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DESIGNAÇÃO DA ÁREA DO PROJECTO

Biotecnologia médica

TÍTULO DISSERTAÇÃO/MONOGRAFIA (riscar o que não interessa)

Preimplantation genetic testing for Huntington's disease: the perspective of one portuguese center.

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Preimplantation genetic testing for Huntington's disease: the perspective of one Portuguese centre

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Abstract

Background: Huntington disease (HD) is an autosomal dominant late onset neurodegenerative disease caused by an unstable cytosine-adenine-guanine (CAG) trinucleotide repeat expansion in the huntingtin (*HTT*) gene. Preimplantation Genetic Testing (PGT) is a diagnostic procedure available for these individuals, since they carry a high risk of transmitting this genetic condition to their offspring.

Methods: Information about fifteen HD couples referred for PGT and twenty-one cycles performed from 2009 to 2018 was collected retrospectively.

PGT provide direct testing of embryos obtained after intracytoplasmatic sperm injection (ICSI), using PCR multiplex as the genetic testing protocol.

Results: PGT for HD was performed in 15 couples, with no history of previous attempts, in a total of twenty-one cycles. The mean number of biopsied embryos per cycle was 4,9. The amplification efficiency in blastomeres was 87,4%. From the 90 amplified embryos, 32 were normal and suitable for transfer. The mean number of transferred embryos per couple was 1,2.

Overall, 3 positive hCG tests were obtained in 3 couples, resulting in 2 clinical pregnancies. The clinical pregnancy rate was 14.3% per embryo transfer. The 2 ongoing clinical pregnancies had normal evolution, and culminated in 2 deliveries, resulting in the birth of two healthy children.

Conclusions: PGT for Huntington disease is considered an effective and safe reproductive option for couples who are at risk of transmitting HD, when proper genetic and reproductive counselling is warranted.

Introduction

Huntington disease (HD) is an autosomal dominantly inherited, late onset neurodegenerative disease caused by a dynamic mutation in the huntingtin (*HTT*) gene: an expanded cytosine-adenine-guanine (CAG) triplet repeat.[1] *HTT* gene is responsible for the synthesis of the huntingtin protein. Normally, the CAG segment is repeated 10 to 35 times within the gene. In HD patients the CAG segment is repeated 36 to more than 120 times. HD is observed at reduced penetrance for repeat ranging between 36-39 and at full penetrance for repeat counts over 40.[2] Repeat length is not stable during meiosis, and it can expand in the subsequent generations, particularly when mutation is paternally derived.

Normal HTT plays a vital role in brain development, being mainly found in striatum and cerebral cortex. Mutated HTT has larger dimensions, due to a polyglutamine repeat in its structure. The elongated protein is fragmented in smaller toxic portions, which attach to each other and accumulate in different tissues, mainly in neurons, inducing their dysfunction and, ultimately, their death.

HD prevalence in Western Europe varies between 5 and 10 per 100.000, similar to what is observed in Portugal.[3,4]

Clinically, symptoms begin at age of 35-44 years and rapidly progress, significantly affecting patients' quality of life. CAG repeat length is inversely correlated to age of onset. In fact, juvenile HD (JHD), a variant form of HD in young adults, is characterized by a large number of repeats, usually greater than 60. Overall survival stands at 15-18 years and there is no current effective treatment.[3]

Preimplantation genetic testing (PGT) is performed for couples at a high risk of transmitting a known genetic condition to their offspring and allows the diagnosis of chromosomal abnormalities and monogenic diseases (PGT-M). PGT-M is one of the reproductive options available for these individuals, since there is a 50% risk of a carrier transmitting the mutation to the offspring.[4] It requires a multidisciplinary approach by a team of experts in gynecology/obstetrics, embryology and medical genetics, which will follow the couple from the adequate genetic counselling until the birth of a healthy child.

PGT-M is an alternative to prenatal diagnosis, involving the biopsy and genetic testing of single or few cells from preimplantation embryos and transfer of unaffected embryos for the genetic condition being tested to the patient's uterus. PGT-M avoids the risk of induced abortion, the psychological burden associated to termination of pregnancy, and it is the most suitable option for couples with an increased genetic risk combined with infertility.

Despite its numerous advantages, this procedure presents some risks and ethical and legal issues. It is technically complex and misdiagnosis may occur due to allele drop-out (ADO), an event in which one of the alleles is not properly amplified, and mosaicism, in case the biopsied blastomere is not representative of the total embryo. Main ethical problems relate to the moral

status of the human embryo, embryo manipulation via assisted reproductive techniques and eugenics.[5]

The Department of Genetics in the Faculty of Medicine/Centro Hospitalar Universitário São João has been the only Portuguese public centre, since 1998, performing this technique. Since then, the range of chromosomal disorders and monogenetic disorders for which PGT is available has expanded enormously. In 2009, for the first time, it was performed PGT-M in a HD couple in Portugal.

The main goal of this work is to provide an overview about the uptake and outcome of PGT-M techniques in HD couples, in the perspective of a Portuguese public centre.

Methods

All therapeutic procedures were done in accordance with the National Ethical Committee and National Council for Assisted Medical Reproduction. Informed consent was obtained from both partners after careful explanation of the treatment technique. This study was approved by the Ethical Committee from Centro Hospitalar S. João (Protocol nº 357/18).

From 2009 to 2018, fifteen HD couples were referred for PGT-M for Huntington disease. Twenty-one cycles were performed and six couples repeated the cycle once corresponding to a mean of 1.4 cycles per couple. Mutation was paternally derived in six couples, while in the other nine couples it was maternally derived.

Couples obtained genetic and reproductive counselling by a clinical geneticist before being referred for PGT-M. All patients had normal karyotypes and were considered suitable candidates for this procedure.

Controlled ovarian hyperstimulation was done by a GnRH agonist or antagonist protocol on female patients. After this treatment, oocytes were collected by ultrasonography guided folicular aspiration. Oocytes were fertilized by Intracytoplasmatic Sperm Injection (ICSI), which is preferred to conventional in vitro fertilization (IVF), since it prevents DNA contamination with sperm and/or cumulus cells during embryo biopsy. Embryo's development was carefully evaluated every day.

Embryos of type A (no anucleated fragmentation), type B (1-20% fragmentation) and type C (21-50% fragmentation) were biopsied in day 3 after ICSI. One (embryos with 6 cells) or two (embryos with 7 or more cells) blastomeres were removed from each embryo.

There are different strategies to perform genetic analysis on single cells, but the most widely procedure is a multiplex PCR. In this protocol, amplification of the disease-associated locus along with different informative polymorphic markers, known as "short tandem repeats" (STRs), which flank the mutated *HTT* gene is done. This strategy overcomes the potential threat of ADO and allows the detection of contamination.

After genetic diagnosis, one or two unaffected embryos were selected and transferred into the uterus, on blastocyst stage (day 5 post-ICSI).

Results

A total of 15 couples were submitted to PGT-M for HD, with a male:female ratio of carriers/at risk persons of 1:1.5. Twenty-one cycles were performed and 6 couples repeated the treatment cycle once, which represents a mean of 1.4 cycles per couple. None of these couples had a previous PGT-M attempt. The mean female age at beginning of each cycle was 34.5 years (Table 1).

One couple had a reported male infertility history and so the sperm was obtained by Testicular Sperm Extraction (TESE).

Following ovarian stimulation, the mean number of cumulus-oocyte complexes (COC) retrieved per cycle was 9.5 (ranging from 3 to 19). Overall, a mean of 7.6 oocytes per cycle were considered mature (metaphase II oocytes) and a mean of 5.6 oocytes, per cycle, were successfully fertilized (Table 1).

The mean number of biopsied embryos per cycle was 4.9. Laser drilling was the preferred method for zona breaching, during embryo biopsy. Embryos were biopsied at cleavage-stage, on day 3, and 1 or 2 blastomeres were retrieved for genetic analysis.

Multiplex PCR was the genetic testing method used for DNA amplification of each biopsied embryo. In average, 4.3 embryos per PGT-M cycle obtained a positive signal in the PCR reaction (Figure 1). The amplification efficiency in blastomeres was 87.4%. From the 90 amplified embryos, 32 were genetically transferable, while 58 were genetically not transferable (including mutated, haploid and inconclusive embryos). ADO was detected in 3 embryos, from 3 different cycles.

In 13 cycles it was possible to perform embryo transfer, with 17 embryos being transferred (including a frozen-thawed embryo transfer cycle from couple 8), corresponding to a mean number of transferred embryos per couple of 1.2 (in the range 1-2).

Overall, 3 positive hCG tests were obtained in 3 couples, resulting in 2 clinical pregnancies. One of the positive hCG ended as an ectopic pregnancy, and termination of pregnancy (couple 13). The clinical pregnancy rate was 14.3% per transfer. Amniocentesis confirmed PGT-M result in one couple, while the other couple decided not to perform prenatal diagnosis.

The 2 ongoing clinical pregnancies had normal evolution, and culminated in 2 deliveries (delivery rate/embryo transfer of 14.3%). The 2 caesarian sections deliveries resulted in the birth of two healthy children, one girl and one boy (Table 2).

Table 1. PGT-M cycles performed for HD couples.													
Couple	Clinical cycle	Female age (years)	Nº oocytes	Nº MII oocytes	Nº fertilised oocytes	Nº biopsied embryos	Nº amplified embryos	Nº genetically transferable embryos	Nº genetically not transferable embryos*	Nº Embryos transferred	hCG result	Clinical pregnancy	Delivery
1	1	29	10	9	8	8	8	4	3	0			
2	1	36	7	5	4	4	4	1	3	1	-	-	No
	2	37	8	6	4	4	4	1	3	1	-	-	No
3	1	34	7	5	5	5	5	3	2	1	-	-	No
	2	35	12	9	3	2	2	1	1	0			
4	1	35	5	4	3	3	3	1	2	0			
5	1	35	4	3	3	2	2	2	0	2	+	+	Yes
6	1	33	5	3	3	3	3	0	3	0			
7	1	37	14	12	4	3	2	1	1	0			
8	1	38	13	12	9	8	0	0	0	0			
	2	39	11	10	9	7	7	4	2	1 / 1	- / - **	- / -	No
9	1	36	13	11	8	8	6	2	4	1	-	-	No
	2	37	10	7	6	6	5	1	3	1	-	-	No
10	1	32	19	12	11	10	10	4	6	2	+	+	Yes
11	1	33	13	11	4	3	3	0	3	0			
12	1	36	9	8	5	5	5	2	3	2	-	-	No
	2	37	3	3	1	1	1	1	0	1	-	-	No
13	1	29	12	10	7	7	7	2	5	1	+	-***	No
	2	30	6	5	2	2	2	1	1	1	-	-	No
14	1	28	14	11	11	11	10	0	7	0			
15	1	39	5	4	2	1	1	1	0	1	-	-	No

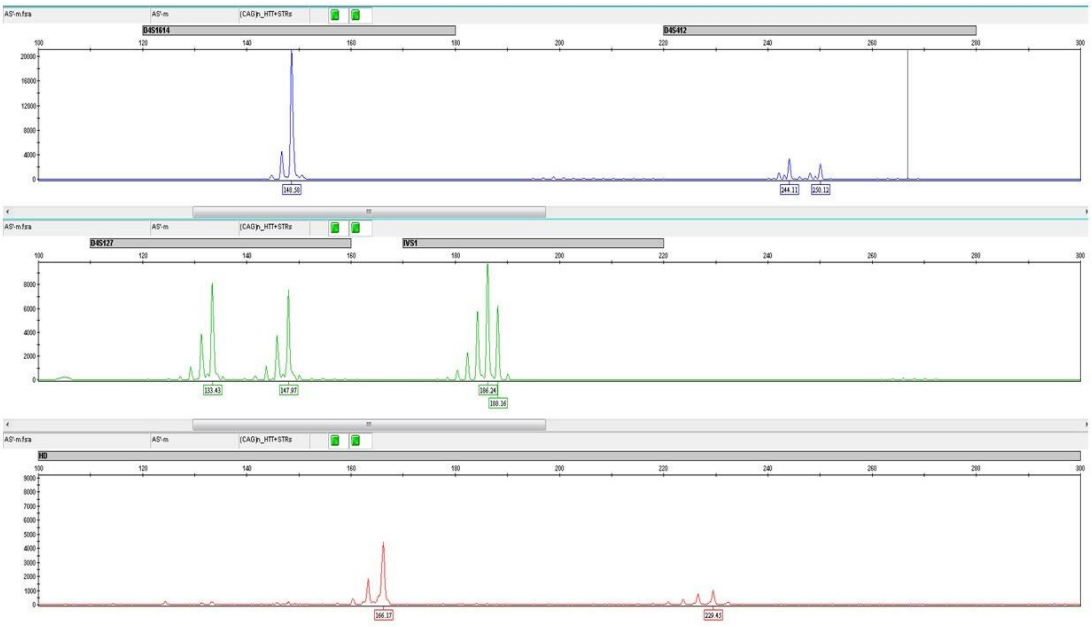
* Mutated, haploid and inconclusive embryos

** Frozen-thawed embryo transfer cycle

*** Ectopic pregnancy

Table 2. Clinical information of the two babies at birth.		
	Baby 1	Baby 2
Couple	5	10
Gender	Male	Female
Delivery Mode	Caesarian	Caesarian
Weight (g)	3045	3290
Height (cm)	48	50
Head circunference (cm)	34,5	35,5
Apgar score (1-minute/5-minutes)	9/10	9/10

Figure 1: Electropherogram of the multiplex PCR amplifying simultaneously the CAG repeat (red) and the polymorphic markers D4S1614 (blue), D4S412 (blue), D4S127 (green) and IVS1-intronic marker (green).



Discussion

Reproductive options available for couples with genetic disorders should be analysed in each individual case, concerning their advantages and disadvantages. Although prenatal diagnosis has higher success rates, the possibility of termination of pregnancy in case of an unfavourable result represents one of the major concerns. Whether the introduction of PGT for HD has reduced or not the use of prenatal diagnosis in these patients is an interesting question, and should be studied in further investigations in our population.

Preimplantation Genetic Testing has specific indications and its implementation is regulated by law and subject to the National Council for Medically Assisted Procreation approval. One of the indications accepted by law is Huntington disease. PGT is one of the available reproductive options for these couples, acknowledging the possibility of analysis for the presence of the triplet

expansion, and/or genetically linked markers associated with the dynamic mutation, in human embryos.

This analysis can be performed in two different modalities: direct testing of embryos or exclusion testing. The latter is not approved in Portugal, but it is performed in other European centers, when couple decided not to be informed about their HD carrier status, and do not want to be subjected to presymptomatic testing[6].

European Society of Human Reproduction and Embryology (ESHRE) PGT Consortium, established in 1997, collects, retrospectively and prospectively, data on PGT cycles, pregnancies, deliveries and children. The later published report, covering monogenetic diseases, HLA typing, and chromosome abnormalities, documented cycles performed from 2011 to 2012.

Our main results on PGT cycles for HD patients were compared to those internationally published, although we were aware on implications of working on different sample sizes.

The mean age of woman at beginning of the first cycle (34.5 years) was a bit higher than that reported in literature (32 years).[7] This may be caused by the delay of Portuguese couples on searching for these treatments, waiting list or by the lack of available information about them.

In our study, male:female ratio of HD carriers or at-risk persons was 2:3, matching the 40:60 ratio reported for couples opting for presymptomatic testing[3].

Although all couples were selected for ICSI procedure, most parents who undergo PGT do not have fertility problems, except one couple with a reported male infertility history. None of the 15 couples have benefited on this technique in the past.

According to literature, day 3 cleavage stage embryo biopsy remains the preferable biopsy method for PGT-M cycles, although it may switch to day 5 biopsy in the near future.[7] In our study, 100% of embryos were biopsied in day 3 after ICSI.

From the embryos successfully biopsed, 87.4% gave a diagnostic result (vs. 91%, from literature)[7]. Multiplex PCR may be subjected to several problems, including sample contamination, total PCR failure and ADO. The latter phenomenon was detected in 3 (3.3%) embryos, from 3 different cycles. Increasing number of linked informative polymorphic markers are being used, in order to reduce the risk of misdiagnosis.

The mean number of transferred embryos per couple was 1.2 (ranging from 1-2), comparable with that found in the literature[8].

The clinical pregnancy rate and the delivery rate were both 14.3% per embryo transfer, which significantly differs from what is depicted in literature, although we must be aware about the small sample size.[7]

The success rates of the Department of Genetics in the Faculty of Medicine/Centro Hospitalar Universitário São João differ from international data on pregnancy rates in PGT. One potential reason, which may explain this difference, is the small sized PGT-M sample for Huntington disease in our study. Most international PGT data collection includes results from thousands of PGT cycles performed on different monogenic diseases.

Despite the reported differences, we concluded that PGT-M for Huntington disease is considered an effective and safe reproductive option for couples who are at risk of transmitting HD, when proper genetic and reproductive counselling is warranted.

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None

Presentation: none

Conflicts of Interest

The authors report no conflicts of interest.

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Annex

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These should describe fully, but as concisely as feasible, the results of original clinical, laboratory or biomedical research. *Special note regarding case studies:* Case studies will be considered for publication only in the Letters to the Editor section of the Journal. The average Original Article fills 7 pages in the printed journal, although manuscripts that exceed this may be occasionally accepted for publication at the Editors' discretion. In general, an Original Article should not exceed 3500 words, not including the abstract, figure legends, and references. Abstracts should be 250 words or less. If possible, each figure legend should be held to 60 words or less. Each Original Article may be accompanied by no more than 8 graphic presentations (tables and/or figures)-for example, 3 tables + 5 figures. (Additional text, tables, or figures can be designated as "supplemental" material, which will be included in the PBJS Online Repository. Please

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Letters to the Editor

Letters to the Editor are brief reports of clinical or laboratory observations, substantiated by controlled data but limited in scope, and without sufficient depth of investigation to qualify as Original Articles. These may include a brief description of a particular condition that provides insights into diagnosis and clinical management or images that impart important clinical information. Like Original Articles, these manuscripts are subject to peer review. A Letter to the Editor must:

1) Be brief. The average Letter to the Editor fills 2 pages in the printed journal, although manuscripts that exceed this may be occasionally accepted for publication at the Editors' discretion. In general, a Letter to the Editor should not exceed 1000 words, not including the figure legend(s) and references. If possible, the figure legend(s) should be held to 60 words or less. Please note: Letter to the Editor manuscripts that are determined to significantly exceed these limits may be returned to the authors for shortening prior to review.

2) Have a short, relevant title. Please see the suggestions that appear above (under "A. Original Articles").

3) Have a complete title page (see section A1).

4) Be accompanied by a short summary that encapsulates the report's findings for a clinically oriented audience (see above).

5) Begin with the salutation "To the Editor:"

6) Close with the author's name(s), academic degree(s), institutions(s), and location(s).

7) Have no more than nine references.

8) List the references as complete bibliographic citations following the closure of the letter (see section above for formatting).

9) Present lists of Key words, as relevant (see sections above).

10) Be limited to a total of 2 figures and/or tables. (Additional figures or tables may be placed in the article's Online Repository; please see the relevant section below.)

Correspondence and replies

Correspondence concerning recent publications in the Journal will be considered for publication and accepted based on their pertinence, their scientific quality, and available space in the Journal. If the correspondence is considered acceptable, a response will be requested from the authors of the referenced PBJ article. Upon review and approval by the Editor, the Correspondence and relevant Reply will both be published together. Both Correspondence and Reply manuscripts must:

1) Be no longer than 500 words.

2) Have a short, relevant title, distinct from the title of the referenced article. Please note that all Replies should have the title "Reply to [Corresponding author's name]."

3) Have a complete title page (see section above).

4) List the references as complete bibliographic citations at the end of the letter with the journal article being discussed as the first reference (see section above). The total number of references should be no more than seven. Replies should include the Correspondence to which they are replying as one of the references.

5) Have no more than one graphic presentation (table or figure). (See the section on Graphic Presentations below).

6) Begin with the salutation "To the Editor:" and close with the author's name(s), academic degree(s), institutions(s), and location(s).

Review articles

Definitive, in-depth, state-of-the-art reviews of clinical and research subjects. Unsolicited reviews are not generally published in PBJ. Before submitting any unsolicited reviews, please forward an outline to the Editor for consideration. Systematic reviews and meta-analyses should follow the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (seehttp://www.prisma-statement.org/). A PRISMA flow diagram (http://www.prisma-statement.org/documents/PRISMA%202009%20flow%20diagram.pdf) should be used to describe the steps of the systematic review, and a complete PRISMA checklist (http://www.prisma-statement.org/documents/PRISMA%202009%20checklist.pdf) should be provided during submission.

Clinical Guidelines

Official recommendations from professional organizations on issues related to clinical practice and health care delivery. PBJ is most interested in publishing the primary guideline documents but will also consider synopses of guidelines when the primary document is published elsewhere. Synopses should focus on those issues of most relevance to generalist clinicians. Manuscripts must:

1) Have an equal or less than 275 words, structured abstract (use the following subheadings: Description, Methods and Recommendations)

2) Include the name of the responsible organization in the title and identify the article as a clinical guideline.

3)Primary Guideline Reports: PBJ is flexible with length, reference, and other format requirements given the variability in the format of guidelines developed by different organizations. However, if guidelines are lengthy (more than 4000 words), we may require the production of an executive summary document with the full document published as a digital-only appendix. A concise table or concise graphic summarizing the recommendations and other key points is desirable.

Guideline Synopses

Text of synopses include the following sections and subheads:

Rationale, Guideline Focus, Target Population, Guideline Development Process, Evidence Review and Grading, Comments and Modification, Clinical Recommendations,

Research Recommendations, Applicability and Implementation Issues, and Summary. Guideline Group members followed by key references should be listed at the end.

Rostrum articles

Opinion articles about subjects of particular interest and/or debate may be accepted for peer review after preliminary review by the Editor. Proposals for rostrum articles may be emailed to the Editorial Office; they will be evaluated based on level of interest, novelty, and the current needs of the Journal.

MANUSCRIPT PREPARATION AND FORMATTING INSTRUCTIONS

Manuscripts must be written in clear, grammatical English (see English Language Assistance above). Manuscripts not conforming to Journal format will be returned to authors for modification. Please double space the entire main body document and number each page. Do not add line numbers as the system will generate those when the PDF is built.

Title page, footnotes, abbreviations, and abstract pages must be included in the main body file. Please do not upload separate copies of these documents.

Acceptable document file types for text and tables include .DOC and .DOCX; do not submit a PDF.

Page 1:

Title Page. The following elements are required for every submission:

Title. Include a descriptive title of the work; the title should not be a sentence. No proprietary or brand names for drugs or agents may be used in article titles. Please, include the study design in the title; for instance, “randomised controlled trial”, or “systematic review”. Titles should be as informative and complete as possible.

Authors. The full first name, middle initials, and family name of each author, as well as the name(s) of the department(s) and institution(s) to which the work should be attributed.

Address for Correspondence. A current email and full mailing address for the corresponding author must be provided.

Page 2:

Abstract. Original articles should include a structured abstract of no more than 300 words using the following headings: Background; Methods; Results; and Conclusions. They should briefly describe, respectively, the problem being addressed in the study, how the study was performed, the salient results, and what the authors conclude from the results. Conventional non-systematic, reviews should include an unstructured abstract of no more than 250 words.

Main Body: *Introduction*. The introduction contains a statement of the purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations.

Methods. Avoid detailed description of previously published methods and cite the appropriate reference. Include appropriate ethical and statistical information.

Results. The results should be concise, avoiding redundant tables and figures illustrating the same data.

Discussion. This section should follow the results and is used to interpret results, with minimal recapitulation of findings.

Acknowledgments: The acknowledgements section should be headed 'Acknowledgements relating to this article' and contain the following distinct statements in separate paragraphs:

- Assistance with the study. Acknowledgements should be made only to those who have made a substantial contribution to the study. Authors are responsible for obtaining written permission from people acknowledged by name in case readers infer their endorsement of data and conclusions. If there was no assistance state: 'Assistance with the study: none.'
- Financial support and sponsorship. You must make reference to all relevant sources of funding concerning this article. If there were no sources of funding please state: 'Financial support and sponsorship: none.'
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- Presentation (for original articles only). Presentations of preliminary data at, for example, international meetings should be acknowledged separately. If preliminary data was not previously presented please state: Presentation: none.

References: Use the Vancouver reference system as adopted by the U.S. National Library of Medicine ensuring that all journal titles conform to Index Medicus approved abbreviations. Number references consecutively in the order in which they are first mentioned in the text. Identify references in the text, tables and legends using superscripted Arabic numerals that are placed after the punctuation. References cited only in tables or in legends to figures should be numbered in accordance with the sequence established by the first identification in the text of the particular table or illustration.

Avoid citing abstracts unless from a MEDLINE or EMBASE indexed journal. Unpublished observations and personal communications should not be used as references, although references to written (not verbal) communications may be inserted (in parentheses) in the text. Manuscripts that have been accepted but not yet published (e.g. Epub ahead of print) should be included in the list, followed by (in press). Information from manuscripts not yet accepted may be cited only in the text as (unpublished observations). Authors should verify references against the original documents before submitting the article.

Electronic or online references should be cited in the reference list only if the material referenced is a specific article (e.g. a paper published in a web-based journal); see below for correct style. Less specific references (e.g. the web pages of societies, organisations

and university departments) should not appear in the references; instead the URL should be cited in full in the text.

Authors must confirm that the details of these references are accurate and complete. In the full list of references give the names and initials of all authors. If there are more than six, cite only the first three names followed by et al. The authors' names are followed by the title of the article: the title of the journal (italics) abbreviated according to the style of Index Medicus: the year of publication: the volume number (in bold): the first and last page numbers in full followed by a full stop. Titles of books should be followed by the town and country of publication, the publisher, the year and inclusive page numbers. See the following examples:

Journal articles:

Pollard BJ, Bryan A, Bennett D et al. Recovery after oral surgery with halothane, enflurane, isoflurane or propofol anaesthesia. Br J Anaesth 1994; 72:559–566.

Books:

Korttila K. Recovery period and discharge. In: White P, ed. Outpatient Anaesthesia. New York, USA: Churchill Livingstone Inc, 1990: 369–395.

Chapter in a book:

Pessayre D, Feldmann G, Haouzi D, Fau D, Moreau A, Neumann M. Hepatocyte apoptosis triggered by natural substances (cytokines, other endogenous molecules and foreign toxins). In Cameron RG, Feuer G (editors): Apoptosis and its Modulation by Drugs. Handbook of Experimental Pharmacology. Berlin: Springer-Verlag; 2000, pp. 59-108.

Electronic articles:

Margolis PA, Stevens R, Bordley WC, Stuart J. From concept to application: the impact of a community-wide intervention to improve the delivery of preventive services to children. Pediatrics [online serial] 2001; 108:e42.

http://www.pediatrics.org/cgi/content/full/108/3/e42. [Accessed 20 September 2001].

Tables: References to tables should be made in order of appearance in the text and should be in Arabic numerals in parentheses, e.g. (Table 1). Each table should be typed on a separate sheet in 1.5 spacing. Tables should not be submitted as photographs. Each table should have a brief title as a heading. Vertical rules should not be used. Place explanatory matter in footnotes, not in the heading. Authors are discouraged from using abbreviations in tables. If abbreviations are necessary then please explain them in the table's footnotes. Be sure that each table is cited in the text. If you use data from another published or unpublished source, obtain permission and acknowledge the source fully.

Authors are encouraged to submit non-essential tables as supplemental digital content for publication online only. See Supplemental Digital Content section for more details.

Figures and Legends: Figures should be uploaded in the highest resolution available. Legends should be supplied for all figures. They are numbered to correspond with the figures and typed double-spaced on a separate page. Figure legends for any

supplemental figures being submitted are to be provided separately; see section, Supplemental Digital Content (SDC).

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