 2012; Schapira, 2006; DiMauro and Davidzon, 2005; Schon and Manfredi, 2003). Moreover, mtDNA has a high mutation rate, which can be explained since this molecule is almost totally encoding, it is localized near the main source of ROS production, it does not have protective histones and its repair mechanisms are limited. For those reasons, spontaneous mutations are frequent, mainly being neutral polymorphisms (DiMauro and Davidzon, 2005).

Even though mtDNA plays an important role in mitochondrial function, mitochondria do not work autonomously. In fact, most MRC subunits are encoded by nuclear genes. Furthermore, nDNA also has the genes necessary to the synthesis of other proteins needed for replication, transcription and stabilization of mtDNA and for the import, transport and assembly of MRC components (Greaves *et al*., 2012).

Many diseases with mitochondrial involvement have been reported, affecting mainly the nervous system, heart and skeletal muscle, since these are the tissues with higher energy needs (Greaves *et al*., 2012; Schapira, 2006; Zeviani and Donato, 2004; Schon and Manfredi, 2003). OXPHOS abnormalities that are characteristic of these disorders can be caused by alterations in nuclear or mitochondrial genes, namely substitutions, deletions, duplications, insertions or depletion of mtDNA (DiMauro and Schon, 2008; Schapira, 2006; Zeviani and Donato, 2004; Schon and Manfredi, 2003).

Beyond mitochondrial OXPHOS syndromes, there are neurological diseases in which physiopathological mechanisms are considered to involve an interaction between genes and environmental factors (Mattson *et al*., 2008), with reports of structural and functional mitochondrial abnormalities (DiMauro and Schon, 2008; Mattson *et al*., 2008; Lin and Beal, 2006; Schapira, 2006; Beal, 2005; Wallace, 2005; Schon and Manfredi, 2003). This is the case of Alzheimer´s disease, Parkinson´s disease, Huntington´s disease and amyotrophic lateral sclerosis. mtDNA polymorphisms and specific haplogroups have also been associated to neurodegenerative diseases, dementia and longevity (Mancuso *et al*., 2008; Grazina et al., 2006; Wallace, 2005). Mitochondrial dysfunction is related to ROS production and mtDNA somatic mutations that accumulate throughout the years, leading to energy insufficiency, signaling defects, apoptosis and replicative senescence, culminating in loss of cell function (Taylor and Turnbull, 2005). Moreover, accumulation of somatic mutations can exacerbate inherited defects of MRC.

FTLD is among the neurodegenerative disorders, with pre-senile onset, more commonly associated with dementia (Seelaar *et al*., 2011). The etiology of this pathology seems to include genetic, behavioral and environmental factors, accounting for the clinical and histological heterogeneity of this disease (Seelaar *et al*., 2011; Sleegers *et al*., 2010; Mackenzie et al, 2010, 2009). Clinically, the designation FTLD includes behavioral variant frontotemporal dementia, semantic dementia and progressive nonfluent aphasia (Neary *et al*., 2005). About 15% of the patients are also diagnosed with amyotrophic lateral sclerosis or Parkinson's disease (Sleegers *et al*., 2010). Clinical overlap with Alzheimer´s disease (van der Zee et al., 2008), corticobasal syndrome and supranuclear palsy (Rabinovici and Miller, 2010) is not uncommon. Various nuclear genes mutations are associated with the different variants of FTLD, namely *microtubule associated protein tau* (*MAPT)* and *progranulin* (*GRN)* genes and, less frequently, *valosin containing protein (VCP), charged multivesicular body protein 2B (CHMP2B), TAR-DNA binding protein (TARDP) and fused in sarcoma (FUS)* genes (Seelaar *et al*., 2011; Sleegers *et al*., 2010) but these fail to explain most cases.

Grazina *et al*. (2004) have already reported two homoplasmic mtDNA variants in one patient with diagnosis of FTLD, in nucleotides 3316 and 3337 of the *MTND1* gene, correlating with complex I deficiency. Recently an association has been described between FTLD and mtDNA haplogroup cluster IWX (Krűger et al., 2010), which has a higher non-synonimous/synonymous rate in the *MTND* genes than other European haplogroup clusters. However, as for most correlations between specific mtDNA haplogroups and neurological disorders, results are not consensual and a previous study did not come with the same conclusion (Rose *et al*., 2008).

In this perspective, the aim of the present work is to analyze mtDNA genes encoding MRC complex I (or NADH dehydrogenase) subunits in patients diagnosed with FTLD, in order to

determine whether *MTND* genes variants are involved in this disease, contributing for pathology.

Material and methods

We have studied 17 patients (eleven female and six male) diagnosed with probable FTLD according to standard criteria (McKhann et al., 2001), 16 with frontotemporal dementia – behavioral variant and 1 with semantic dementia, followed at the Department of Neurology of "Centro Hospitalar e Universitário de Coimbra". The mean age at onset was 60.7 years (range 41 – 79).

Written informed consent was obtained from all the participants and the study was approved by the local Ethical Committee.

Total DNA was extracted from venous peripheral blood using standard methods and quantified by UV spectrophotometry (λ=260 nm). Automated sequencing analysis was performed according to manufacturer's instructions (3130 ABI Prism sequencing system), using BigDye® Terminator Ready Reaction Mix v3.1 (Applied Biosystems). We have studied mtDNA regions to screen *MT-ND1*, *MT-ND2*, *MT-ND4L* and *MT-ND4* genes coding for complex I subunits for confirmed pathogenic mutations, polymorphisms and novel sequence variations. The database MITOMAP (www.mitomap.org) was used to classify the variants found. All sequences were analyzed using Sequencing Analysis® v5.4 and SeqScape® v.2.5 software (Applied Biosystems), by comparison with reference sequence obtained from database GenBank. *In silico* analysis was performed for all non synonymous sequence variations found, using PolyPhen-2® to predict the possible impact of amino acid substitutions on the structure and function of the protein translated.

Results

A total of 29 different alterations were identified and 59% of the patients had multiple alterations, in multiple genes. 82% of the patients had at least one mtDNA sequence variation in the genes studied.

We have found 9 alterations in *ND1* gene, 7 in *ND2* gene, 2 in *ND4L* gene and 11 in *ND4* gene (Table 1). In *ND1* gene, there were 5 polymorphisms, 3 variants reported in disease (one of which is a haplogroup marker) and 1 alteration not reported in the literature. In *ND2* gene, there were 6 polymorphisms (2 are haplogroup markers) and 1 variant reported in disease. In *ND4L* gene, there were 2 polymorphisms (one is a haplogroup marker). In *ND4* gene, there were 5 polymorphisms (one is a haplogroup marker), 3 variants reported in disease (one is a haplogroup marker) and 3 novel alterations. In total, 62.1% of the different alterations corresponded to polymorphisms, 27.1% to variants reported in disease and 13.8% to novel variants.

Each alteration was detected in one patient only, except the ones in positions 4580, 5460, 10550, 11299, 11467 and 11914, found in 2 patients each, and in position 11719, identified in 7 patients.

In silico analysis predicts that all the non-synonymous variants identified are possibly benign.

Table 1 – Characteristics of the mtDNA variants detected (according to MITOMAP)

Nucleotide Position	Locus	Nucleotide Change	Amino Acid Change	Reported mtDNA Base Substitution Diseases	mtDNA Somatic Mutations	*In silico* analysis prediction (score)
3316	MT-ND1	G-A	A-T	DM II/ LHON/ PEO (status unclear)		Benign (0.001)
3337	MT-ND1	G-A	V-M	Cardiomyopathy (Possibly synergistic)		Benign (0.020)
3483	MT-ND1	G-A	syn			
3772*	MT-ND1	A-G	M-V			Benign (0.421)
3847	MT-ND1	T-C	syn			
3915	MT-ND1	G-A	syn			
4025	MT-ND1	C-T	T-M			Benign (0.017)
4104	MT-ND1	A-G (het)	syn			
4216	MT-ND1	T-C	Y-H (hg JT)	LHON/Insulin Resistance	Acute leukemia platelets, leukocytes & bone marrow	Benign (0.006)
4529	MT-ND2	A-T	syn (hg I)			
4580	MT-ND2	G-A	syn (hg V)		Pancreatic cancer cell line	
4727	MT-ND2	A-G	syn			
4745	MT-ND2	A-G	syn			
4976	MT-ND2	A-G	syn			
5046	MT-ND2	G-A	V-I			Benign (0.024)
5460	MT-ND2	G-A	A-T	AD/PD		Benign (0.000)
10550	MT-ND4L	A-G	syn (hg K)		Endometrium control tissue	
10589	MT-ND4L	G-A	syn			

Nucleotide Position	Locus	Nucleotide Change	Amino Acid Change	Reported mtDNA Base Substitution Diseases	mtDNA Somatic Mutations	*In silico* analysis prediction (score)
10771*	MT-ND4	A-G	syn			
10899*	MT-ND4	A-G	N-S			Benign (0.017)
11251	MT-ND4	A-G	syn			
11299	MT-ND4	T-C	syn			
11467	MT-ND4	A-G	syn	Altered brain pH		
11470	MT-ND4	A-G	syn			
11488*	MT-ND4	A-G	syn			
11674	MT-ND4	C-T	syn		Pancreatic cancer cell line, prostate tumor	
11719	MT-ND4	G-A	syn (hg H=G)			
11914	MT-ND4	G-A	syn			
11947	MT-ND4	A-G	syn (hg W)		Prostate tumor	

* Novel alterations; het – alteration detected in heteroplasmy; Syn – synonymous; hg – haplogroup marker; DM II – diabetes mellitus type II; LHON - Leber's hereditary optic neuropathy; PEO – progressive external ophthalmoplegia; AD – Alzheimer´s disease; PD – Parkinson´s disease

Discussion

Many of the mtDNA alterations found (69%) were synonymous and hence are not expected to be the major cause of disease or to have repercussions on mitochondria function, even though that may not always be the case, with some synonymous mutations reported in several diseases (Rollins *et al*., 2009; Jeronimo *et al*., 2001).

Concerning the 4 novel variants not previously reported in literature, two were non synonymous and *in silico* analysis predicts that they are possibly benign.

The non synonymous polymorphisms detected were also submited to *in silico* analysis, given the fact that, even if so far no disease has been associated to those nucleotide variants, it does not mean that they are not potentially pathogenic. However, the analysis of the variants that we have detected predicts that they are possibly benign (table 1). However, the polymorphisms detected may modulate the effects of other DNA variants and be indirectly involved in disease by that mechanism. The accumulation of polymorphisms can also reveal a tendency for the occurrence of mtDNA alterations, since this may mean that repair systems are less effective in these subjects.

Regarding the 7 mutations that have been reported in other diseases (three synonymous), none of the patients has been diagnosed with those disorders. According to the *in silico* analysis, the non synonymous mutations are not expected to be the cause of significant alterations in the proteins translated. Therefore, they are not predicted to be a major etiological factor in any disease, at least if not associated with other mutations. Accordingly, the status of alteration in position 3316, detected in two mitochondrial syndromes and in diabetes mellitus, is unclear. The mutation in nucleotide 3337 is reported to have a possibly synergistic role in cardiomyopathy (Zifa *et al*., 2008). The T-C transition in position 4216 has been found in leukemia (Linnartz *et al*., 2004). It has also been considered a secondary mutation in Leber's hereditary optic neuropathy (Johns and Berman, 1991) but Lodi *et al*. (2000) found no further impairment in mitochondrial oxidative metabolism in patients with this mutation. It is currently classified as a polymorphism and it is also a marker of haplogroup JT. One of the mutations (in position 5460), which was present in two patients, has been reported in two neurodegenerative disorders (Kosel *et al*., 1996; Schnopp *et al*., 1996; Lin *et al*., 1992; Petruzzella *et al*., 1992) in which dementia is one of the symptoms. Moreover, Parkinson´s

disease can be associated to FTLD and Alzheimer´s disease has been proposed to be part of a spectrum of disorders which also includes FTLD (van der Zee *et al*., 2008). These data are in favor of an eventual association of this mutation with FTLD. On the other hand, it can also mean that these two patients are more likely to have the FTDL subtype associated with Parkinson´s disease, even though they have not experienced any parkinsonian symptoms so far. The *in silico* analysis predicts the variant in position 5460 to be benign, suggesting that it is more likely just a polymorphism frequent in the neurological diseases in which it has been reported rather than part of the primary etiology of those disorders. Another possibility is that this alteration may increase the penetrance of other pathogenic mutations. The synonymous mutation in position 11467 has been associated with altered brain pH (Rollins *et al*., 2009). This is one of the three positions that define the super- haplogroup U, K, UK. The other two are located in genes not included in the current study. Haplogroups U and UK have less coupled mitochondria, leading to a lower level of ROS production, which could explain the increased postmortem brain pH (Rollins *et al*., 2009). These haplogroups have been reported to have a protective effect against aging and neurodegeneration. Two mutations (in positions 11674 and 11947) have been reported in prostate tumor cells (Jeronimo *et al*., 2001). As they are synonymous, their role in oncogenesis is not clear. The first one, as well as a polymorphism in position 4580, have been detected in a pancreatic cancer cell line (Jones *et al*., 2001), but they have not been identified in patients with pancreatic cancer so far.

Besides the alteration in positions 5460 and 11467, three other variants were detected in more than one patient, and these are markers of haplogroups common in Europe. Concerning the variant in position 11719, although it is not the defining polymorphism for any haplogroup, it is more frequent in haplogroups G and H, which is the most frequent haplogroup in Western Europe, explaining why it was found in 41% of the patients. Interestingly, a study on the association between haplogroups and longevity in a Finnish population (Niemi *et al*., 2003)

reported a lower frequency of haplogroup H in the elderly, comparing to middle-aged subjects and infants, which supports a possible haplogroup related predisposition to neurodegenerative diseases and early aging. This is consistent with the hypothesis that this haplogroup is associated to tightly coupled mitochondria, with higher levels ATP production rates but also of ROS (Wallace, 2005). Therefore, haplogroup H might be associated to an increased susceptibility to mtDNA mutations, explaining the reports of a high frequency in subjects with neurodegenerative and psychiatric disorders. Interestingly, Niemi *et al*. (2003) also found a higher frequency of cluster WIX in nonagenarians comparing to younger people, implying a possible protective role of this mtDNA lineage against aging. This is contrary to the correlation found between the same cluster and FTLD (Krűger et al., 2010), also in Finland. Further studies are needed to evaluate whether these associations are valid and reproducible in other European populations.

An important feature of most pathogenic mutations (Montoya *et al*., 2009; DiMauro and Davidzon, 2005; Zeviani and Donato, 2004) is their presence in heteroplasmy, which, in our study, was only detected for one of the variants, that was synonymous.

Even though synonymous mRNA variants are considered to be functionally neutral, their selection in some diseases, including neurodegeneration and cancer, may challenge this assumption. In fact, it is possible that nucleotide variants that do not change an amino acid might change some yet unidentified functions in the mRNA. As an example, internal mRNA sequences seem to be required to initiate mRNA translation and these could be rendered non-functional by synonymous variants (Brandon *et al*., 2006).

Regarding the non synonymous alterations detected, the prediction of benignancy does not exclude their involvement in FTLD. Even without causing major changes in protein structure, the mtDNA variants may interact with other mutations, leading to the disease, worsening its expression or influencing age of onset. The reports of some “benign” mutations in several

disorders are consistent with this hypothesis. Nevertheless, we cannot exclude that mtDNA alterations might be an epiphenomenon of the diseases.

The *in silico* analysis of non synonymous variants takes into account the sequence, philogenetic and structural information characterizing the substitutions. However, there are other criteria to evaluate the pathogenicity of mtDNA variants (DiMauro and Davidzon, 2005; Zeviani and Donato, 2004). Therefore, studies with cybrids and determination of MRC activity should be a complementary approach to our study. However, since the variants found are not expected to be directly pathogenic, it would probably be more useful to compare the frequency of these alterations in a healthy population of the same age, in order to evaluate whether there is a significant difference in frequencies between FTLD patients and controls. This might be difficult to investigate due to the small number of patients. This problem could be overcome by increasing the number of subjects.

The high mutation rate of mtDNA also makes it more complex to determine whether mtDNA alterations have significant effects in the cell. It is also necessary to take into account the extension of tissues affected, haplotype, environmental factors and nDNA background (DiMauro and Davidzon, 2005; Zeviani and Donato, 2004). Moreover, the presence of alterations in blood does not predict whether the same alterations are present in the nervous system. One possibility is to study other tissues, for example by performing a muscle or skin biopsy, since the presence of an alteration in multiple tissues makes it more likely to be also present in brain. This is only valid for inherited mtDNA alterations. Even so, we have to consider the possibility of heteroplasmy and the possible variability in levels of mutant mtDNA in different organs. Therefore, the best way to evaluate mtDNA involvement in FTLD, especially of sporadic cases, is through post-mortem study of the brain, which is often not possible.

Conclusion

Our analysis revealed a considerable number of mtDNA alterations in the FTLD population studied, present in 82% of the patients. 59% had multiple alterations, in several genes. This may be an indicator of mtDNA involvement in FTLD, not yet known whether as an etiologic agent or as consequence of other alterations. The polymorphisms and mutations can also be solely associated with aging, according to the mitochondrial theory of aging.

Similarly to what has been proposed in Alzheimer´s disease (Grazina *et al*., 2006; Onyang *et al*., 2006), mtDNA mutations may induce different phenotypic alterations related to MRC dysfunction in FTLD patients, depending on the metabolic characteristics of the subject, the individual antioxidant panel and the exposure to toxic agents. Mitochondrial dysfunction may cause or exacerbate some of the histopathological characteristics of neurodegenerative diseases, according to the "Mitochondrial Cascade Hypothesis", which has been proposed in Alzheimer´s disease (Swerdlow *et al*., 2010; Swerdlow and Khan, 2009) and also in Parkinson´s disease (Domingues *et al*., 2008). This hypothesis is also supported by the fact that neurodegeneration caused directly by mitochondrial dysfunction is common in primary mtDNA disorders, with the specific neuropathological findings depending on the underlying genetic defect (Greaves *et al*., 2012).

Further studies are needed to clarify the possible contribution of mtDNA to FTLD, including sequencing of the remaining mtDNA genes and correlating genetic data with results of MRC enzyme activities.

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Appendix - Guide for Authors from *Neurobiology of disease*

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

Article structure

Subdivision - unnumbered sections

Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.

• Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon

abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract

A Graphical abstract is optional and should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 10 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Database linking

Elsevier aims at connecting online articles with external databases which are useful in their respective research communities. If your article contains relevant unique identifiers or

accession numbers (bioinformatics) linking to information on entities (genes, proteins, diseases, etc.) or structures deposited in public databases, then please indicate those entities according to the standard explained below.

Authors should explicitly mention the database abbreviation (as mentioned below) together with the actual database number, bearing in mind that an error in a letter or number can result in a dead link in the online version of the article.

Please use the following format: Database ID: xxxx

Links can be provided in your online article to the following databases (examples of citations are given in parentheses):

- ASTM: ASTM Standards Database (ASTM ID: G63)
- CCDC: Cambridge Crystallographic Data Centre (CCDC ID: AI631510)
- GenBank: Genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: BA123456)
- GEO: Gene Expression Omnibus (GEO ID: GSE27196; GEO ID: GPL5366; GEO ID: GSM9853)
- MI: EMBL-EBI OLS Molecular Interaction Ontology (MI ID: 0218)
- MINT: Molecular INTeractions database (MINT ID: 6166710)
- NCBI Taxonomy: NCBI Taxonomy Browser (NCBI Taxonomy ID: 48184)
- NCT: ClinicalTrials.gov (NCT ID: NCT00222573)
- OMIM: Online Mendelian Inheritance in Man (OMIM ID: 601240)
- PDB: Worldwide Protein Data Bank (PDB ID: 1TUP)
- TAIR: The Arabidopsis Information Resource database (TAIR ID: AT1G01020)
- UniProt: Universal Protein Resource Knowledgebase (UniProt ID: Q9H0H5)

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes: Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Save text in illustrations as 'graphics' or enclose the font.

• Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Produce images near to the desired size of the printed version.

• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as 'graphics'.

TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

• Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color: in print or on the Web only. For

further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard

reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: All citations in the text should refer to:

1. Single author: the author's name (without initials, unless there is ambiguity) and the year of publication;

2. Two authors: both authors' names and the year of publication;

3. Three or more authors: first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated in wheat (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9.

Reference to a book:

Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.

Reference to a chapter in an edited book:

Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age. New York: E-Publishing Inc; 2009. p. 281–304.

Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by "et al." For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (J Am Med Assoc 1997;277:927–34) (see also http://www.nlm.nih.gov/bsd/uniform_requirements.html).

Journal abbreviations source

Journal names should be abbreviated according to

Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;

List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;

CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

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of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

• Telephone and fax numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print

• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes

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